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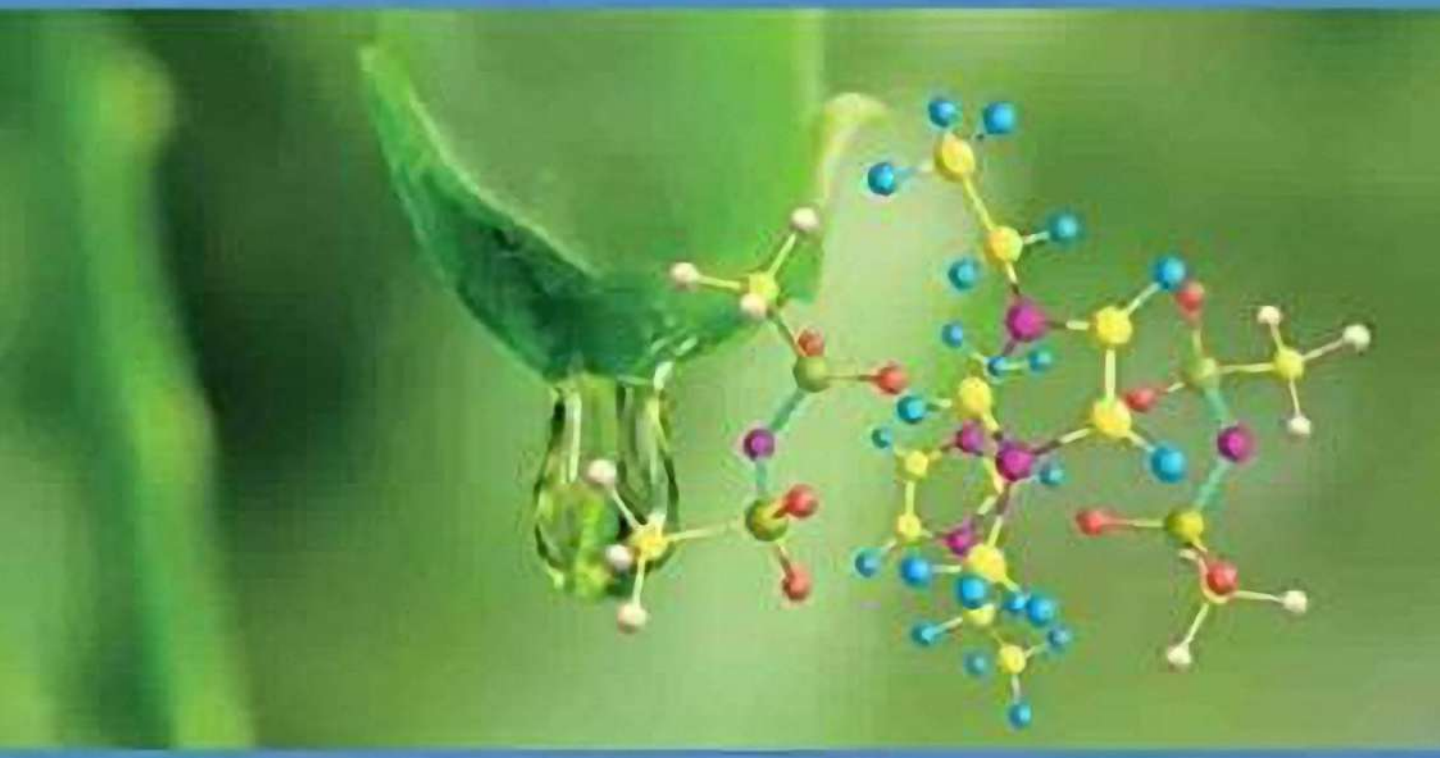
Biocatalysis in Green Solvents



Edited by
Pedro Lozano

Biocatalysis in Green Solvents

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Foundations and Frontiers in Enzymology

Biocatalysis in Green Solvents

Edited by

Pedro Lozano

Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Chemistry, University of Murcia, Murcia, Spain

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Dedication

To my wife Berta Goberna

To my daughters Marta, Paula and Berta Lozano

Foreword

The world is facing a serious threat from the climate change crisis, and hence more sustainable solutions for state-of-the-art manufacturing (circular economy) are urgently needed. In nature, microorganisms routinely use their enzymes and metabolic pathways to convert organic and inorganic compounds to synthesize biochemicals and obtain energy required for their growth in aqueous environments. In the last few decades, tremendous advances have been accomplished on biocatalysis in nonconventional media as well as fundamental understanding of how to select enzymes to convert feedstocks into special (bio)chemicals. These achievements can serve as an important basis from which it can be extended to build new applications in biocatalysis, metabolic engineering, and synthetic biology to enable biobased processes that are greener and cleaner for the environment. Prof. Lozano has put together an impressive team of experts in biocatalysis in nonconventional media such as ionic liquids, deep eutectic solvents, sub- and supercritical fluids, as well as "smart" engineering devices. Besides the inherent greener approach, biocatalytic reactions in these fluids have also shown higher selectivity, faster reaction rates, greater enzyme stability, as well as the possibility to use substrates that are not soluble in water. In summary, this book highlights the current state of knowledge regarding the enzymatic reactions that have potential applications in different industries using green and sustainable approaches and provide valuable information to those researchers from academia or industry, currently working either permanently or for a limited amount of their time in different areas that require the use of biocatalysis.

Jairton Dupont

List of contributors

Belen Altava
Inorganic and Organic Chemistry Department, E.S.T.C.E., Universitat Jaume I, Castelló, Spain

Inês O. Baptista
CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

Marie-Pierre Belleville
European Membrane Institute, UMR 5635, CNRS, ENSCM, Montpellier University, Montpellier, France

João A.P. Coutinho
CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

Pablo Domínguez de María
Sustainable Momentum, SL., Las Palmas de Gran Canaria, Spain

Antonio Donaire
Department of Inorganic Chemistry, Faculty of Chemistry, University of Murcia, Murcia, Spain

Ana M. Ferreira
CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

Mara G. Freire
CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

Eduardo García-Verdugo
Inorganic and Organic Chemistry Department, E.S.T.C.E., Universitat Jaume I, Castelló, Spain

Vicente Gotor-Fernández
Organic and Inorganic Chemistry Department, University of Oviedo, Oviedo, Spain

María J. Hernáiz
Biotransformation Group, Department of Chemistry in Pharmaceutical Science, Faculty of Pharmacy, Complutense University, Madrid, Spain

Eric Husson
Unité de Génie Enzymatique et Cellulaire, UMR 7025 CNRS Université de Picardie Jules Verne, Amiens, France

Hasan Tanvir Imam
School of Chemistry and Chemical Engineering, Queen's University Belfast, Belfast, Northern Ireland

Toshiyuki Itoh
Toyota Physical and Chemical Research Institute, Nagakute City, Aichi, Japan

Željko Knez
University of Maribor, Faculty of Chemistry and Chemical Engineering, Maribor, Slovenia; University of Maribor, Faculty of Medicine, Maribor, Slovenia

Maja Leitgeb
University of Maribor, Faculty of Chemistry and Chemical Engineering, Maribor, Slovenia; University of Maribor, Faculty of Medicine, Maribor, Slovenia

Pedro Lozano
Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Chemistry, University of Murcia, Murcia, Spain

Santiago V. Luis
Inorganic and Organic Chemistry Department, E.S.T.C.E., Universitat Jaume I, Castelló, Spain

Flávia F. Magalhães
CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

Hamayoun Mahmood
Department of Chemical, Polymer & Composite Materials Engineering, University of Engineering and Technology (UET), Lahore, Pakistan

Andrew Craig Marr
School of Chemistry and Chemical Engineering, Queen's University Belfast, Belfast, Northern Ireland

Patricia Catherine Marr
School of Chemistry and Chemical Engineering, Queen's University Belfast, Belfast, Northern Ireland

Muhammad Moniruzzaman
Department of Chemical Engineering, Universiti Teknologi PETRONAS, Perak, Malaysia; Centre of Research in Ionic Liquids (CORIL), Universiti Teknologi PETRONAS, Perak, Malaysia

Susana Nieto
Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Chemistry, University of Murcia, Murcia, Spain

Caroline Emilie Paul
Department of Biotechnology, Delft University of Technology, Delft, The Netherlands

Raul Porcar
Inorganic and Organic Chemistry Department, E.S.T.C.E., Universitat Jaume I, Castelló, Spain; Organic and Bio-Organic Department, Faculty of Science, UNED, Las Rozas-Madrid, Spain

Mateja Primožič
University of Maribor, Faculty of Chemistry and Chemical Engineering, Maribor, Slovenia

María Catalina Quesada-Salas
Unité de Génie Enzymatique et Cellulaire, UMR 7025 CNRS Université de Picardie Jules Verne, Amiens, France

Sikander Rafique
Department of Chemical, Polymer & Composite Materials Engineering, University of Engineering and Technology (UET), Lahore, Pakistan

Jose Sanchez-Marcano
European Membrane Institute, UMR 5635, CNRS, ENSCM, Montpellier University, Montpellier, France

Catherine Sarazin
Unité de Génie Enzymatique et Cellulaire, UMR 7025 CNRS Université de Picardie Jules Verne, Amiens, France

Ahmad Shakeel
Department of Chemical, Polymer & Composite Materials Engineering, University of Engineering and Technology (UET), Lahore, Pakistan; Faculty of Civil Engineering and Geosciences, Department of Hydraulic Engineering, Delft University of Technology, Delft, The Netherlands

Roger Arthur Sheldon
School of Chemistry, University of the Witwatersrand, Johannesburg, South Africa; Department of Biotechnology, Technical University of Delft, Delft, The Netherlands

Anamika Sindhu
Department of Chemistry, University of Delhi, Delhi, India

Yumiko Takagi
Department of Science, Faculty of Education, Kagawa University, Takamatsu, Kagawa, Japan

Ana P.M. Tavares
CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

Pannuru Venkatesu
Department of Chemistry, University of Delhi, Delhi, India

Rocio Villa
Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Chemistry, University of Murcia, Murcia, Spain

Marie E. Vuillemin
Unité de Génie Enzymatique et Cellulaire, UMR 7025 CNRS Université de Picardie Jules Verne, Amiens, France

Hua Zhao
Department of Chemistry, Howard University, Washington, DC, United States

Preface

Since the launch of the United Nations (UN) Sustainable Development Goals (SDGs) in 2015, the 17 SDGs have been widely adopted by governments and corporations in an effort to improve their sustainability (see Fig. 1). In agreement with these goals, our society needs to address important challenges through actions on mitigation and adaptation to the climate change, the sustainable use and protection of water and marine resources, the circular economy, the prevention and control of wastes, as well as the protection and the recovery of biodiversity and ecosystems. And the best weapon to face these challenges is scientific knowledge through which we can educate and train our young people who are amed to build our future.

From its origin, chemistry as a science is the engine that has allowed the humanity to advance, contributing continuous improvement to the quality of life and life expectancy (e.g., by means of pharmaceutical drugs, safer and more nutritious foods, clean drinking water, materials, and fertilizers and insecticides to improve agricultural productions, fuels). Molecules and materials created by chemists have provided a permanent improvement of our quality of life, allowing us to enjoy levels of comfort unexpected a century ago. Our quality of life depends entirely and positively on chemistry, and chemistry plays a significant role toward solving the great challenges of humanity by providing appropriate solutions based on sustainability criteria in the near future. In this context, Green Chemistry is the scientific approach that instigates and ensures efficient use of renewable raw materials and their selective transformation through (bio)catalysis, eliminating wastes and avoiding the use of toxic and hazardous reagents and solvents in the manufacture and application of chemical products, as P.T. Anastas and J.C. Warner summarized in 1998, based on the *Twelve Principles of Green Chemistry*, that converge directly in many of the 17 SDGs. Thus, the goal of Green Chemistry is the design (or redesign) of products and manufacturing processes to reduce their impact on human health and the environment, being considered the concept of sustainability by means of reducing environmental impact and preserving natural resources for future generations, as essential milestones.

The greenness in chemical processes stands on two essential actions, to minimize, or even to eliminate, the use and the production of hazardous substances that are not fully recoverable, and to maximize the selectivity of chemical transformations. The impossibility of full recovery for volatile organic solvents (VOSs) in chemical processes involves an important contribution to environmental deterioration in terms of health, safety, or impact on air, water, and land, as well as it significantly defines the cost of the process. In the same way, the lack of selectivity in chemical transformations may be considered as more responsible for the economic, environmental, and social impact of chemical processes, because of the undesirable by-products and wastes generated. Enzymes are considered as the most efficient catalysts for developing green chemical processes, not only by their biocompatible, biodegradable, and renewable nature but also because of its suitability for designing multicatalytic processes of industrial interest, mimicking in reactor metabolic paths as they do in living organisms. Additionally, their exceptional activity, stability, and selectivity (stereo-, chemo-, and regioselectivity) for chemical transformations can be improved by the continue progress in genomics and directed evolution. Green solvents and biocatalysts are the most suitable tools to tackle the essential actions in Green Chemistry, the former as a result of its easy recovery and

FIGURE 1

Strategic convergence between the UN 17 Sustainable Development Goals and the 12 Principles of Green Chemistry.

reusability, and the latter is supported by the highest selectivity of enzymes as catalysts, even improving the efficiency for chemical transformations shown in living systems.

Biocatalysis in Green Solvents is a book devoted to introduce MSc and PhD students, as well as scientists or professionals in Chemical Sciences, into the knowledge and applications of Green Chemistry, where amazing and synergic opportunities resulting when green solvents and biocatalysts are combined.

All chapters of this book are contributed by internationally recognized scientists and professors with many years of experience in the design and development of green biocatalytic processes, many of them being pioneers in this field. The book contains 17 chapters with over 2000 references and more than 200 figures, tables, and chemical schemes. The first chapter of the book concerns the biocatalysis, green solvents, and green metrics relationships as an attempt to define the limits of the book, being contributed by Prof. Roger A. Sheldon (TUDelft-The Netherland) as a pioneer and important author in biocatalysis and Green Chemistry.

Ionic liquids (ILs) play a leading role in this work as green and neoteric solvents. This green label is mainly due to their nonvolatile characteristics that permit an easy and full recovery for further reuse. Furthermore, by the appropriate selection of cations and anions, many solvent characteristics of ILs, such as polarity, hydrophilicity/hydrophobicity, miscibility with molecular solvents (i.e., water, organic solvents, etc.) can be tuned. These result in a plethora of unique properties that include over activation and overstabilization of enzymes, which have been successfully applied for developing smart and green approaches of biotransformation and recovery of nearly pure products from the reaction mixture. The opportunities of nonaqueous biocatalysis for chemical transformations are summarized by Prof. S. Nieto (Univ. Murcia-Spain) in Chapter 2, being then insightfully presented many of the amazing synergies that result when enzymes work in ILs, such as enzyme activation (Prof. T. Itoh, Toyota Res. Inst.-Japan), protein refolding (Prof. P. Venkatesu, Univ. Delhi-India), biocatalysts stabilization (Prof. H Zhao, Howard Univ.-USA), efficient biotransformation/separation systems by using sponge-like ILs (P. Lozano, Univ. Murcia-Spain) or biphasic systems (Prof. J.A.P. Coutinho, Univ. Aveiro. Portugal), as well as the suitability for biotransformations of carbohydrates (Prof. M.L. Hernaiz, Univ. Complutense Madrid-Spain).

Three additional chapters emphasize the unique properties of ILs for sustainable chemical processes, such as the extraction and processing of biopolymers (Prof. M. Moniruzzaman, U.T. Petronas-Malaysia), biomass biotransformation (Prof. C. Sarazin. Univ. Jules Verne-Picardie – France), and the key role of biocatalysis for a low carbon future (Prof. A.C. Marr, Queen Univ. Belfast-UK). This section also includes another chapter concerning the new frame that ILs have opened in pharmaceutics and medicine fields (Prof. B. Altava, Univ. Jaume I Castellon-Spain).

Two consecutive chapters analyze the excellent suitability of supercritical fluids for biocatalysis (Prof. Z. Knez, Univ. Maribor-Slovenia), as well as the ILs/supercritical carbon dioxide biphasic systems (P. Lozano, Univ. Murcia-Spain), for developing green and clean continuous biotransformation systems. Next, the book provides an essential description on the applications of enzymatic membrane reactors in these systems (Prof. J. Sanchez-Marcano, Inst. Europeen des Membranes, Montpellier-France), as well as, on the excellent suitability and possibilities of deep eutectic solvents (Prof. V. Gotor-Fernandez, Univ. Oviedo-Spain) as green biocatalytic systems. The last chapter is focused on a detailed analysis about the trends, needs, and opportunities of biocatalysis and green solvents (Dr. P. Dominguez, Sustainable Momentum, S.L-Spain).

It is my pleasure to express my sincere appreciation to all the contributors for their excellent contributions to *Biocatalysis in Green Solvents*. I also acknowledge Sara Pianavila and Peter B. Linsley for their support during the preparation of this book. I would like to give my sincere thanks to my wife Berta and my daughters Marta, Paula, and Berta for their support and encouragement while working on this book.

Pedro Lozano

CHAPTER

Biocatalysis, solvents, and green metrics in sustainable chemistry

1

Roger Arthur Sheldon[1,2]

[1]*School of Chemistry, University of the Witwatersrand, Johannesburg, South Africa* [2]*Department of Biotechnology, Technical University of Delft, Delft, The Netherlands*

1.1 Introduction to green chemistry and sustainability

Thirty years ago, there was growing environmental concern regarding the generation of waste and the use of toxic and hazardous materials by the chemical industry. In particular, the production of fine chemicals, for example, flavors and fragrances, and active pharmaceutical ingredients (APIs) primarily involved the use of stoichiometric inorganic and organic reagents that generated copious amounts of waste. Catalytic processes, widely applied in bulk commodity chemicals manufacture, were almost totally absent. Synthetic organic chemists preferred the more familiar stoichiometric reagents for example, metal hydrides for reductions and stoichiometric inorganic oxidants such as Cr^{VI} reagents for oxidations, rather than for example, catalytic hydrogenation and aerobic oxidation. An important reason for clinging to these well-established reagents, such as chromium (VI) oxidants, was their broad scope in organic synthesis.

However, increasing environmental awareness created a pressing need for alternative, cleaner processes that are more resource efficient and generate less waste, with emphasis on waste prevention rather than waste remediation. A paradigm shift was clearly needed, from traditional concepts of reaction efficiency and selectivity, focusing largely on chemical yield, to one that assigns value to maximizing raw materials utilization, eliminating waste and avoiding the use of toxic and/or hazardous substances [1]. Furthermore, the US Pollution Prevention Act of 1990 [2] focused regulatory attention on the need to reduce environmental pollution and acknowledged that waste prevention at source not only eliminates the cost of waste remediation but also strengthens economic competitiveness through more efficient use of raw materials. It caused a fundamental shift in the strategy for environmental protection—from "end of pipe" waste treatment to waste prevention—leading to the emergence, in the early 1990s, of the term Green Chemistry at the US Environmental Protection Agency [3].

Green chemistry focuses on waste prevention rather than waste remediation. That is, *Green chemistry efficiently utilizes (preferably renewable) raw materials, eliminates waste and avoids the use of toxic and/or hazardous reagents and solvents in the manufacture and application of chemical products*. The overall guiding principle is "benign by design." Green chemistry gained formal recognition with the publication of the 12 principles of green chemistry (Table 1.1), by Anastas and Warner in 1998 [4]:

Biocatalysis in Green Solvents. DOI: https://doi.org/10.1016/B978-0-323-91306-5.00012-1

Table 1.1 The 12 principles of green chemistry.	
1. Waste prevention not remediation	7. Preferably renewable raw materials
2. Atom efficiency	8. Avoid derivatisation (shorter synthesis)
3. Less hazardous materials	9. Catalytic not stoichiometric reagents
4. Safer products by design	10. Design products for degradation
5. Innocuous products by design	11. Analytical methods for pollution prevention
6. Energy efficient by design	12. Inherently safer products

The Brundtland report, *Our Common Future*, published *in 1987* by the World Commission on Environment and Development, launched the phrase sustainable development [5]. While acknowledging the need for industrial and societal development to provide a growing global population with a satisfactory quality of life, it emphasized that such a development must be sustainable over time. Sustainable development was defined as "*development that meets the needs of the present generation without compromising the ability of future generations to meet their own needs.*" According to Graedel [6], to be sustainable, a technology must fulfill two conditions: (i) natural resources should be used at rates that do not unacceptably deplete supplies over the long term and (ii) residues should be generated at rates no higher than can be assimilated readily by the natural environment. It is abundantly clear that an economy based on nonrenewable fossil resources—oil, coal and natural gas—consumes natural resources at a much higher rate than they are produced in Nature and that carbon dioxide is being generated at a much higher rate than can be assimilated by the natural environment, leading to undesirable climate change.

A balance has to be found between societal equity, environmental impact, and economic development, often referred to as the three Ps—people, planet, and profit—or the triple bottom line. Sustainable development, in contrast to green chemistry, comprises an economic component. A technology will only be sustainable in the long term if it is cost-effective. Increased interest in green and sustainable growth has focused attention on resource efficiency and is driving the transition from a "take-make-use-dispose" economy based on a linear flow of materials, to a greener, more sustainable circular economy [7].

1.2 The role of catalysis

Most of the waste produced in the industrial production of organic compounds, for example, pharmaceuticals, consists of inorganic salts, a consequence of the use of stoichiometric inorganic reagents. As mentioned earlier, this primarily involves oxidants, such as Cr(VI) reagents, permanganate, and manganese dioxide, and reductants such as metal hydrides, in addition to stoichiometric amounts of mineral and Lewis acids, and bases such as NaOH and KOH. Hence, the generation of copious amounts of waste can be alleviated by the widespread replacement of stoichiometric reagents with catalytic alternatives [8,9]. This applies to all segments of the chemical industry.

A pertinent example from the bulk chemicals arena is provided by propylene oxide with an annual production of 9−10 mio tonnes. Traditionally this involved the reaction of propylene with hypochlorous acid (essentially chlorine in water). For every kg of propylene oxide ca. 3 kg of $CaCl_2$ is produced (Fig. 1.1). One could say that it is a process to make calcium chloride with

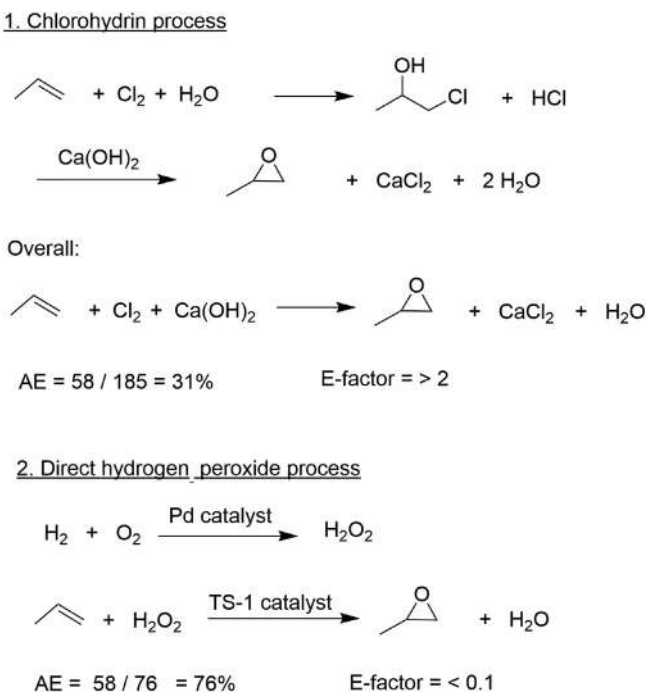


FIGURE 1.1

Manufacture of propylene oxide.

propylene oxide as the byproduct. More recently, this process has been largely superseded by greener alternatives involving catalytic oxidations with alkyl hydroperoxides or, in the HPPO process, hydrogen peroxide [10]. Combination with the direct production of hydrogen peroxide by catalytic reaction of hydrogen with oxygen [11] affords a process for the production of propylene oxide from propylene, hydrogen and oxygen with coproduction of one equivalent of water.

Another regulatory change, with far-reaching technological consequences for the pharmaceutical industry, was implemented at roughly the same time as the Pollution Prevention Act. In 1989, the FDA passed legislation that required pharmaceutical companies to market chiral drugs as the pharmacologically active enantiomer, or prove that the "wrong enantiomer" exhibited no detrimental side-effects [12]. This regulatory measure was long overdue [13]. It meant that the "wrong enantiomer" should be considered as waste which should be recycled or, preferably, not formed in the first place. It created a pressing need for clean, cost-effective, that is catalytic methods for the synthesis of pure enantiomers and led to the development of, for example, catalytic asymmetric hydrogenation. Subsequently, as a result of modern advances in molecular biology, in particular (meta) genome sequencing and directed evolution through protein engineering, biocatalysis emerged as an environmentally attractive and commercially viable technology for the highly enantioselective synthesis of pure enantiomers.

1.3 Advantages and limitations of biocatalysis

Biocatalysis conforms to 10 of the 12 principles of green chemistry [14] and has clear economic and environmental benefits:

- Enzymes are derived from readily available and inexpensive renewable resources and are biocompatible and biodegradable.
- Enzyme costs are essentially stable and predictable in contrast with the disruptive price fluctuations observed with scarce precious metals and the serious environmental costs associated with their mining.
- Costly removal of traces of noble metals from end products, to the very low levels demanded by regulatory authorities, is avoided.
- Enzymatic reactions are performed under mild conditions (near ambient temperature and atmospheric pressure) in water in standard multipurpose reactors.
- Enzymatic methods proceed without the need for functional-group activation or convoluted protection and deprotection protocols and are, hence, more step economic [15], generate less waste and are more cost-effective than conventional organic syntheses.
- Enzymes generally exhibit high chemo-, regio- and stereo- selectivities that are difficult to achieve with chemo-catalytic methods. In particular, the (near) perfect enantioselectivities observed with highly engineered enzymes are practically inimitable [16].
- Enzymatic reactions, in contrast with many chemo-catalytic reactions, usually involve roughly the same temperature and pressure, thus facilitating integration of multiple steps into cost-effective and environmentally friendly one-pot processes [17]. Telescoping multistep processes into one-pot cascades affords several economic and environmental benefits: fewer unit operations, minimized solvent and reactor volume, higher throughputs and space time yields and reduced waste [18] Moreover, it circumvents the need for wasteful and costly separation and purification of intermediates. Additionally, coupling of enzymatic steps can drive equilibria toward product formation, thus enhancing overall yields.

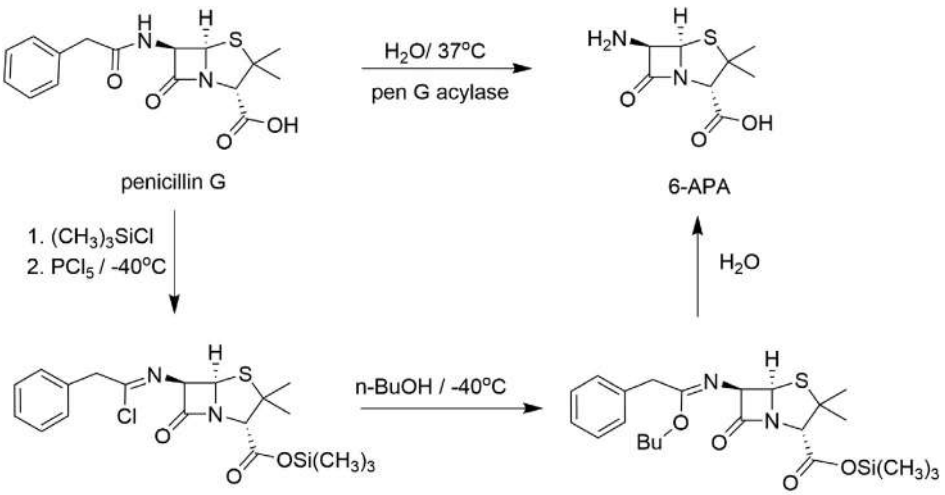
Biocatalysis can involve metabolically active and growing cells, that is fermentations, or isolated enzymes or metabolically inactive cells where generation of the enzyme and the biotransformation are uncoupled. In this case, deactivated enzymes have to be replaced with a fresh batch and cofactors have to be independently regenerated. Nonetheless, the significant investments and long lead times associated with the development of fermentation processes, and the preference of organic chemists for catalysts that can be stored, motivated the adoption of isolated enzymes as the preferred biocatalysts for the pharmaceutical industry [19].

An early example of replacing conventional chemistry with biocatalysis, that is green chemistry *avant la lettre*, dates from the late 1980s. It concerns the manufacture of 6-aminopenicillanic acid (6-APA), a key raw material in the industrial synthesis of semisynthetic penicillin and cephalosporin antibiotics, with an annual production of more than 20,000 tonnes, from penicillin G (Pen G). The latter is produced by fermentation. Until the late 1980s a conventional chemical procedure was used for the selective deacylation of Pen G, which is not a trivial reaction as it involves competition between a secondary and a more reactive tertiary amide function. It was accomplished in a one-pot process by protecting the carboxyl function with a trimethylsilyl group followed by transforming

the less reactive secondary amide into an imine chloride by reaction with phosphorus pentachloride at −40°C. Subsequent reaction with *n*-butanol at −40°C afforded an enol ether, which was hydrolyzed to 6-APA at room temperature (Fig. 1.2). The process involved the use of environmentally unattractive reagents in dichloromethane: 0.6 kg of Me_3SiCl, 1.2 kg PCl_5, 1.6 kg $PhNMe_2$, 0.2 kg NH_3 and 8,41 kg of *n*-BuOH were used to produce 1 kg of 6-APA.

The overall process is the epitome of what is wrong with many conventional organic syntheses. It is a multistep process involving protection, deprotection and functional-group activation steps. These involve stoichiometric amounts of environmentally unattractive reagents and suspect solvents, under energy intensive conditions, to produce, in addition to the desired product, copious quantities of toxic waste. This process, developed and commercialized by the Gist-Brocades company, is often referred to as the "Delft Cleavage."

In stark contrast, enzymatic hydrolysis of Pen G is a one-step process, requiring no activation or (de)protection steps and conducted in water at 37°C. The only reagent involved is 0.9 kg NH_3 per kg 6-APA to adjust the pH [20,21]. The enzyme, penicillin G amidohydrolase (E.C. 3.5.1.11),



Chemicals used to produce 1 kg 6-APA:

Chemical process		Enzymatic process	
PCl_5	1.2 kg	NH_3	0.09 kg
$(CH_3)_3Cl$	0.6 kg		
$PhN(CH_3)_2$	1.6 kg		
n-BuOH	8.0 kg		
CH_2Cl_2	11.0 kg		
NH_3	0.2 kg		

FIGURE 1.2

Enzymatic versus chemical deacylation of penicillin G.

otherwise known as Pen G acylase, was already known and used in the early 1960s [22]. However, the production of the enzyme was very expensive, and it had poor thermal stability, low volumetric productivity, and was discarded after a single use. Hence, 6-APA was produced using the chemical procedure for the next two decades. However, Pen G acylases with improved stability were developed by screening, and cost-effective production was eventually achieved using recombinant DNA technology. Moreover, effective immobilization of the enzyme enabled multiple recycling, up to 1000 recycles [23] of the enzyme and ensuing dramatic reductions in the enzyme cost contribution [24].

1.4 The metrics of waste minimization

As Lord Kelvin (1824–1907) so wisely observed: "If you can't measure it you can't improve it." Since a primary goal of green chemistry is to reduce or, preferably, eliminate waste generation in the production and application of chemicals, it is essential to have metrics for measuring the mass efficiency of chemical processes. The oldest two mass efficiency metrics—atom economy (AE) [25] and the E-factor [1]—dating from 30 years ago, are the most accepted and widely used.

1.5 Atom economy: every atom counts

AE is the molecular weight of the product divided by the sum of the molecular weights of the starting materials. Calculation of the AE assumes the use of stoichiometric amounts of starting materials and a 100% chemical yield. It doesn't take auxiliary reagents and solvents, not occurring in the stoichiometric equation, into account but it is very useful for comparing different routes to a target molecule before any experiments are performed. AEs of multistep processes are derived from the overall stoichiometry and are not simply the addition product of the AEs of the individual steps.

1.6 The E-factor: the environmental footprint of chemicals

In contrast, the E-factor is the actual amount of waste produced per kg of product, succinctly defined as "everything but the desired product" and including solvent losses and chemicals used in work-up. E-factors of individual steps are additive and are readily calculated for single- or multistep processes. The E-factor is essentially the environmental footprint of a process, the higher the E-factor the greater the environmental impact. Lower E-factors translate to a more efficient utilization of raw materials and show a strong positive correlation with reduced manufacturing and waste disposal costs [26,27]. The ideal E-factor is zero in line with the first principle of Green Chemistry: "It is better to prevent waste than to treat or clean up waste after it is formed." Using the E-factor places emphasis firmly on designing cleaner, waste-free processes, and the ideal E-Factor of 0 clearly reflects the ultimate goal of zero waste manufacturing. Typical examples of high AE, low E-factor catalytic processes are ketone hydrogenations, alcohol carbonylation, aerobic oxidation of alcohols, and olefin hydroformylation (Fig. 1.3).

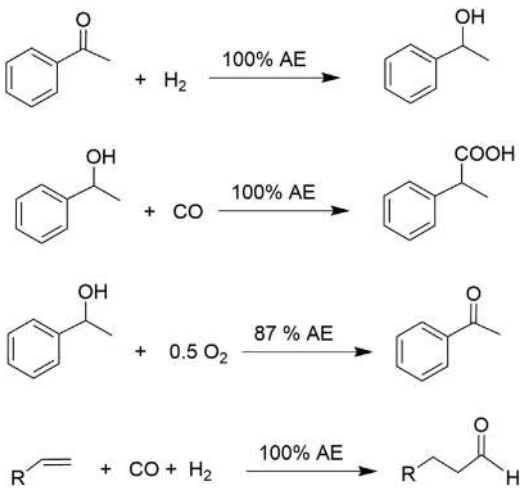


FIGURE 1.3

High atom economy catalytic processes.

Table 1.2 E-factors in the chemical industry.

Industry segment	Product tonnage (p/a)	E-factor (kgs waste/kg product)
Oil refining	10^6–10^8	<0.1
Bulk chemicals	10^4–10^6	<1–5
Fine chemicals	10^2–10^4	5–50
Pharmaceuticals	10–10^3	25–>100

The publication, in 1992, of the average E-factors of processes in various segments of the chemical industry (Table 1.2), from oil refining to pharmaceuticals, was a turning point [1]. It provided an important challenge to the industry, particularly the Fine Chemicals and Pharmaceuticals segments, to reduce the copious amounts of waste generated in their manufacturing processes. The Pharmaceutical industry accepted the challenge and has spent the last 2–3 decades cleaning up their manufacturing operations [28].

The E-factor basically includes all reagents used, in both the process and the down-stream processing. We assumed that solvents would be recycled and if data were not known we assumed that 90% would be recovered and, hence, only 10% of the solvent used was counted as waste. In hindsight this was too optimistic. The penchant of organic chemists for optimizing the particular solvent used in individual steps in a multistep synthesis leads to cross-contamination and difficulties in solvent recycling.

We excluded water based on the argument that inclusion would create a skewing of E-factors. However, disposal or reuse of processed water will inevitably involve some sort of pretreatment, and the current method is to calculate and compare E-factors both including and excluding water

[29,30]. This led to the introduction of simple E-factors (sEF), that disregard solvents and water for use in early route scouting, and complete E-factors (cEF) that include solvents and water assuming no recycling [26]. The true commercial E-factor will lie between the sEF and cEF and can be calculated when reliable data for recycling and solvent losses are known.

Inclusion of the energy requirements of a process in the E-factor was always implicit as energy consumption generates waste, which can be accounted for as carbon dioxide equivalents. In practice fine chemicals and pharmaceuticals are generally produced in multipurpose facilities and energy consumption is not allocated to particular processes. This makes assigning waste derived from energy to individual products difficult. In contrast, bulk chemicals are produced in dedicated units and energy forms an important part of total resources used. To improve the energy accounting of processes, the E^+ factor, which considers the greenhouse gas emissions generated from electricity used for unit operations such as cooling, heating, stirring and pumping, was recently proposed [31].

The strength of the E-factor is its simplicity—in terms of both concept and use—and, because it was introduced 30 years ago, it is familiar to many and is widely used. Other mass-based green metrics have been proposed and they can be divided into two types: those constituting a percentage of the ideal analogous to AE and those expressed as kg/kg analogous to the E-factor. An example of the former is reaction mass efficiency [32], a refinement of AE taking reaction yield and excess of reagents into account. An example of the latter type is process mass intensity (PMI) which is the mass ratio of the total input of materials (including solvents and water) to product [33]. However, such metrics have not reached the same broad acceptance as the E-factor [34,35], and their use is largely confined to small-molecule pharmaceuticals [26]. The E-factor has been widely adopted in chemistry courses at undergraduate and postgraduate level [36–41] and high-school chemistry classes [42] and consistently used as a yardstick for assessing the greenness of competing synthetic routes to various chemicals [43–48].

1.7 Intrinsic E-factors and system boundaries

Traditionally, the E-factor is based on gate-to-gate system boundaries, that is the process conducted at the manufacturing site, rather than cradle-to-grave. This means that the E-factor is dependent on the starting point of the synthesis and the E-factor of a multistep synthesis can be significantly reduced overnight by farming out an early intermediate instead of making it in-house. This means that it is necessary to agree on a definition of starting material. For example, in pharmaceuticals manufacture this has been defined as being readily available at a price of $<$ \$100 per kg from a reputable commercial supplier [24]. Since E-factors are additive (in contrast to PMIs), the intrinsic E-factor for the ASM synthesis may simply be added to the main synthesis E-factor to obtain an unbiased E-factor value for a complete synthetic pathway.

For example, Dunn and coworkers at Pfizer [49] calculated the E-factor of the commercial process for the production of sildenafil citrate (Viagra). The traditional E-factor, excluding water and including 10% of the mass of solvents used, was found to be 6.4 and the sEF (excluding solvents and water) and cEF were 3.9 and 50.3, respectively. However, one of the starting materials did not meet the requirement of being commercially available at a price of $<$ \$100 per mole, and its inclusion led to significant increases: the sEF increased from 3.9 to 9.9, the cEF from 50.3 to 85.5, and

the E-factor from 6.4 to 13.8. Hence, an agreement is required on an industry-wide starting point concept for assessing process greenness.

1.8 The climate factor

Thirty years ago, the number one environmental problem was ozone-depleting chemicals in the atmosphere. Thirty years later greenhouse gases, in particular carbon dioxide, have become the number one priority for climate change mitigation. This is motivating the transition from an economy based on fossil resources to a bio-based economy based on renewable energy and raw materials, which is manifest in the defossilization of chemicals manufacture.

Christensen and coworkers [50] proposed the use of the climate factor, defined as the total mass of CO_2 emitted divided by the mass of product formed (kg CO_2/kg product), to compare the CO_2 burdens of different processes. It is the sum of kg CO_2 emitted in the production of the raw material(s) and conversion of the latter to the product(s). It is useful for comparing biomass- versus fossil resource-based processes [51] but system boundaries will need to be defined and accepted. It should not be confused with carbon economy which is the % of carbon in a molecule that is derived from renewable resources. C factor is to carbon economy as the E-factor is to AE.

1.9 The nature and environmental impact of wastes

Not only the amount of waste generated but also the environmental impact of that waste is of paramount importance. The E-factor assigns the same weighting to all types of waste and the E-factor must, therefore, be considered in conjunction with other metrics [52]. We recognized this when we introduced the E-factor thirty years ago and proposed [53] the environmental quotient where Q is an "unfriendliness" multiplier representing the nature of the waste. The problem then becomes how to quantify Q. A decade later, Eissen and Metzger [54] developed the simple and easy-to-use Environmental Assessment Tool for Organic Synthesis software to assess the potential environmental impact, that is the Q value, of waste by assigning penalty points based on human and ecotoxicity.

Graedel [55] noted, in 1999, that "adding a life-cycle perspective to green chemistry enlarges its scope and enhances its environmental benefits." However, conducting a full scale cradle-to-grave Life Cycle Assessment (LCA) in the design or development phase of a process is generally too difficult and time consuming. Nonetheless, many groups [33,56–59] have addressed the problem of integrating mass efficiency with LCA-based assessment of the environmental impact of waste. However, the application of the LCA methodology to fine chemicals and pharmaceuticals is a challenge owing to the paucity of life cycle inventory data. Consequently, many chemical companies have adopted their own customized metrics, for example, BASF's eco-efficiency [60].

Pharmaceutical and Flavor and Fragrance companies have been particularly active in this respect. For example, GSK developed FLASC (Fast Life Cycle Assessment of Synthetic Chemistry) [61] and the cosmetic ingredients producer Chimex, a subsidiary of L'Oreal, introduced Eco-footprint which covers the supply chain from the supplier's gate to the product leaving the

production facilities [62]. It comprises a manufacturing footprint and an eco-design footprint. The former is based on five indicators: water footprint, carbon footprint of the transportation of raw materials to the production site, aqueous waste and used solvents valorization and energy consumption. The eco-design footprint comprises the E-factor, synthetic pathway efficiency, raw materials of renewable origin, and environmental impacts of raw materials and waste.

Similarly, Green Motion, a gate-to-gate green metric tool for evaluating the efficiency and health, safety, and environmental impacts of manufacturing processes, was developed by flavor and fragrance company, Mane [63]. Their starting point was to group the 12 Principles of Green Chemistry into seven fundamental concepts: raw material, solvent selection, hazard and toxicity of reagents, reaction efficiency, process efficiency, hazard and toxicity of final product and waste generation. Penalty points are then allocated within each category based on well-defined criteria, such as origin of raw materials (renewable or synthetic), yield, number of steps and solvents involved, and amount of waste as expressed by the E-factor. The latter was favored over PMI because it fitted better with the objective of zero points for an E-factor of zero. For a given criterion, the higher the impact on health, safety and environment, the higher the number of penalty points. Hazard and toxicity ratings are based on the hierarchy of GHS pictograms used on labels and safety data sheets that denote various hazards and toxicity.

Each process is assessed by means of a questionnaire requiring simple yes/no answers, pictograms, numerical values or a selection of multiple choice options that then assigns penalty points based on the answers. Deduction of the penalty points from 100 affords an overall score, meaning the higher the score the more sustainable and the lower the environmental impact of the process. The method was used to rate more than a thousand products in 12 months, thus building a useful database for benchmarking, and each new product which was scaled to production level was rated. The authors concluded that Green Motion is a simple and quantitative method that is well suited to the evaluation of flavor and fragrance ingredients, noting that a full assessment can be made in only half an hour.

We recently used the E-factor and other metrics in selecting a route for the synthesis of a key intermediate for HIV protease inhibitors [64]. The inclusion of so-called intrinsic E-factors to account for the synthesis of advanced starting materials was essential for comparison on a level playing field. Green motion was the most useful and conceptually simple method for comparing the three routes. Radial polygon is a useful visual tool that provides an overview of multivariable performance indicators [65]. An ideal green synthesis corresponds to a regular polygon, while distortions toward the center identify weak points in a synthesis and provide guidance for optimization.

1.10 The role of solvents: the medium is the message

In 1992 we remarked that "*So many of the solvents favored by organic chemists are now on the black list that the whole question of solvents in organic synthesis requires rethinking. Not only do organic chemists generally use too much solvent, they very often choose the wrong ones. In the first place, is a solvent really necessary? If a solvent (diluent) is needed it should preferably be water*" [1]. The last two decades have been devoted to rethinking the question of solvents in chemical

processes [66]. The best solvent is no solvent. Reactions can be performed with neat liquid substrates, for example, in biodiesel production from triglycerides [67].

Solvents constitute 80%–90% of the total mass of nonaqueous material used in pharmaceutical manufacture and account for the majority of waste formed and 75%–80% of the environmental life cycle impacts [68]. A survey of solvent usage, described in 388 publications in the period 1997–2012, revealed much room for improvement across the global pharmaceutical industry [69].

The eight most commonly used solvents of concern were three chlorinated hydrocarbons (dichloromethane, 1,2-dichloroethane, chloroform), four ethers (diethyl ether, diisopropyl ether, 1,2-dimethoxyethane and 1,4-dioxane) and one hydrocarbon (n-hexane). Many of these solvents were still being selected despite the availability of greener alternatives. The clear need for the greening of organic solvents encouraged pharmaceutical companies to develop Solvent Selection Guides (SSGs) to stimulate replacement of environmentally undesirable solvents, in particular chlorinated hydrocarbons [70]. Traffic-light inspired color coding was used, whereby green, amber and red signify "preferred," "usable" and "undesirable" solvents, respectively, [71–75]. GSK was the first to incorporate LCA into solvent selection [76]. Solvents were assigned a score of 1–10 in four categories, waste disposal, environmental impact, health and safety (EHS) issues, each of which consisted of multiple subcategories. Each solvent is given a composite score corresponding to the geometric mean of the four scores: <3.5 is red, $3.5- <7.5$ is amber and 7.5–10 is green.

The concept of scores based on EHS statements was adopted by the ACS Green Chemistry Institute's Pharmaceutical Round Table consortium [77] and the European collaborative research project known as CHEM21 [78]. 51 solvents were ranked and placed in four categories: recommended, problematic, hazardous and highly hazardous. In contrast with the GSK method, greener solvents have a lower score in the CHEM21 SSG. As Welton [79] remarked, appropriate solvent selection can improve both the environmental and commercial performance of a process and, therefore, its overall sustainability. Hence, the quest for improved solvents continues, for example, aided by in silico machine learning methods [80]. In addition to the need for replacing chlorinated solvents there is a pressing need to replace many hydrocarbons and ethers that are classed as hazardous or highly hazardous on the basis of toxicity and/or flammability issues. In addition, the four most commonly used dipolar aprotic solvents—dimethyl formamide, dimethylacetamide, N-methylpyrrolidone and sulfolane—are reproductive toxicity hazards the first three of which are classified as substances of very high concern.

1.11 Bio-based solvents

A variety of mono- and bi-phasic solvent systems have been proposed as alternatives for volatile organic solvents with undesirable ecological properties [66,81,82]. Various less common organic solvents, many of which are derived from renewable feedstocks [83], have also been ranked in the CHEM21 project as "recommended" or "problematic" [78]. These include a variety of bio-based alcohols, such as bio-ethanol [84], isobutanol and glycerol [85], bio-based ethers such as 2-methyltetrahydrofuran (Me-THF) [86], dimethyl tetrahydrofuran (diMeTHF), cyclopentyl methyl ether (CPME), dimethylsorbide, cyrene (dihydrolevoglucosenon) [87] and the cygnet [88] family of ethers, and bio-based esters such as isobutyl acetate, ethyl lactate, ethyl levulinate, diethyl

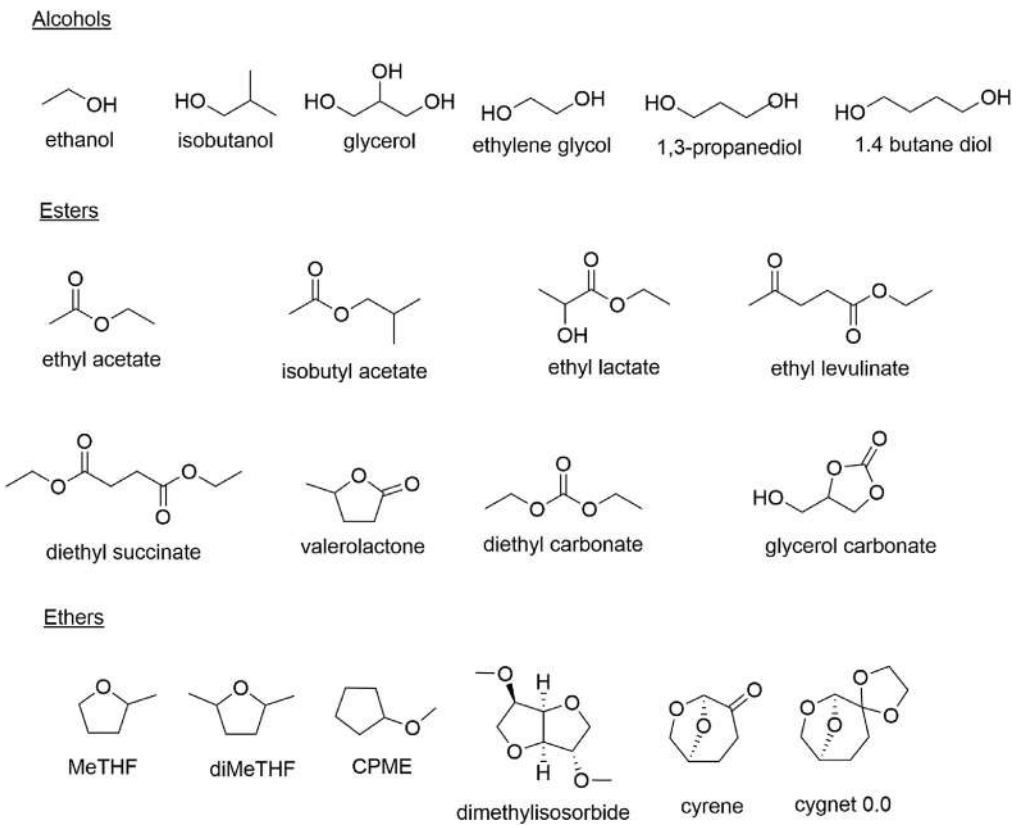


FIGURE 1.4

Bio-based solvents.

carbonate, glycerol carbonate and γ-valerolactone [89] (see Fig. 1.4 for structures). Interestingly, lipases dissolve in glycerol and glycerol carbonate [90] with retention of activity. Bio-based ethers, such as Me-THF, CPME and cyrene [91], are also useful solvents for biocatalysis [92].

1.12 Water as a reaction medium

Significant reductions in waste produced in chemicals manufacture have been achieved by replacing archaic stoichiometric methodologies with atom efficient, low E-factor, catalytic alternatives, notably hydrogenation, hydroformylation, carbonylation and oxidation [34]. However, inefficiencies associated with the containment, recovery, and reuse of solvents still remain. Alternatives for classical organic solvents should, therefore, not only have acceptable EHS properties. Moreover, both the catalyst and the solvent should be readily recovered and recycled to afford an environmentally acceptable and cost-effective process.

Water has much to offer: it is nontoxic, noninflammable, abundantly available and inexpensive and can be used in catalytic processes by employing water soluble catalysts. Indeed, in Nature many reactions are conducted with both water miscible and immiscible substrates in aqueous media. However, when water is used as the reaction medium, after the removal of the product(s) and catalysts, the remaining water will be contaminated with small amounts of organics and hence need to undergo some sort of treatment before it can be discharged to effluent treatment facilities. This problem is often aggravated by using organic solvents to extract the product from the water [93]. Another problem is that water has the highest heat capacity of all liquids. While this property is very useful for maintaining life on earth it is a serious disadvantage for chemical processes.

Nonetheless, there is a noticeable trend toward the use of water as a reaction medium, and with remarkable results. For example, Bailey and coworkers [94] at Takeda Pharmaceutical developed a five step synthesis of an investigational 5-HT4 receptor conducted almost exclusively in an aqueous medium with dramatic improvements in process efficiency compared with the first generation process in organic solvents. The overall yield was improved from 35% to 56%, the overall PMI from 350 to 79, and the solvent intensity from 223 to 14, representing a 94% reduction in organic solvent use. Even more remarkable was the reduction in water intensity from 106 to 55, that is the process in water used 48% less water than the organic solvent-based process. Both processes delivered the API with an overall purity of ca. 99.8%.

1.13 Aqueous biphasic catalysis

Catalytic reactions can be performed in water by employing water soluble catalysts. When the substrate is immiscible with water the reactions are performed as aqueous biphasic catalysis whereby the catalyst is dissolved in the water phase and the substrate in a separate organic phase. The reaction takes place in the aqueous phase which means that the substrate needs to be slightly soluble in water. The product is recovered by phase separation and the water phase, containing the catalyst, can remain in the reactor and be recycled. All of the atom economical catalytic processes discussed earlier can be performed as aqueous biphasic catalytic processes. The most well-known process is probably the Ruhr-Chemie/Rhone Poulenc process for propylene hydroformylation employing a water-soluble Rh (I) tppts complex (Fig. 1.5) as the catalyst [95]. This process works well because propylene is slightly soluble in water. The principle is also readily translated to other noble metal

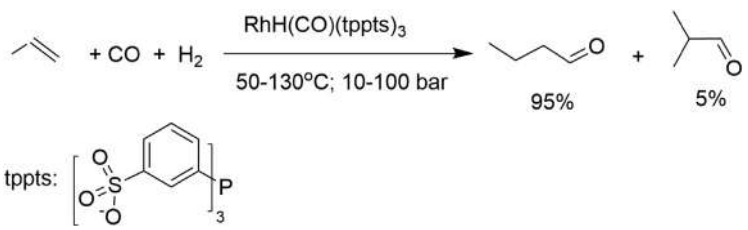


FIGURE 1.5

The RuhrChemie/Rhone Poulenc hydroformylation process.

catalyzed processes, such as hydrogenation, carbonylation, olefin metathesis and aerobic oxidations of alcohols [96,97].

1.14 Surfactants in water: aqueous micelles as nanoreactors

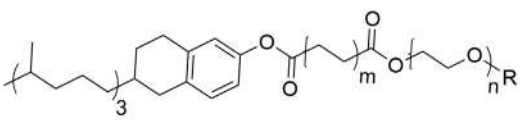
The rates of the biphasic Rh/tppts catalyzed hydroformylations of olefins decrease dramatically with increasing molecular weight of the olefin, for example, the practically water insoluble 1-tetradecene afforded only traces of aldehydes [97]. The envisaged approach to circumvent this problem was to use tenside phosphines that were specifically designed to combine the functions of a ligand and an amphiphile (surfactant) to create micelles in which the water insoluble substrate is solubilized. Although the concept was sound it did not lead to broad application.

Lipshutz and coworkers [98] used a different strategy that took the concept to a new level of sophistication and has proven more successful. They used small amounts (e.g., 2 wt.%) of designer amphiphiles above their critical micelle concentration (ca. 10^{-4} M) that spontaneously self-assemble in water to form nanomicelles with lipophilic interiors in which reactions can occur between water-insoluble substrates and catalysts. The choice of amphiphile is critical as it determines the size, shape and internal lipophilicity of the nano-micellar reactors.

Amphiphiles, PTS and TPGS-750-M (see Fig. 1.6 for structures), derived from vitamin E, for example, were used to perform a variety of noble metal catalyzed reactions [99], [100–102], such as hydrogenation [103] and palladium catalyzed C-C coupling reactions [104] at room temperature in organic solvent free water. The product was recovered by extraction with a minimum amount of an environmentally acceptable solvent, such as ethyl acetate, or precipitated as an insoluble solid with the amphiphile remaining in the water phase. However, it will be necessary to remove traces of amphiphiles, such as TPGS-750-M, remaining in the aqueous effluent [105].

Water is an excellent solvent for conducting enzymatic reactions and the product can be extracted into an environmentally acceptable solvent, such as ethyl acetate. Alternatively, the extraction can be back-integrated into the reaction step by conducting the process as aqueous biphasic catalysis. The company Codexis, for example, developed cost-effective biocatalytic processes to a variety of pharmaceutical intermediates using highly engineered enzymes in this way [106].

Alternatively, enzymatic reactions of hydrophobic substrates in water could be facilitated by designer amphiphiles. For example, ketoreductases (KREDs) were fully compatible with aqueous



PTS m = 4, n =14, R = H
TPGS-750-M m = 1, n = 17, R = CH_3

FIGURE 1.6

Structures of designer amphiphiles.

solutions containing micelles derived from TPGS-750-M [107]. This enabled the one-pot production of chiral secondary alcohols in high enantioselectivities through a combination of noble-metal catalyzed synthesis of ketones and subsequent enantioselective KRED catalyzed reductions (Fig. 1.7A). Moreover, the activity of the KRED was increased in the presence of the amphiphile,

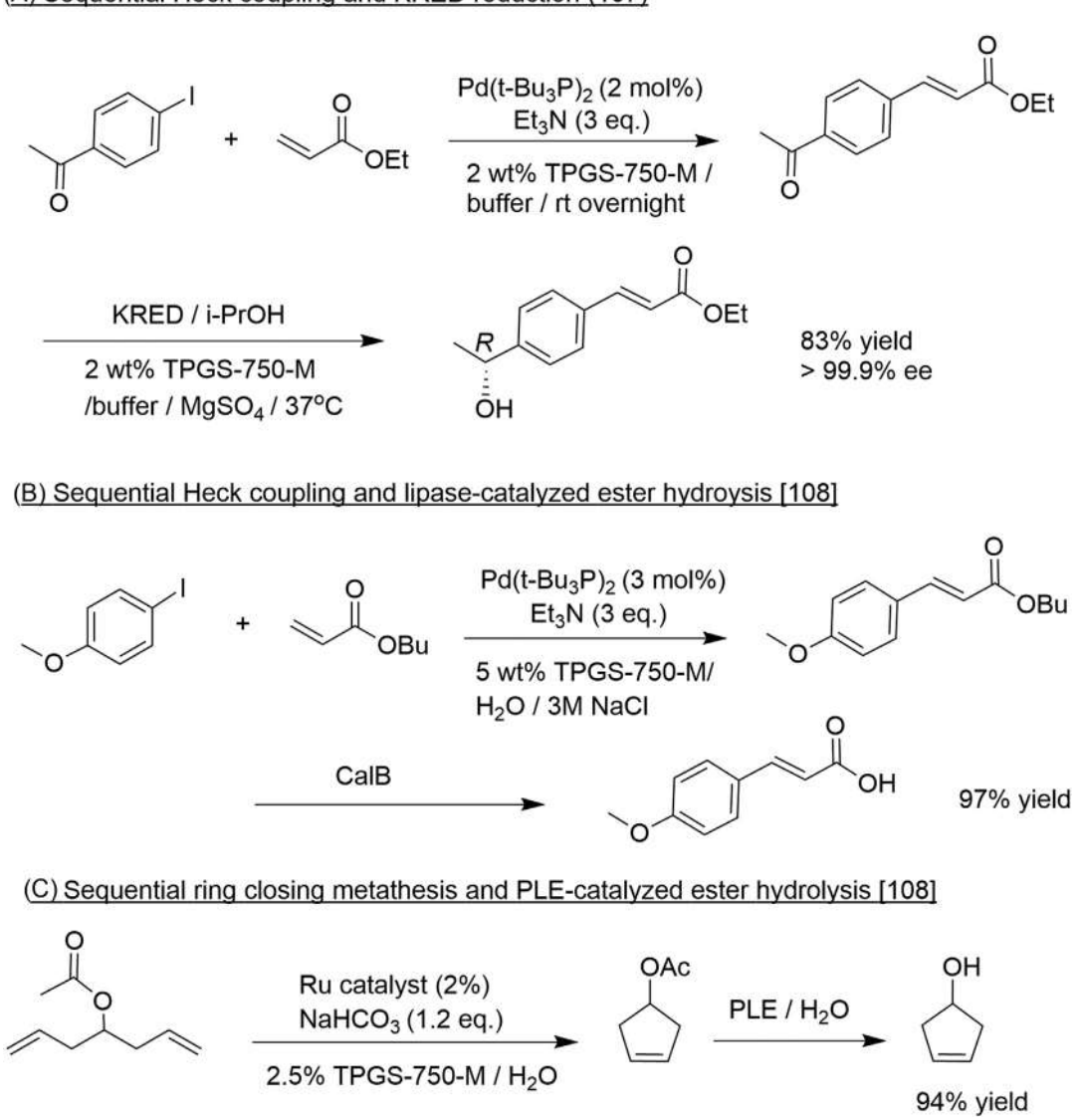


FIGURE 1.7

One-pot chemo-enzymatic conversions in water containing designer surfactants.

compared to in buffer alone. This was attributed to a "reservoir" effect in which the micelles control the supply of both substrate and product to the active site of the enzyme, thereby limiting substrate and product inhibition.

Hastings and coworkers [108] similarly reported one-pot combinations of chemo- and biocatalysis, involving Heck coupling and olefin metathesis reactions with enzymatic hydrolysis catalyzed by CaLB and pig liver esterase in water containing 2 wt.% of TPGS-750-M as the surfactant (Fig. 1.7B and C).

1.15 Neoteric solvents: ionic liquids and deep eutectic solvents

The last decade has witnessed the emergence of the use of neoteric solvents—ionic liquids (ILs) and Deep Eutectic Solvents (DESs), alone or together with water—as reaction media for conducting biocatalytic reactions. Following initial reports in 2000, biocatalysis in ILs, including protic ILs, has been widely studied and is the subject of numerous reviews [109–117]. The introduction of DESs, as novel reaction media for biocatalysis, was a decade later than that of ILs, but they have also been widely used as reaction media for biocatalysis with both whole cells and isolated enzymes [118–121]. In particular, natural deep eutectic solvents, prepared from combinations of relatively simple, primary metabolites, including sugars, amino acids and organic acids, such as citric, itaconic, malic, lactic and succinic acids, are potentially interesting green solvents for conducting biocatalytic processes. They play the role of reaction media in the intracellular synthesis of secondary metabolites, such as flavonoids and steroids, that are sparingly soluble in water [122] and have been called "Solvents for the 21st century" [123].

1.16 Concluding remarks

To be green and sustainable, a process must generate minimum waste, avoid the use of toxic and hazardous reagents and solvents, and efficiently utilize (preferably) renewable raw materials. Biocatalysis is green and sustainable, and biocatalysts perform admirably in water as solvent. However, this can lead to problems with hydrophobic substrates owing to their low water solubility. As discussed in this chapter, various approaches are possible for solving this problem. Moreover, the ongoing transition from a linear, fossil resource-based economy to a circular bio-based economy based on renewable raw materials will facilitate further widespread application of biocatalytic processes.

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CHAPTER

Nonconventional biocatalysis: from organic solvents to green solvents

2

Susana Nieto[1], Rocio Villa[1], Antonio Donaire[2] and Pedro Lozano[1]

[1]Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Chemistry, University of Murcia, Murcia, Spain [2]Department of Inorganic Chemistry, Faculty of Chemistry, University of Murcia, Murcia, Spain

2.1 Introduction

From the beginning of the past century, conventional chemical methodologies for organic synthesis have been around to produce high-value products and commodities. Along this time, those approaches were benefited from the research and acquired experience to polish the shortcomings and improve their efficiency. However, the incursion of enzymes, or biocatalysts, in the processes has meant a revolution in the industrial proceedings, surpassing the prevalence of conventional chemistry and adapting the approaches to the enzyme's operational requirements. Of course, there was a powerful reason to discard part of the knowledge coined in the experience on conventional chemistry and invest in the expensive biocatalysts and the implementation of new procedures. Thus, enzymes are catalysts with highest efficiency and selectivity affording more cost-effective transformations, without forgetting their sustainable characteristics.

Nature has always been a source of inspiration for chemists, and the design of living organisms is the best representation of efficient chemical processes where the maximum use is made of matter and energy, completely excluding undesired transformations. This is because the existence of enzymes and their excellent performance as the catalysts of the life. Their relevance becomes even more evident if we consider that the only information transmitted between generations in any living system, because of the genetic material replication, only encodes the synthesis of proteins (and certain RNAs with catalytic activities).

Enzymes are proteins with catalytic activity (so they are also referred as biocatalysts). Whether inside the cell or in extracellular surroundings, enzymes are designed to operate in a complex environment crowded with plenty of molecules and where numerous reactions occur at the same time, which involves a high efficiency despite low substrates concentration. Their performance is highly dependent on their tridimensional structure that configures a catalytic site to accommodate a substrate and drive it to is transition state, thus decreasing the activation energy and improving the transformation. Even more, the specific location of the amino acids (building blocks of proteins) defines the high selectivity of transformations, even affording the discrimination of optically active isomers.

Undoubtedly, these features (high efficiency and selectivity, and low energy requirements) are pursued in every industrial process to increase its yield. Even more, their protein nature demands soft reaction conditions (neutral pH and a temperature average of 37°C), which is the icing of the cake to reduce the cost-effectiveness of processes.

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Nevertheless, the use of enzymes to meet the needs of the society is not cutting edge. Without even knowing, enzymes contained in whole living organisms have been used since ancient times, for example, for food processing (fermentations of bread or alcoholic beverages) [1]. At the beginning of the 19th century, the isolation of the first enzymes (invertase and diastase) by Berthelot, Anselme Payen and Jean François Persoz and the confirmation of their catalytic activity regardless a living organism by Edward Buchner, were key facts for their later exploitation as free catalysts.

The incorporation of enzymes to chemical industry in the mid-19th century began the first wave of biocatalysis, boosted by their catalytic excellences, the simplification of methodologies and the reduction in solvents demand, thus increasing the profit margins. A pioneering and significative example of biocatalytic synthesis was the production of cortisone, where its synthetic pathway was dramatically simplified from 31 steps to 11, by using a living fungus able to selectively hydroxylate progesterone at position 11 (Fig. 2.1) [2]. Soon, whole organism fermentation was aimed to the synthesis of other valuable metabolites such as lactic acid, amino acids or vitamins [3].

Another fundamental reason that has propelled the use of biocatalysis is the change to a bio-based industry driven by the depletion of fossil feedstocks and the concerns about the

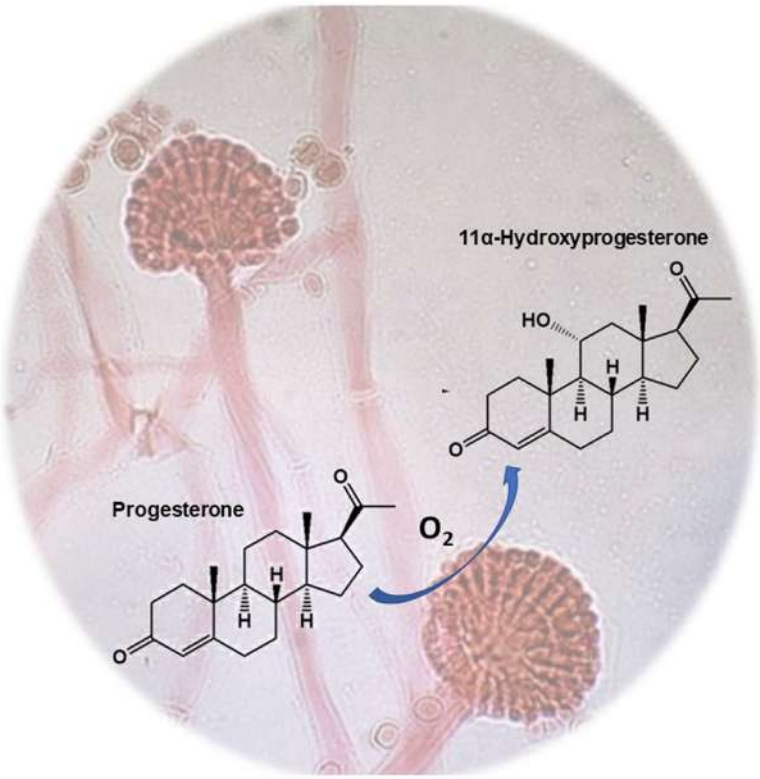


FIGURE 2.1

Pioneering biocatalytic reaction: the selective hydroxylation of progesterone at C_{11} position to produce a precursor of cortisone, catalyzed by the fungus *Rhizopus nigricans* (background) [2].

Table 2.1 Industrial applications of enzymes.

Enzymes	Applications	Field
Nitrile hydratase, transaminase, monoamine oxidase, lipase, penicillin acylase	Synthesis of APIs intermediates	Pharmacy
Trypsin, papain, pectinases, amylases, invertase, glucose isomerase	Conversion of starch to glucose, production of high fructose corn sirup, production of prebiotics, clarification of fruit juice	Food processing
Protease, lipase, amylase, cellulase	Removal of fats, oils, stains	Home cleaning-detergent
Lipase, esterases, cellulase xylanase	Production of fatty acid methyl esters and degradation of lignocellulosic material for bioethanol production	Biofuels
Lipase, cellulase xylanase	Lignin degradation for bleaching and improve fiber properties	Pulp and paper

Adapted from J. Chapman, A.E. Ismail, C.Z. Dinu. Industrial applications of enzymes: recent advances, techniques, and outlooks, Catalysts 8 (2018) 238–264.

environmental footprint [4]. From the sustainable and green standpoint, renewable raw materials are highly available, biodegradable and low toxic carbon and energy sources that permit the design of greener processes. Since organic molecules and polymers are intermediates in the flux of carbon in the biosphere, their CO_2 emissions are already contemplated in the carbon cycle which reduces the industrial footprint and greenhouse emissions. In this regard, the enzymes are the best catalysts for biomass transformations, as they have been specifically designed for their synthesis and degradation, affording the harnessing of even recalcitrant polymers. At this point, technologies based in biocatalytic transformations of biomass can be categorized into biomass processing (starch, oils, cellulose, proteins) or transformation of biomass derived molecules (sugars, organic acids, alcohols, etc.). For example, amyloglucosidases and cellulases have allowed the use of starch, lignocellulose or cellulose as carbohydrate source for further fermentation to produce ethanol and other commodities like lactic acid, 1,3-propanediol, aromatic amino acids and vitamins, dyes or antibiotics building blocks [5–7]. Fats and oils are important raw materials as well, used for the synthesis of biodiesel and glycerol release [8,9] or the synthesis of fatty acid esters for food, fine chemistry and cosmetic additives [10–13] through (trans)esterification reactions performed preferentially by lipases and some proteases. Table 2.1 compiles the major applications of the enzymes at different industrial sectors [14].

The huge interest for applied biocatalysis in the aforementioned sectors and others like wastewater, animal feed, personal care and cosmetics, etc. has boosted the demand of the enzyme market, with a value of USD 9.9 billion in 2019 and a growth rate forecast of 7.1% to 2027 in United States because of the developments in protein engineering is expanding their range of applications [15].

2.2 Biocatalysis strengths and weaknesses at industrial level

As mentioned earlier, the functionalities of biocatalysts are pursued by the industry to improve the yield of organic synthesis. However, enzymes are not intended to operate in those unnatural

conditions and their implementation has highlighted certain hiccups that need to be refined. This section points out several advantages and constraints of industrial biocatalysis.

The enzymes are designed to display a high affinity and specificity toward their specific substrate as result of the complex environment where they normally operate. Their excellent performance derives from the configuration of their catalytic site, specially designed to accommodate and transform their natural substrate, although it is also responsible for their limited scope. However, other more promiscuous biocatalysts have also been described, like lipases and oxidative enzymes, which low specificity, far from being a disadvantage, broadens their range of performance even toward nonnatural substrates [16–18].

Indeed, the most valuable property of enzymes is their excellent selectivity (i.e. enantioselectivity, regioselectivity and stereoselectivity) which involves not only the higher atom economy to avoid wastes generation, but also the specific transformation of one of two optically active isomers. This ability to distinguish between two enantiomers, despite their similar Gibbs free enthalpy (1–3 kJ/mol difference), has positioned the enzymes in a preferent place in asymmetric reactions for the synthesis of chiral intermediates of high value in fields like fine chemistry [19,20].

On the other hand, it concerns about the impact of industrial footprint in the environment have driven the revision of conventional chemical methodologies and the emergence of Green Chemistry [21], a philosophical stream that promotes the design of benign synthetic procedures and more respectful with the environment. At this respect, the natural origin and biodegradability of enzymes and their performance excellences under soft reaction conditions afford a conscious use of renewable feedstocks and allow the design of benign processes and products, fitting in with the vast majority of the 12 Principles of Green Chemistry [22,23]. The excellent efficiency of biocatalysts is reflected in an improved use of substrates (atom economy) avoiding unwanted reactions and the accumulation of byproducts to prevent wastes. Even more, their selectivity circumvents additional steps of protection/deprotection which reduces the number of steps with significant savings in solvents and energy inputs. For example, the incorporation of immobilized glucose isomerase to the industrial synthesis of high fructose corn sirup (HFCS) resulted more economically competitive than the chemical approach, because the better yield and product quality and the reduction of other undesired sugars [24]. Codexis and Merck have engineered a transaminase from *Arhrobacter sp*. to achieve an improved yield and enantioselectivity in the synthesis of sitagliptin (a drug for diabetes treatment) in a simpler synthesis strategy that avoids the use of energetically costly metal-catalyzed high-pressure hydrogenation processes and subsequent metal removal steps [25].

The enzymes afford new strategies of accessibility to raw materials, reducing the energy and material requirements of processes and decreasing the emissions and wastes. In biodiesel synthesis the milder reaction conditions of enzymes performance has afforded considerable energy savings [26]. Also, the enzymatic degumming of vegetables has improved the yield of oils production, reducing the feedstock requirement and the CO_2 emissions (44 tons of equivalent CO_2 per 1000 tons of oil produced) [27].

However, the natural conditions of enzyme performance (e.g., temperature, pH, substrate/enzyme ratio, aqueous solvent, etc.) differ from those of industrial processes, highlighting several drawbacks that have to be polished. The major issues attend to their high cost and reduced availability and stability, limited range of applications or the need of expensive cofactors. Besides, most of the industrial processes are performed in organic media, since aqueous environment is not suitable for dissolving hydrophobic substrates and promotes unwanted side-reactions (like hydrolysis) that decrease the final productivity. Although biocatalysts may operate in organic solvent media

[28], most of them show reduced activity and instability [19]. In this regard, the successful marriage between biocatalysis and industry requires a series of adaptations concerning the biocatalysts improvement and solvent engineering. In the next sections, different resources for biocatalysis optimization and industrial implementation are being discussed.

2.3 Whole cell biocatalysis

At the beginning, the high cost of production and isolation of enzymes and their instability were a brake for the expansion of free enzymes in the industry. Nonetheless, the use of whole cells permitted to overcome those drawbacks, as the living systems not only preserve the enzymes stability and activity because of their cell wall, but also provide the enzyme regeneration and more importantly, the recycling of the expensive cofactors. In this manner, microorganisms are used as economic biofactories for the synthesis of drugs or fuels. One example of a successful and still prevalent approach using microorganisms is the production of citric acid by *Aspergillus niger*, that in the mid-20th century, came to reach an annual production of 26,000,000 pounds in the United States [29]. Also the synthesis of acrylamide by *Rhodococcus rhodochrous*, whose efficiency (7 kg acrylamide per gram of cells) has made this process one of the most settled at industrial scale [30]. In this case, the existence of the whole cell is indispensable to preserve the quaternary structure of the enzyme nitrile hydratase, which otherwise undergoes the dissociation of its subunits.

These reasons explain the faster implementation of whole cell biocatalysis in industry over the free enzymes, and the interest in search and catalog new microorganisms to expand the range of biocatalytic transformations. In a step further, the development of molecular biology and protein engineering tools has overcome the limits of Nature providing a plethora of new microorganisms to meet the industry demands. Technologies like mutagenesis or recombinant DNA (see section 2.5) have allowed the genetic modification of microorganisms either expressing foreign or improved biocatalysts to perform the transformation of (un)natural substrates. In this manner, Colombié et al. have overcome the low production of lactic acid of bacteria sensible to low pH, by inserting the lactate dehydrogenase gene in a more tolerant yeast strain, leading to a production of 55 g/L lactic acid at pH 3.6 [31]. An upper stage involves the engineering of the whole metabolic pathways at will to push certain routes over the control imposed by the cell homeostasis or even creating new synthetic ones. A representative example of the last case is the synthesis of 1,4 butanediol, an intermediate in the synthesis of plastics and fibers, by a metabolically engineered strain of *Escherichia coli* that has resulted very efficient and more sustainable and competitive than the petrochemical approach, reducing the emissions of CO_2 by 83% and the energy requirements by 67% [32].

2.4 Isolation of new biocatalysts

In extreme habitats, microorganisms have established a diversity of molecular strategies to survive in extreme conditions. Biodiversity prospection of such microorganisms in extreme pH, temperature, high salinity or even organic media has allowed the identification of more robust enzymes or with other valuable attributes for their industrial implementation [33]. Biocatalysts isolated by these organisms, also named extremozymes, possess extraordinary properties of salt allowance,

thermostability, and cold adaptivity, resulting very resistant to extreme conditions owing to their great solidity, and they offer new opportunities for biocatalysis and biotransformations, as well as for the development of the economy and new line of research, through their application. Enzymes from thermophilic microorganisms are capable of accepting proteolysis and extreme situations like the presence of denaturing agents and organic solvents and high salinity. The use of these enzymes includes the possibility to reduce the risk of contamination, keeping a low adhesiveness, and greater solubility of substrates [34].

Organic solvent-tolerant bacteria and halophilic and alkaliphilic organisms, have provided a profusion of enzymes suitable for their use in organic solvents or high salt concentrations. For example, *Pseudomonas aeruginosa* has been a source of numerous solvent-tolerant enzymes like proteases and lipases [35,36]. Even more, the studies performed with those enzymes are very useful to identify the structural basis of their increased stability and performance in such harsh conditions. This knowledge allows deepening in the structure-function relationships and is also a powerful tool for the conscious modification of other biocatalysts. In the food industry, cold-adapted enzymes are very important owing to their high activity and their low structural stability In this way, cold-active enzymes have great potential applications for biotransformations, including volatile substrates, cosmetic industry, and pharmaceutical industry, such as for production of enantiomer peptides, lipids, and sugars [34].

2.5 Recombinant technologies and enzyme evolution

Before the implementation of the revolutionary tools of molecular biology, industry had to deal with low availability, high cost and solvent sensitivity of biocatalysts. However, the increased understanding of the relation DNA-protein and the disposal of technological advances to modify the coded information in this molecule have allowed the use of modified microorganisms as biocatalysts factories (instead of animal or vegetal traditional sources), increasing their availability and reducing the cost of production and purification.

Insulin was the first recombinant protein to be produced and commercialized [37], but soon this technology was extended to the synthesis of enzymes aimed to the production of antibiotics (e.g., penicillin amydase was expressed for the synthesis of amoxicillin and ampicillin [38]), food processing (by 2006, 80% of rennet market was replaced by recombinant chymosin in United States [39], pectinases are used to clarify juices [40]), or detergents (i.e. lipolase and subtilisin [3]).

Once solved the problem of enzyme availability, methodologies like mutagenesis and recombination enabled their tailoring and improvement toward the industrial demands and led to the second wave of biocatalysis. The third wave came with the development of protein direct evolution because of the upgrading of molecular biology and protein engineering tools [i.e. site-directed mutagenesis, saturation site mutagenesis, recombinant DNA, shuffling DNA, error-prone PCR (epPCR)...]. Direct evolution aims to force the natural evolution of enzymes for their adaptation to the industrial operation conditions, concerning a better stability (like a carbonic anhydrase stable at 107°C and pH $>$ 10 [41]), solvent or substrate concentration tolerance, improved activity, broaden substrate specificity [42], modified enantioselectivity [43], or to introduce novel functionalities [44,45]. Fig. 2.2 shows the steps followed in any strategy of protein/enzyme evolution.

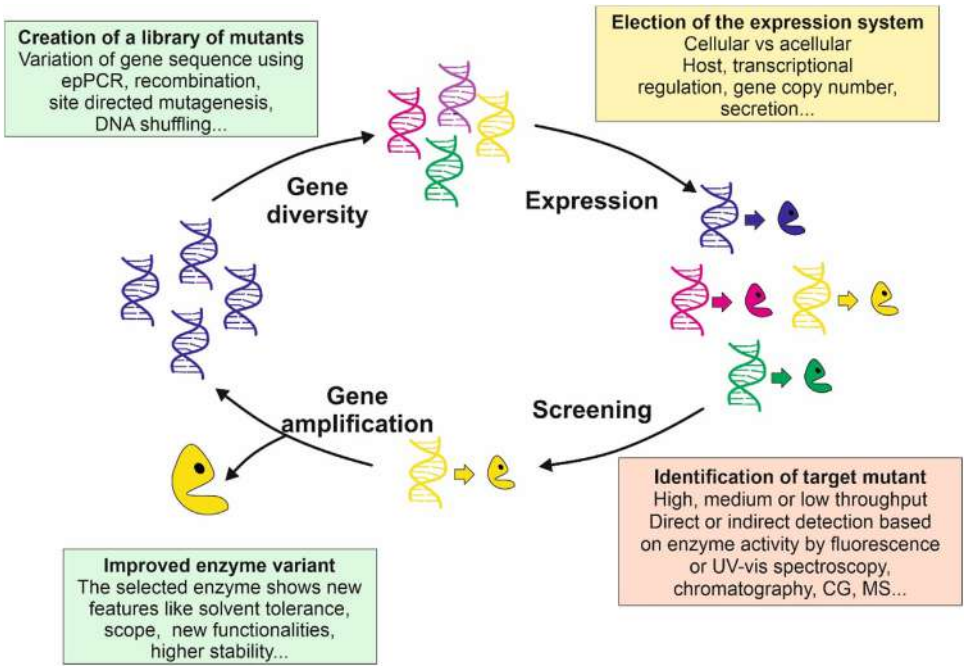


FIGURE 2.2

Scheme of the different stages involved in the forced evolution of enzymes to tailor their properties [46].

Frances Arnold (Nobel Prize in Chemistry 2018) is considered a pioneer of direct evolution and because the impact of her work in this field. In 1993 Arnold et al. [47] reported the first strategy of random mutagenesis to improve the activity of the protease subtilisin in organic medium. The application of several rounds of epPCR improved 256-fold the hydrolytic activity of subtilisin E in dimethylformamide, almost reaching the activity of the wild enzyme in aqueous media. This work laid the groundwork for the improvement of other catalytic activities like *p*-nitrobenzyl esterase, used for the synthesis of loracarbef (a cephalosporin-derived antibiotic), which activity increased almost 60-fold after four rounds of epPCR and the random pairwise gene recombination of two positive mutants [48].

However, the strategy of random mutagenesis results in large libraries of mutants giving rise to the need of developing new methodologies for high-throughput screening. Other more conscious strategies, like rational design and semirational design, deal with more easy-handling libraries. Both involve a previous knowledge of the structure-function of enzymes to introduce specific mutations, although the second combines this information with the strategy of random evolution (Fig. 2.3).

The Reetz's group could be considered as the major representative in the development of rational design as strategy to improve the performance of biocatalysts by the implementation of both the "combinatorial active site saturation test" [42] and the B-FIT approaches [49]. Both genetic engineering approaches are based in the selection of different sites (A, B, C...) at the amino acid

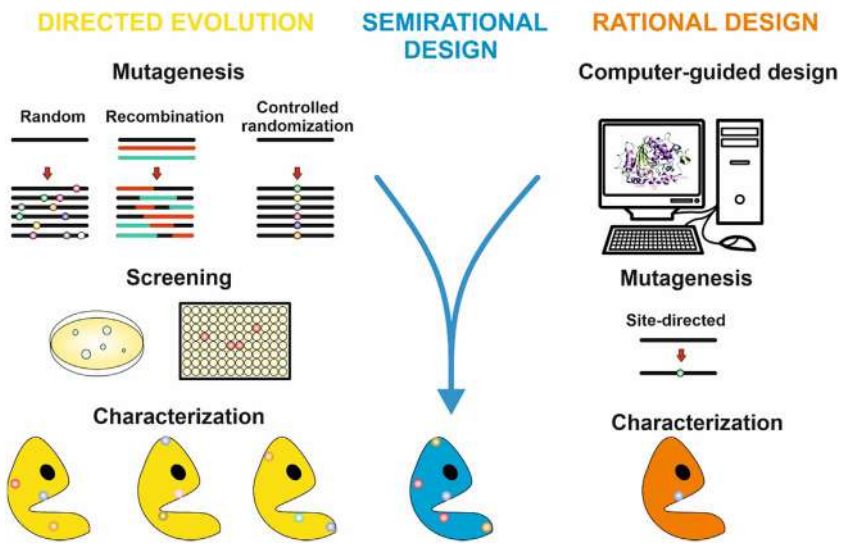


FIGURE 2.3

Comparison of the strategies of protein evolution [33].

backbone of the enzyme, and then to perform saturation mutagenesis, each one containing one or more residues. The selection of biocatalyst variations is not fortuitous as is guided by X-ray crystallographic information, and the modifications proceed sequentially. So, the efforts are first focused on a specific site and the best mutant is then used as starting template for the next one. This approach of iterative saturation mutation significantly reduces the size of mutant libraries and it has proven to be a successful tool to expand the range of enzyme substrates, and the modification of enantioselectivity and improved thermostability (by focusing on residues with high B-factors, that is, with high flexible positions).

In this context, Vazquez-Figueroa et al. [50,51] used semirational design to engineer thermostable glucose 1-dehydrogenase variants that were combined to yield a mutant that showed a >2500-fold resistance in organic cosolvents (acetone, acetonitrile and 1,4-dioxane). Koudelakova et al. [52,53] also used this approach to improve the stability of enzymes with buried active site in organic solvents, by pointing out to the residues lining the gorge of the active site. Their results unveiled that the higher the packing of the residues of the active site tunnel, the lower the destabilization caused by the organic molecules that have entered. However, Yang et al. [54] have provided the smartest illustration of semirational design application. They have enabled the reversion of a nucleotide salvage route for the synthesis of the anti-HIV drug islatravir, because of the evolution of five out of the nine participating enzymes. This manner, they have developed a simplified biocatalytic pathway for the efficient and enantioselective synthesis of islatravir from 2-ethynylglycerol.

In the strategies of a powerful (semi)rational design, bioinformatic tools for macromolecular modeling and docking are needed to target the residues. The key residues are preferentially located at the surface, hydrophobic active site, active site gorge, allosteric sites or interfacial side of

subunits, or may be individual residues involved in covalent interactions (disulfide bonds) or electrostatic interactions (surface charges or isolated charges) [33]. As can be assumed, the major bottleneck of these methodologies so far is the low availability of protein structures. In this regard, the recently reported computational program for protein structure prediction, AlphaFold 2, has revolutionized the field of biochemistry because of its accurate results [55], uncovering more than 350,000 structures (by august 2021, conventional X-ray diffraction and NMR methodologies had only provided 180,953 structures to Protein Data Bank, [56]). The major prospects of this program for unravel the protein structure are the better comprehension of protein folding and discover the functionality of new enzymes that have been identified by DNA sequencing methodologies. No doubt, this groundbreaking methodology is becoming a powerful tool for (semi)rational design and to provide more accurate predictions of the effect of mutations.

The newest goal of direct evolution of enzymes is to expand the field of biocatalysis through the design of new biocatalysts, the improvement of the enzyme promiscuity or introduction of new functionalities. In this sense, the methodologies of direct evolution are combined with studies of metagenomic comparison (a methodology that permits the identification of new enzymes based in the similarity of their sequence with others already known) or the introduction of noncanonical amino acids or metal ions (to create new metalloenzymes or dual catalysts) [57].

2.6 Immobilization of biocatalysts

Notwithstanding all the advantages of biocatalysts, industrial application of enzymes is often hampered by a lack of long-term operational stability and difficult recovery and re-use of the enzyme. These drawbacks can generally be overcome by immobilization of the enzyme [58]. Immobilization methodologies arose as powerful tools to ease the products separation and enable the enzyme's recovery and reusability, with the subsequent impact in cost reductions. But soon their application revealed other unexpected improvements for biocatalysis like, increased activity, stability and solvent tolerance, which together with the suitability of immobilized preparations to batch or flow continuous systems, became the final push for the use of immobilized enzymes [59]. Enzyme immobilization provides improved enzyme performance and repeated re-use, being reflected in higher catalyst productivities (kg product per kg enzyme) which, in turn, determine the enzyme costs per kg product [59]. As historic examples, it could be considered the synthesis of HFCS by immobilizd glucose isomerase-catalyzed the isomerization of glucose [60], which has an annual productivity of $>$10,000 kg HFCS per kg of catalyst [61], or the synthesis of 6-aminopenicillanic acid (6-APA), an intermediate for semisynthetic antibiotics, catalyzed by immobilized penicillin amidase [62]. The latter is a representative example of the improvement achieved by switching from chemical to a biocatalytic approach despite the cost of enzymes, as the new conditions (aqueous medium and 37°C) significantly reduce the investment in solvents and energy [3].

The higher enzymés stability provided by immobilization is due to an increased rigidity in the protein structure, that also improves their solvent tolerance, particularly in organic solvents, preventing their deactivation by denaturation, unfolding or aggregation [3]. For example, ketoreductases and transaminases have been reported to show improved performances in organic media [63,64]. Although a partial loss of activity has been detected, because the increased rigidity due to

the immobilization and the reduced hydrating shield in organic solvents, this constraint is counterbalanced by the increased stability and reusability of enzymes.

To date, there is available a wide variety of immobilization methodologies. Their selection must attend to the biocatalyst's features, the impact of the attachment to the biocatalytic activity, the reaction media and operational conditions and even, the physicochemical properties of the support (if present). Basically, methods of enzyme immobilization can be divided into three categories, binding to a support (carrier), entrapment (encapsulation) and cross-linking (Fig. 2.4). The suitability of each immobilization procedure for the immobilization of free enzymes or whole cell organisms depends on the kind of attachment (weak or strong forces), or the interaction of the support with the enzyme (heterogeneous vs homogeneous biocatalysts).

The entrapment or encapsulation (Fig. 2.4A) is a mechanical immobilization that retains the biocatalyst in an agarose gel or a polyacrylamide matrix. This kind of immobilization preserves the enzyme conformation, but may present different shortcomings like limited mass diffusion, particularly in the inner zones of the matrix [66], and risk of enzyme leaching [67], especially in aqueous media. Thus, this immobilization is more convenient for whole cell organisms, while free enzymes

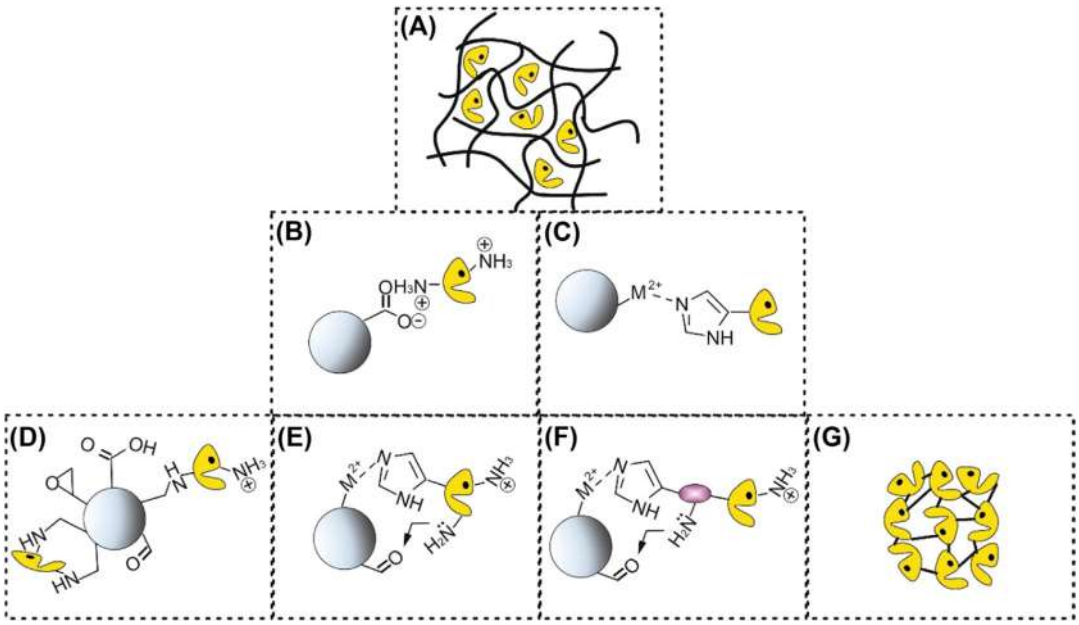


FIGURE 2.4

Different methodologies for whole cell and free enzyme immobilization, with or without a support. (A) Mechanical entrapment of biocatalysts within a polymer. (B) Adsorption through ionic interactions (e.g., with a Lys residue). (C) Adsorption through affinity tags with a His residue permits to control the enzyme orientation. (D) Different ligands for covalent attachment, including a case of loss of enzyme conformation by multipoint attach. (E) Covalent attachment through affinity tags or (F) a fusion protein. (G) Direct covalent cross-linking between biocatalysts [65].

are preferentially linked through physicochemical attractions to a support that acts as a carrier. Nevertheless, recent progresses are made in this sense to improve the entrapment of enzymes, like the use of new microgels or μ-Gelzymes. These colloidal polymers of reduced size (nano or micrometers) permit important enzyme loadings [68]. Besides, their properties, like porosity, size, swelling degree or hydrophilicity/hydrophobicity, can be tuned according to their composition. Even in some cases, the structure of these polymers is switchable attending to external stimuli like temperature, pH, ionic strength or kind of solvent, which can be used to develop the enzyme immobilization strategy [69]. In this manner, the ability of a p-NIPAM microgel to change its morphology through a sequential transition from aqueous buffer to isopropanol and hexane, permitted to Gawlitza et al. [70] the creation of hydrophilic cages for the immobilization of CALB lipase, with a 2.5% enzyme loading efficiency.

Adsorption of the enzyme to the solid support (Fig. 2.4B) can be achieved by hydrophobic/hydrophilic or ionic interactions. Novozym 435 is one of the major representatives of a commercially available adsorbed enzyme, where the lipase B of *Candida antarctica* is retained in an acrylic resin through hydrophobic interactions [65]. Because ofthis derivative, different processes have been scaled up to industry, like the synthesis of polyol acrylates in neat organic solvents (e.g., acetone, *t*-butanol or methyl *t*-butyl ether) developed by BASF [71].

Notwithstanding, adsorption has two important drawbacks, the enzyme leaching due to the weak interactions (only relevant in aqueous media, not in organic solvents) and the poor control of enzyme immobilization. The latter may lead to restricted accessibility to the active site, thus decreasing the enzyme activity. This can be due to physical restraints (difficult localization of biocatalysts, i.e., inside pores) or a wrong arrangement of the enzymes, which orient their active center toward the support. To address these issues, the use of different tags (e.g., his-tag, streptavidin, lectins, etc.) may help to control the enzyme orientation during immobilization and improve the strength of attachment to the support (Fig. 2.4C). Different solvents, like ionic liquids (ILs), can also be used to functionalize the support surface. ILs are neoteric solvents with tunable properties that can affect the biocatalyst performance, thus enhancing their activity or modulating their enantioselectivity (see section 2.9). In addition, ILs have been proven to improve biocatalysts stability and exert a protective effect against deactivation by high temperatures, solvents (i.e. hydrophilic solvents or supercritical CO_2) [72] or substrate poisoning [9]. The polymeric surfaces functionalized with ILs are known as supported IL-like phases (SILLPs), and result very suitable smart supports for enzyme adsorption because of the improvement in biocatalysts performance (see Fig. 2.5). For example, García-Verdugo et al. [73] have reported the co-immobilization of an alcohol dehydrogenase and its cofactor, revealing high efficiency in the enantioselective reduction of ketones and a high stability for long-term storage, reusability in operational cycles or use in flow systems.

However, to reduce the enzyme leaching, covalent attachment (Fig. 2.4D–F) offers the strongest immobilization. Although it also shares the problem of uncontrolled orientation and adds the risk of enzyme distortion and loss of activity when the biocatalyst suffers a multipoint attachment. Here again, the use of different tags may help to overcome those shortcomings, for example, by initially adsorbing the enzyme with the right orientation before the covalent attachment or by using a small fusion protein as anchorage point [75]. However, it is important to point out that unlike adsorption, the resin is not reusable after enzyme deactivation, which may affect the cost-effectiveness of processes at high scale [76].

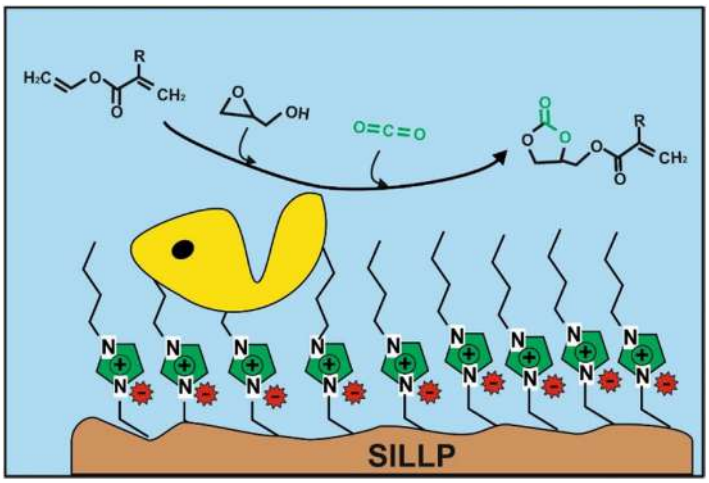


FIGURE 2.5

Chemoenzymatic synthesis of glycerol carbonate (meth)acrylates. The biocatalysts, lipase B from *Candida antarctica*, is immobilized onto a SILLP support. The product is the result of an enzymatic transesterification and a CO_2 cyclo-addition performed by the SILLP [74].

Another advantage of the immobilization, whatever the strategy used, is the possibility to immobilize different enzymes in close proximity on the same support to perform cascade reactions [77]. This approach affords the multistep transformation of substrates without the need for prior purification steps, improves the efficiency, as avoids the accumulation of intermediates and their inhibitory effects, and increases the selectivity by reducing unwanted side reactions. Also, the volume of the reaction system is reduced, and increases the space-time yield leading to more cost-effective conditions. Even more, the combination of enzymes from different hosts affords the design of new synthetic pathways of unnatural products. Though, the biggest challenges of this multienzyme immobilization are the control of the degree of individual immobilization and stability as well as the control of their spatial location to achieve the "substrate channeling." This is a difficult issue, taking into account that key aspects as the optimum distance between biocatalysts in multicascade transformations, are yet to be clarified. While geometrical studies suggest that the distance should range between 0.1−1 nm [78], a study with precise glucose oxidase (GOx)/horseradish peroxidase (HRP) co-immobilization have evidenced an improved efficiency with a larger distance, as much as 10 nm [79]. This can be the explanation of the good performance of nonspecific co-immobilization strategies, which together with their higher feasibility, have led to their prevalence over site-specific immobilization [80].

A key factor to consider when carrying out an immobilization is the selection of the support. The size, surface and geometry of the support affects the enzyme loading and may be determinant for the implementation of batch or flow processes. Other considerations like its pore-diameter or physico-chemical characteristics (hydrophobicity/hydrophilicity) also may have an impact in the enzyme performance and efficiency, introducing sterical constraints or affecting the diffusion or

partitioning of substrates and products. An example of the latter is the case of penicillin amidase, which preferentially carries out synthesis reactions when immobilized on hydrophilic resins instead of hydrolysis reactions [81]. Other properties of the support can be relevant for other stages after the synthesis process, such as the recovery of the biocatalyst. For example, the use of magnetic supports has proven to be very convenient at industrial scale for the recovery of biocatalysts.

In addition, noninert resins may also contribute to the catalytic activity. Recently, Lozano et al. have developed a dual chemo-enzymatic catalyst (lipase immobilized onto a SILLP) for the synthesis of glycerol carbonate (meth)acrylates, where the lipase catalyzes a transesterification, and the covalently attached ILs catalyzes the capture and cycloaddition of carbon dioxide into the epoxide moiety (Fig. 2.5) [74]. It has also been reported that the conductive properties of carbon-based nanoparticles improve the electron transference in the catalytic performance of the immobilized oxide-reductases, leading to the design of different biosensor devices, for example, for the determination of glucose [82].

However, the need of a support increases the cost of biocatalysts which has led to the emergence of new approaches based on direct crosslinking between biocatalysts (Fig. 2.4G). This methodology, known as CLEAs (cross-linked enzyme aggregates), improves the enzyme loading and has revealed high productivity and stability [83]. Equally, different biocatalysts can be combined in the same aggregates (Combi-CLEAs) to perform synthetic cascades, acting as a confined space where the intermediates are funneled between the active sites to improve the speed and effectiveness of transformations. In this approach, there is no need for control of spatial location of biocatalysts, as the high density of enzymes improve the chance for an intermediate to be transformed by the downstream enzyme with high efficiency [78]. This issue was demonstrated by Nguyen et al. [84] using a CLEA based in GOx and HRP. They showed that even in the presence of catalase in the medium, the enzyme responsible for the reduction of the hydrogen peroxide produced in glucose oxidation by GOx, was the co-immobilized HRP (Fig. 2.6). No doubt, this excellent performance of CLEAs is very convenient for the regeneration of cofactors, thus optimizing the performance of enzymes like oxidoreductases, and improving the cost-effectiveness of the processes where they participate [85]. But this strategy is not only aimed to enzymes, since it also allows the co-immobilization of chemical catalysts, giving rise to hybrid CLEAs. In this way, the combination of Pd and CALB enabled to carry out the enzymatic resolution of secondary alcohols in one pot [86].

2.7 Volume-confined biocatalysis

In living organisms, the confinement of biocatalytic reactions in separated organelles permits to increase the efficiency of metabolic pathways, since they provide the reaction physico-chemical conditions (i.e., the low pH of lysosomes or gradient of protons in mitochondria) increase the concentration of substrates and avoids any detrimental interference. The volume-confinement of biocatalysts mimic the nature to pursue the same goals. In this way, advanced applications of enzymatic cascade reactions with immobilized enzymes include enzymatic fuel cells and enzymatic nanoreactors, both for in vitro and possible in vivo applications have been developed [77].

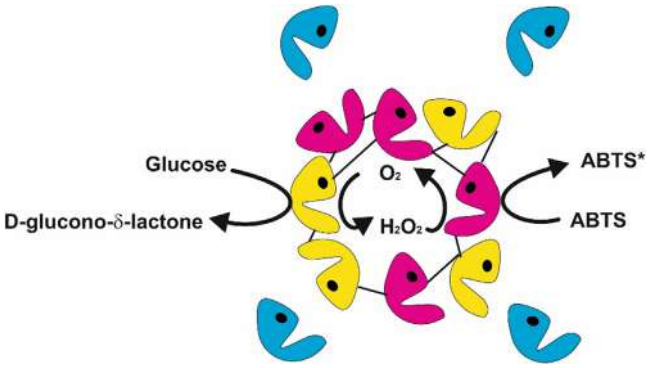


FIGURE 2.6

Scheme of the efficient sequential transformation by a Combi-CLEA based on glucose oxidase (*yellow*) and horseradish peroxidase (*purple*). The proximity of HRP improve its efficient H_2O_2 reduction despite the presence of free catalase (*blue*) in the medium [84].

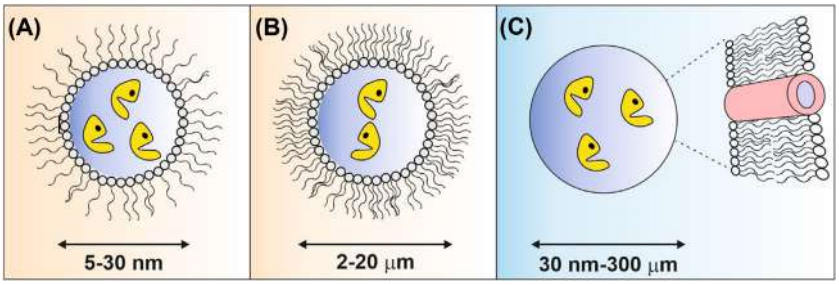


FIGURE 2.7

Different models of volume-confined biocatalysts. (A) Reverse micelles, where the polar head of surfactant molecules create an aqueous environment for biocatalysts within a hydrophobic solvent. (B) Aqueous microdroplets of amphiphilic molecules have a reduced size. Cascade reactions can be performed through the coexistence of droplets containing different biocatalysts. (C) Lipidic vesicles or liposomes. The structure mimics the biological bilayers. The permeability can be tailored at will through changes in their composition or by introducing protein pores or channels [77].

There are different models for enzyme confinement (Fig. 2.7). The most common ones use different molecules with surfactant properties, like reverse micelles (Fig. 2.7A), water microdroplets (Fig. 2.7B), or lipidic vesicles, also known as liposomes (Fig. 2.7C). However, new models using polymers, proteins or fiber glass have also been developed. These structures permit the existence of an aqueous environment for biocatalysts in a water immiscible solvent. In the particular case of reverse micelles, the lower availability of water inside these structures has been reported to have a beneficial effect in enzymatic activity. For example, an increased activity of HRP [87] and

chymotrypsin [88] give rise to different arguments related to the altered properties of confined water and its interaction with the biocatalysts.

Vesicles are attractive systems to study enzymatic reactions because of the large variability of their size, as well as the possibility to design multivesicular-based systems. Moreover, because vesicles do not spontaneously fuse or exchange their aqueous interiors, it is possible to create multivesicular systems in which large vesicles contain smaller vesicles in their interior. In principle, the chemical composition of any membrane in the system can also be changed by design. This large variability allows investigations of cascade reactions with enzymes that are located in different internal vesicles mimicking eukaryotic cells and their enzyme-specific organelles [77].

The substrates can be coimmobilized with the enzymes during the synthesis of compartments, be delivered after the merging of compartments, or simply diffuse across the boundary or a protein pore/channel. Precisely, the high permeability of vesicles and the different tools to grant the entry of substrates is one of the main reasons of their preferent use. For example, Yoshimoto et al. have designed a nanoreactor for glucose oxidation based on a liposome [89]. The presence of protein channel OmpF afford glucose transport. Inside the vesicle, GOx oxides glucose to glucono-δ-lactone which subsequently undergoes a nonenzymatic hydrolysis meanwhile the presence of a coimmobilized catalase prevents GOx inactivation by dissipating the byproduct hydrogen peroxide.

A further step on volume-confined biocatalysis concerns the use of self-assembled protein cages, such are the apo-ferritin or the capsids, because they provide nanosized internal spaces which are capable of encapsulating metal ions/complexes, enzymes/proteins have great potential for use as catalytic nanoreactors in efforts to mimic confined cellular environments for synthetic applications. Because of their restricted size, the protein cages are excellent candidates for use as vessels to exert control over reaction kinetics and product selectivity. Virus capsids with larger internal spaces can encapsulate multiple enzymes and can mimic natural enzymatic reactions. The apo-ferritin cage is known to accommodate various metal ions/complexes and suitable for organic transformation reactions in an aqueous medium [90].

2.8 Solvent engineering: organic media

The nature of enzymes involves an important condition for their use, an aqueous medium, since water molecules contribute to shape and maintain their structure and activity. But this medium is not able to solubilize the reaction components in most organic processes and difficult the purification of the products, increasing the cost of production. This handicap was also an important constraint for the application of enzymes in industrial organic chemistry till Zaks and Klibanov demonstrated in 1984 the suitability of organic media to support the enzymatic catalysis [28]. This fact revolutionized the preconceived ideas about the enzyme performance and set the starting point of biocatalysis in nonaqueous media. At first, the idea of an active biocatalysts in organic media might result nonsensical as it contradicts their hydrophilic nature. It would be expected that the three-dimensional structure of the biocatalyst should be lost provoked by the different miscibility of the residues. However, as long as nonaqueous organic solvents are able to retain a critical amount of water housed within the enzyme and on its surface (approximately 0.0003 g H_2O/g of protein), the catalytic activity is not disturbed [91]. However, if the water is drastically and

completely removed from the enzyme, the distortion of the native conformation leads to the enzyme deactivation.

It is important to consider and study the effects of organic solvents on the properties and conformation of enzymes, since biocatalysts can be stabilized or destabilized as a consequence of a change in the secondary or tertiary structure (e.g., alpha helix or beta helix structure) [92]. High concentrations of nonaqueous solvents destabilize the enzymes, while low concentrations promote their stabilization. Conformational studies of different enzymes in organic solvents have shown that solvents capable of stabilizing the structure of the enzyme exclude contact with the surface of the protein. In a more detailed analysis of the preferential interactions between the enzymes and the stabilizing solvents, they show that this stabilization is due to a microphase separation of the solvent on the surface of the protein, preserving water molecules on its surface. In this sense, the layer of water molecules is found in an orderly way, keeping the enzyme active and being able to demonstrate its activity and stability in nonaqueous media [93]. Even more the low dielectric constant of the organic solvent leads to strong electrostatic interactions that maintain the native conformation which becomes even more rigid, explaining the enzyme stability but also lower rates of activity because the reduced flexibility of the active center [92]. However, the configuration of the active center of the enzymes can be modified prior the transfer to the organic solvent to prevent the decay of activity. The molecular memory of enzymes co-lyophilized with their substrates analogs or competitive inhibitors retain the catalytic optimal configuration in anhydrous media [94]. This approach has allowed increasing the activity of subtilisin more than 100 times [95] or tune the enantioselectivity of chymotrypsin [96]. It is important to highlight that this molecular printing of the active site is not observed in aqueous media, as water acts as a protein lubricant that return the original flexibility.

The combination of organic media (as nonaqueous media) and biocatalysts have resulted in an excellent synergy, extending the biocatalytic performance toward new substrates and revealing new synthetic activities [16]. Table 2.2 lists a selection of the most commonly used enzymes grouped by family and the reactions they usually perform in organic solvents.

Organic media improve the solubilization of hydrophobic reactants and prevent hydrolytic reactions as well as other water dependent secondary reactions (like racemization or decomposition), improving the catalytic efficiency of the enzyme [104]. As the enzymes are not soluble in these media due to their hydrophilic nature, it is easy to recover them through filtration after their catalytic action [105]. It is also important to highlight that microorganisms are not able to survive in

Table 2.2 Biocatalysts and reactions catalyzed in organic solvents.

Class	Enzyme	E.C. number	Reaction	References
Oxido-reductases	Yeast ADH	1.1.1.1	Ethanol oxidation	[97]
	Alcohol oxidase	1.1.3.13	Ethanol oxidation	[98]
	HR peroxidase	1.11.1	Sulfoxidation	[99]
Hydrolases	PP lipase	3.1.1.3	Transesterification	[100]
	α-chymotrypsin	3.4.21.1	Transesterification	[101]
	Urease	3.5.1.5	Hydrolysis of urea	[102]
Lyases	*(R)*-oxynitrilase	4.1.2.10	HCN addition to C = O	[103]

organic media. This avoids the high problem of contamination associated to aqueous reactions which leads to important economic losses. In addition, pH is not a critical parameter in organic solvents since the enzymes retain the conformation at the pH of their aqueous solution. Moreover, lyophilized enzymes also preserve the native structure they had in the solution. This has come to light that enzymes are able to keep the memory of their conformation what is called the pH memory or molecular memory, and is based on the preservation of the ionization state of its ionogenic groups [94].

Depending on the miscibility of an organic solvent in water and the concentration of the solvent in the aqueous medium, there are different types of organic solvent systems: (1) water miscible organic solvent system + water (organic cosolvents system), (2) water immiscible organic solvent + water system (biphasic system), (3) nearly anhydrous organic solvent [106,107].

Different studies have come to light the better performance of biocatalysts in water immiscible organic solvents against the hydrophilic ones. For example, Klibanov et al. developed in 1977 a system consisting in a porous glass impregnated with an aqueous buffer solution of chymotrypsin in chloroform that they called as "biphasic water-water-immiscible organic solvent system" for the synthesis of *N*-acetyl-*L*-tryptophan. This system proved to be much more valid than hydrophilic solvents to shift the reaction equilibria toward esterification reactions with generation of water, obtaining a 100% yield, against the 0.01%, in water [108]. This is due to the water partitioning around the biocatalyst surface and the bulk phase of the organic solvent. Meanwhile, solvents such us methanol or acetone remove the essential layer of water causing enzymatic deactivation due to conformational loss. Fig. 2.8 represents the influence of the nature of the solvent on the enzyme stability and its biocatalytic performance.

There is a special interest in correlating the activity of enzymes and organic solvents. Therefore special attention is paid to organic solvent parameters such as dielectric constant, dipole moment, hydrogen bonds, partition coefficient, etc. Others attend to their potential denaturing power like empirical polarity of the solvent [110], hydrophobicity [111], polarity index [112], etc. The study

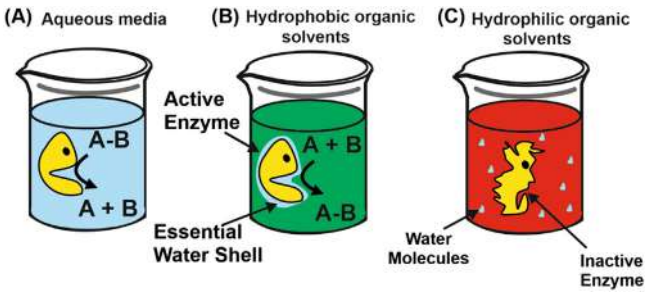


FIGURE 2.8

The role of water in enzymatic transformations and in the structure of proteins in different reaction media. (A) In aqueous media enzymes are fully active, but their reactions are restricted to water participation; (B) In hydrophobic organic solvents the enzymes retain the essential water shell to be active, and the low water content is insufficient to alter the equilibria of the reaction; (C) Hydrophilic organic solvents tend to strip the water shell causing enzyme denaturation [109].

of these parameters can be very useful to describe the behavior and activity of enzymes in water and organic solvents. Thus, for example, despite the enhanced solubility of hydrophobic substrates in organic solvents, their biocatalytic transformation may be reduced by the solvent polarity and log P (partition coefficient) value, as these factors can affect the availability of substrates in the surroundings of the biocatalysts active site.

Furthermore, enzymes in nonaqueous organic solvents are able to perform unusual reactions showing other activities different to the canonical one. For example, lipases are promiscuous hydrolases that in organic solvents catalyze reactions of transesterification esterification, aminolysis, oximolysis, thiotransesterifications, etc. [16]. In this regard, the transesterification of triglycerides to obtain esters is a process widely used in the edible oil industry, as well as for the synthesis of biofuels (e.g., fatty acid methyl esters).

Organic molecules form the solvent can penetrate into the active center and provoke structural modifications to alter the selectivity of enzymes, including the substrate specificity, enantioselectivity, prochiral selectivity, regioselectivity and chemoselectivity [105,113], bringing to light that the solvent engineering is another important tool to tailor a reaction. For example, chymotrypsin, a protease with specificity toward aromatic amino acids in aqueous media, shows preference for serine residues in organic media [114].

The chance of tailoring the enantioselectivity of enzymes in organic media is of particular relevancy in some sector like the pharmaceutical, where there is a special interest in certain stereoisomer over the racemic mixture, because their greater activity and fewer side effects, or the food sector, interested in the organoleptic properties of a specific isomer. Klibanov et al. described the stereoselective esterification of alcohols in organic solvents. For example, they achieved the specific esterification of *R*-2-halopropionic acid with primary alcohols (e.g., *tert*-butanol, *n*-hexanol, *n*-octanol) in chloroform or hexane, not detecting the reaction of the L-isomer [115–117]. Since only the L-isomer has mint taste and smell, the food and pharmaceutical industries are interested in the resolution of DL-menthol and different approaches have been developed so far [118]. Table 2.3 compiles different approaches for the stereoselective transformation of water immiscible steroids [120–122].

Table 2.3 Steroid biotransformation in water-immiscible organic solvents.

Biocatalyst	Biotransformation	Organic solvent
β-Hydroxy steroid dehydrogenase (free or immobilized)	Oxidation of β-hydroxy steroids	Ethyl or butyl acetate
Nocardia sp. (free or immobilized)	Oxidation of cholesterol	Carbon tetrachloride or Toluene
Nocardia rhodochrous (free or immobilized)	Steroid transformations	Benzene or chloroform
Arthrobacter simplex (immobilized)	Δ'-dehydrogenation of hydrocortisone to prednisolone	Heptane or methanol

Data obtained from P.A. Fitzpatrick, A. M. Klibanov, How can the solvent affect enzyme enantioselectivity?. J. Am. Chem. Soc. 113 (1991) 3166–3171.

Table 2.4 Enantioselectivity (E-value) of subtilisin in the transesterification of *rac*-1-phenylethanol in different organic solvents, and the dielectric constants (ε_r) of the solvents.

Solvent	ε_r	E-value
Dioxane	2.2	61
Benzene	2.3	54
Triethylamine	2.4	48
Tetrahydrofuran	7.6	40
Pyridine	12.9	31
Dimethylformamide	36.7	9
Nitromethane	35.9	5
Acetonitrile	35.9	3
Methyl acetamide	191.3	3

Data obtained from P.A. Fitzpatrick, A.M. Klibanov, How can the solvent affect enzyme enantioselectivity? J. Am. Chem. Soc. 113 (1991) 3166–3171.

Other studies have been focused on the solvent effects in the enzyme enantioselectivity. For example, it has been demonstrated a variation of subtilisin enantioselectivity in the kinetic resolution of *rac*-1-phenylethanol according to the nature of the organic solvent used as reaction medium, involving a decrease in the enantioselectivity (E-value) from 61 in dioxane to 3 in methyl acetamide (Table 2.4) [119].

However, another study with the lipase from *Pseudomonas cepacia* does not show a correlation between the enantioselectivity of the enzyme and the polarity or hydrophobicity of the organic solvent [123]. Here, the enantioselectivity value for the resolution of *trans*-sobrerol shift from 69 in THF to more than 500 in *tert*-amyl-alcohol (Fig. 2.9).

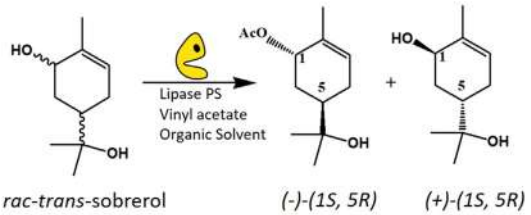
Hirose et al. reported another notable example of the enormous influence of organic solvents on selectivity and stereochemistry in a chemical reaction [124]. They observed how a 99% ee of *S*-monoesters was obtained when the reaction of enantioselective hydrolysis of prochiral dihydropyridine dicarboxylates by *Pseudomonas* sp. lipase was catalyzed in water-saturated diisopropyl ether (Fig. 2.10). However, the *R*-ester was preferably obtained in water-saturated cyclohexane (88%–91%).

2.9 From organic solvents to green solvents

Despite of the great influence and advantages that the organic solvents provide in chemical reactions catalyzed by enzymes it is also very important to consider the disadvantages to obtain improvements and viable alternatives to carry out enzymatic biotransformations. In this sense, the use of certain organic solvents as reaction media may cause enzyme deactivation which has led to the design of expensive alternatives for their stabilization, like immobilization or enzyme evolution. Other constraints are mass transfer limitations in systems highly viscous or heterogeneous or the control of water activity.

On the other hand, most organic solvents are toxic, corrosive and flammable. Besides, most organic solvents belong to the group of Volatile Organic Compounds, being the cause of the

(A)



rac-trans-sobrerol (-)-(1S, 5R) (+)-(1S, 5R)

(B)

Solvent	Log P	ε_r	E-value
***Tert*-amyl-alcohol**	1.4	5.8	518
3-pentanone	0.8	17	212
Dioxane	-1.1	2.2	178
Acetone	-0.23	20.6	142
Vinyl acetate	0.31	-	89
THF	0.49	7.6	69

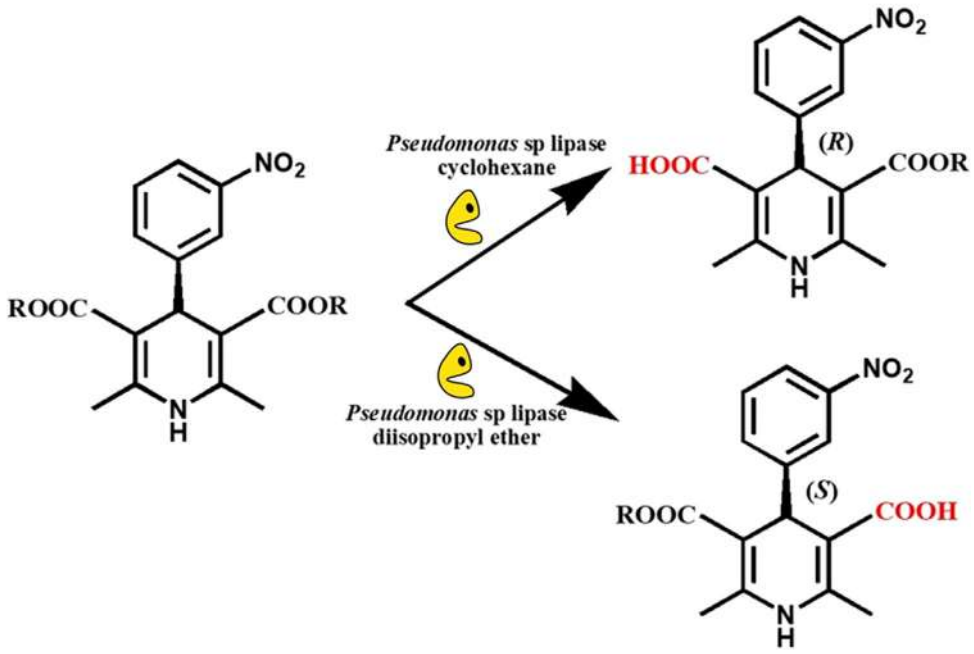
FIGURE 2.9

(A) Lipase-catalyzed the kinetic resolution of *rac-trans*-sobrerol by transesterification with vinyl acetate. (B) Influence of the organic solvent used as reaction medium on the enzyme enantioselectivity [123].

environmental impact caused by the chemical industry throughout over the years which is opposed to the concept of Sustainable Chemistry and the Principles of Green Chemistry. In addition, their inevitable loss due to evaporation difficult the processes of recovery and reuse and lead to high consumption of these solvents. However, reducing or eradicating the use of organic solvents in industrial chemical processes is an enormously difficult task, since these molecular liquids are fully established due to their ability to solvate and transport reagents, homogenize the reaction medium, enable the activation of some catalysts and, in addition, facilitate the separation of products by simple solubility changes [125]. Thus, the need to replace conventional organic solvents has determined an increased interest in solvents with low or no environmental impact and the studies of their properties and techniques for their selection have experienced exponential growth. Solvent selection models are based on a hierarchy of evaluations and tests designed to identify organic solvent replacements and guide toward the development of new alternative solvents [126]. Include the identification of the conventional solvent to be replaced, the selection of potent replacement candidates, the identification of a "green" synthetic pathway, the evaluation of performance and toxicity, and so on. For example, to facilitate the selection of new solvents this model uses Hansen solvatochromic and solubility parameters.

The use of "green solvents" has allowed the design of sustainable processes for the synthesis of highly valued chemical products. The scientific and technological interest to build a green biocatalysis in nonaqueous environment has been focused on several green solvents, such are *ILs*

(A)



(B)

Solvent	R	Configuration	*ee* (%)
Diisopropyl ether	*t*BuC(O)OCH$_2$	*S*	>99
Cyclohexane	*t*BuC(O)OCH$_2$	*R*	88.8
Cyclohexane	EtC(O)OCH$_2$	*R*	91.4
Isopropyl ether	EtC(O)OCH$_2$	*S*	68.1

FIGURE 2.10

(A) Lipase-catalyzed the asymmetric hydrolysis of dihydropyridine carboxylates in organic solvents. (B) Influence of the organic solvent used as reaction medium on the stereochemical preference of the enzyme in this asymmetric hydrolysis.

Data from Y. Hirose, K. Kariya, J. Sasaki, Y. Kurono, H. Ebike, K. Achiwa, Drastic solvent effect on lipase-catalyzed enantioselective hydrolysis of prochiral 1,4-dihydropyridines. Tetrahedron Lett. 33 (1992) 7157–7160.

supercritical fluids, deep eutectic solvents (DESs), biomass-based organic solvents, etc., since all of them shown enormous potential as sustainable tools for the development of chemical processes, because of their easy handling in the laboratory, as well as the recovery and subsequent reuse [127].

ILs are liquid salts made up exclusively of ionic pairs, usually with an organic cation and an inorganic anion and with the particular feature of having melting points below 100°C [128]. Due to their condition as ionic compounds, and not molecular compounds, ILs have been shown to have a unique and genuine combination of properties (i.e. low vapor pressure, high thermal stability, high solvent capacity, etc.) which directly affects their character as environmentally benign solvents, as they never evaporate [129]. Therefore, ILs are considered "green solvents," since they can be recovered and recycled through simple protocols [130]. Furthermore, many of their physicochemical properties (i.e. polarity, solubility, miscibility, viscosity, melting points, etc.) can be tailored easily depending on a suitable selection of the nature of the cation and the anion.

In 2000, two research groups demonstrated for the first time the suitability of ILs to carry out chemical transformations catalyzed by enzymes [131,132], being the starting point of numerous investigations. Since then, multiple reactions with different enzymes (i.e. lipases, dehydrogenases, glycosidases, etc.) have been performed in ILs as reaction medium, due to their ability to dissolve numerous compounds of different polarity [133–135].

Hydrophobic ILs have proved to be better solvents to support the biocatalytic activity over the hydrophilic ones. Despite their hydrophobicity, ILs have a hygroscopic character that enable the co-immobilization of the essential water-shell of enzymes. To understand the excellent solvation properties of ILs and support the biocatalytic activity, it is necessary to know the structure of the ionic network, which is able to incorporate macromolecules, polar substrates and even water molecules. In this sense, ILs based on imidazolium cation have shown to have a structured organization with polar and nonpolar sections in a high degree of directionality in the network, which has led to the conclusion that they are nanostructured systems [136]. The existence of holes of different polarities allows the accommodation and a strong bond of the molecules. If such molecules are biocatalysts, this entails their immobilization in the IL-phase, allowing their recovery and easy extraction of the products by liquid-liquid extraction with immiscible solvents, ultrafiltration, reduced pressure or supercritical fluids. Furthermore, the strongly hydrophobic environment of ILs difficult the increase of the kinetic energy of water molecules with temperature, improving the enzyme stabilization against thermal deactivation. This makes possible to use temperatures above the optimal temperature of the biocatalyst in an aqueous medium, favoring the solubilization and diffusion of the substrates (Fig. 2.11).

Thus hydrophobic ILs have proved to be excellent media for biocatalytic processes. For example, Itoh et al. [137] demonstrated the ability of immobilized lipases to carry out the asymmetric transesterification of an allyl alcohol (*rac*-5-phenyl-1-penten-3-ol) with vinyl acetate in different water-immiscible ILs (e.g., [Bmim] [NTf_2], [Bmim] [PF_6], etc.), while water-miscible ILs (e.g., [Bmim] [TFA], [Bmim] [TfO], etc.) were shown to lead to inactivation of the enzyme.

Conventional methodologies for products extraction from the IL media consist in liquid-liquid extraction using organic solvents such as ethyl ether [138]. However, the use of organic solvents in the extraction stage clouds the green character of the processes. In this sense, different investigations focus on the development of new approaches to integrate sustainable processes of products separation [138]. In this regard, the unique properties of ILs are key enabling technologies, allowing the design of smarts approaches for product separation (e.g., IL/$scCO_2$ biphasic reactors, membrane reactors, nanodrop systems, microfluidic devices, supported IL phases, sponge-like ILs, etc.), incorporating the full recovery and reuse of the biocatalyst and the ILs phase.

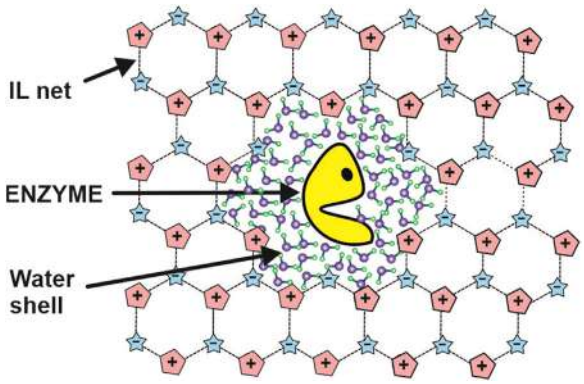


FIGURE 2.11

Scheme of the 3D supramolecular structure of imidazolium ILs, where an imidazolium cation (*pink*) is surrounded by at least three anions (*blue*). The structure is maintained by hydrogen bonding interactions. Within a space of a hydrophobic IL network there is a hydrated enzyme in native conformation [22].

2.10 Solvent-free biocatalysis

The emergence of neoteric solvents like ILs and DESs have meant a step forward to the sustainability of industrial biocatalytic reactions. However, there is still a handicap related to the cost of those solvents and the inversion in the steps of purification of products and the management of solvents for recycling. In this sense, solvent-free biocatalytic reactions come out as the ideal conditions from the sustainable point of view.

Headed by the necessity to maintain the active conformation of enzymes, there are some concerns to consider when performing a solvent-free biotransformation. In this scenario, the optimization parameters to achieve the highest yield are circumscribed to the molar ratio of substrates, enzyme loading and temperature [139]. In particular, the molar ratio of substrates, their polarity and melting point are of utmost relevance for the procurement of a homogeneous reaction media with a defined water activity for the biocatalytic transformation. The main hindrances can be related to the solid character, or even their mutual immiscibility, of substrates. Substrates with high melting point or highly viscous will demand intense mixing or heating to afford mass transfer during the reaction. However, the use of substrates with melting points below room temperature is neither a warranty of succeed since their high concentrations may deactivate the biocatalysts, as in the case of alcohols and carboxylic acids [9,140].

In the literature, there are different strategies reported to perform solvent free reactions overcoming those constraints. As a representative example, Lozano and col. developed a protocol based on the eutectic behavior of certain molecules to achieve a homogeneous reaction media, which they become liquid at temperatures compatible with biocatalysis. By heating up to 60°C the mixture of both solid substrates, such are panthenol (hydrophilic polyol, 69°C m.p.) and lauric acid (hydrophobic free fatty acid, 42°C m.p.), lead a DES system despite their mutual immiscibility, then acquiring a dual role as solvent, as well as reactants for lipase-catalyzed direct esterification [141]. This

mixture was proved to be an excellent reaction media for the biocatalytic performance of Novozym 435, obtaining 83% conversion and 98% selectivity in the synthesis of panthenyl monolaurate. Even more, the biocatalysts remained stable for at least seven cycles of reuse without alteration of the activity nor selectivity. As a result of the combination of the excellent performance of the biocatalyst and the absence of reaction media, the products can be used directly in cosmetic formulations without any additional step of further purification which means important economical savings and improved sustainability.

To overcome the immiscibility between substrates grinders, extruders and millers are used in the so called mechanochemical enzymatic reactions [142]. Also, microwaves and ultrasounds-assistance permit to enhance the mixing of the reaction components and their collision, being especially relevant when working with solid substrates with too high melting points.

Microwaves are an efficient tool for energy transfer, providing a fast increase in the temperature of the reaction mixtures and activating the polar functional groups of the reactants and biocatalysts, which in turn, translate into an improved reaction rate compared to conventional incubation [143]. The high temperatures provided by microwaves are relevant to reduce the medium viscosity and improve de substrates solubility and collisions. Although microwaves have been extensively used with solvents, a new trend promotes their use under solvent-free conditions to improve the sustainability of the processes. Under these conditions, Risso and col. have piloted the transesterification of β-ketoesters with up to 96% yield in just 2 h reaction time [144]. Also, Kahn and col. have stated the superiority of microwaves over other methods of incubation in the esterification of butanol with free palmitic acid, reaching a conversion of 97% in reduced reaction time, 25 min, and at 60°C [143].

Ultrasounds are acoustic waves with a frequency above 20 kHz. They are considered another green tool since their use reduces energy consumption and favors process selectivity, considerably reducing the presence of by-products [145]. Ultrasounds waves provoke an acoustic cavitation phenomenon in the liquid media followed by cycles of compression-expansion of microbubbles. The final collapse of microbubbles results in localized increased pressures and temperatures (1000 atm and 5000K) [146]. This phenomenon accelerates the solubilization and mixing of the components of a reaction and enhances mass transfer [147], and in the case of solid substrates, help to reduce the particle size improving their interaction [148].

Ultrasound-assistance is a very convenient technology in biocatalysis, as the intensified mass transfer promotes a better dispersion of the biocatalyst in the reaction mixture, enhancing the mass-transport rate of substrates to the active center of the biocatalyst, and the product output [149]. As a consequence, there is a positive impact on the efficiency and selectivity of the processes and a reduced inhibition of the enzymes caused by high concentrations of substrates or products in the vicinity. In addition, in the case of lipases, it was reported that ultrasounds can improve their biocatalytic efficiency by inducing changes in their flexible lid toward an open state [145]. These features, together with its lower energy requirement compared to other conventional agitation mechanisms, make this methodology a very attractive green tool (Fig. 2.12).

The efficiency of ultrasound to perform solvent-free biocatalysis has been demonstrated by Nieto and col. in the esterification of xylitol with lauric acid employing a cup-horn device for indirect ultrasonication [150]. Xylitol is a highly hydrophilic polyol with 93°C melting temperature, which hinders the chance to obtain a monophasic liquid media with lauric acid, a highly hydrophobic long alkyl chain free fatty acid with 42°C melting temperature, unless big amounts of solvent

are present. In the strategy reported, the assistance of ultrasounds not only overcomes that constraint and affords the reaction to takes place without solvent, but also provides improved results (>95% yield and 85% selectivity toward the monoester synthesis in just 90 min at 40°C) with lower energy consumption than conventional approaches. Although the reaction starts from a semi-solid mixture where xylitol is scarcely melted, a synergistic action between ultrasounds and the emulsifying role of the product accumulated at short times promotes an improvement in the reaction rate leading to its fast completion. However, the most remarkable results are obtained after scaling up by 200 fold the overall reaction mass, where the net increase in melted lauric acid favors the ultrasound transference eliciting the reduction of the reaction time to 30 min and an improvement in the selectivity.

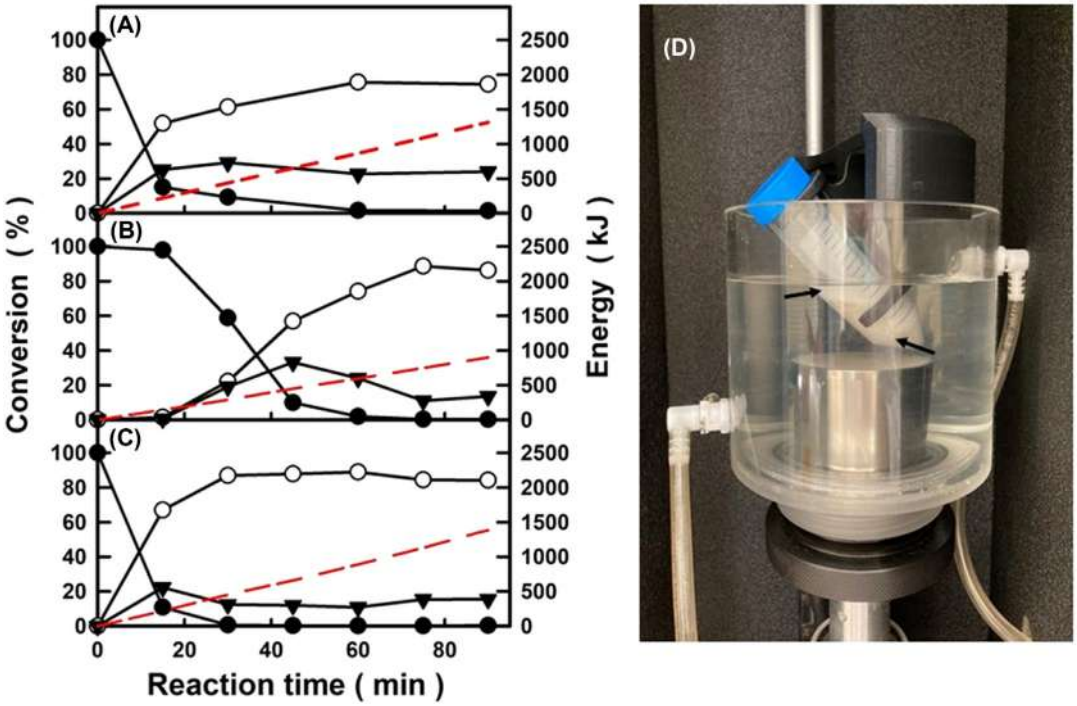


FIGURE 2.12

(A) Kinetic profiles for the novozym 435-catalyzed the synthesis of xylityl monolaurate (○), and xylityl dilaurate (▼) from a (1:1 mol/mol) solid mixture of xylitol and lauric acid (●) at 40% ultrasonic amplitude and at 40°C. A. 0.5 mmol overall mass. (B) 50 mmol overall mass (× 100). (C) 100 mmol overall mass (× 200). The red dash lines show the energy supplied in each assay. (D) Cup horn device and mixture aspect at the initial stage of the reaction. Arrows point to the melted lauric acid (*up*) and the solid particles of xylitol mixed with the immobilized biocatalysts and the dehydrating agent (*bottom*) [150].

2.11 Conclusions

The development of industrial chemical processes is important to improve the society's quality of life through the supply of consumer goods and products. However, the conditions of organic transformations (high temperatures, use of inorganic catalysts, organic solvents as reaction media, restricted solubility of substrates, etc.) limit the range of action of the transformations. In addition, there is some concern about its unsustainable nature due to environmental pollution mainly caused by the volatility of organic solvents and the accumulation and management of unwanted products, as well as the high energy consumption. The discovery of enzymes and their implementation as the most efficient and selective catalysts has made it possible to overcome the deficiencies of organic chemistry. Besides, their natural and biodegradable origin and the features of their catalytic performance permit the design of more benign processes and the use of renewable raw materials for a more sustainable chemistry.

Despite the high cost of biocatalysts, their use rewards with higher activity, selectivity (highlighting their enantioselectivity), operational stability, as well as process simplification and solvents and energy savings, that compensate for the shift from the already established conventional procedures to new synthesis strategies. This has led a large part of the scientific community to focus on improving the biocatalytic performance in such unnatural conditions, paying special attention to enzymatic activity and stability, their availability and recovery in nonaqueous environments.

The first much more rudimentary initiatives relied on nature's supply and were focused on the use of whole organisms or bioprospection of new biocatalysts to overcome the problems of viability and stability. Subsequently, enzyme immobilization was a decisive step to improve enzyme stability and their cost-effectiveness. However, the development of new methodologies in the field of molecular biology has broken the dependence on nature as permit the production and design of biocatalysts with improved activities and stabilities, broaden scope and even new functionalities. These methodologies are not only aimed to single biocatalysts but also allow the reprogramming of microorganisms, which alter the metabolic pathways for the synthesis of (un)natural products.

Alternatively, the medium engineering has also been emerged as an interesting tool to modify the activity and selectivity of enzymes. ILs are neoteric nonaqueous and benign media, which act synergistically with biocatalysts for the design of integrated green protocols of synthesis and purification. Also, biocatalysis under solvent-free conditions is ideal for sustainable chemistry, where interesting opportunities are continuously discovered. Undoubtedly, the growing number of research and publications related to the use of enzymes and their performance conditions is a clear sign that the improvement of the sustainability and efficiency of industrial processes can only be achieved through biocatalysis.

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CHAPTER

Activation and stabilization of enzymes using ionic liquid engineering

3

Toshiyuki Itoh[1] **and Yumiko Takagi**[2]

[1]*Toyota Physical and Chemical Research Institute, Nagakute City, Aichi, Japan* [2]*Department of Science, Faculty of Education, Kagawa University, Takamatsu, Kagawa, Japan*

3.1 Introduction

Enzymes are the key ingredients that enable green chemistry. Numerous biocatalyst-mediated reactions including whole cell systems have been developed for green organic synthesis, and biocatalyst libraries have been developed [1]. Direct evolution is a very powerful tool for tailoring the desired enzyme [2]. Therefore, once an enzyme, which can catalyze a unique reaction, has been discovered from nature, the biocatalysis system could be rapidly improved to the practical level using direct evolution engineering [2–6]. A very impressive result was reported by the Merck team such that they accomplished the synthesis of a key intermediate of Sitagliptin by a new transaminase created by the direct evolution method [3]; they replaced 44 amino acids of the original transaminase from *Arthrobacter* sp. and revealed that the created transaminase displayed an excellent reactivity; the enzyme converted a carbonyl group to the amino group with >99.95% e.e at a 200 g/L loading, while the original enzyme displayed only a 4% conversion at a 2 g/L loading [3]. However, although one property of the enzyme could be increased by the direct evolution, this caused another to drop, thus a vicious circle. To solve this dilemma, chemical process development of biocatalytic reactions, particularly, optimization of the reaction media and supporting materials of enzymes must be performed in parallel with the effort to develop new enzymes by direct evolution.

Ionic liquids (ILs) have very good properties as reaction media for chemical reactions; they are less-volatile, less-flammable, have a low toxicity and unique solubility for organic and inorganic materials [7–9]. ILs are now used as reaction media for biotransformations. The first several examples of enzymatic reactions in ILs were reported from 2000 to 2001, since then, a large number of applications has been reported for biocatalytic reactions in ILs and many reviews from various standpoints in this field have been published [10–16]. In this chapter, I have focused on three topics using ILs for enzymatic reactions from the standpoint of biocatalysts mediating organic synthesis; the first is "how to use an IL as a solvent for enzymatic reactions," and the second is "activation and stabilization of an enzyme by IL engineering," in particular "activation of lipase-catalyzed reactions," and the third is "laccase-catalyzed reactions in ILs." I also discuss in what fields we should focus our investigation for utilizing ILs in biotechnology in the future in the conclusion section.

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3.2 How to use an ionic liquids as a solvent for enzymatic reactions

Lipases (*E*.C.3.1.1.3) are widely found in nature, namely, in microorganisms, animals, and plants. Their main function is catalyzing the hydrolysis of triglycerides to free fatty acids and glycerol [1]. Lipases have a wide substrate acceptance and they can catalyze the enantioselective transesterification of alcohols or amines in a nonaqueous solvent, like toluene, diisopropyl ether, or hexane in the presence of an appropriate acyl donor, that is, vinyl acetate or isopropenyl acetate [1]. Lipases thus works as catalyst for transesterfication in the presence of acyl donor compounds in ILs, since they are nonaqueous liquids. The reaction is typically carried out as illustrated in Fig. 3.1; to a mixture of a lipase in an IL is added a racemic alcohol ((±)-**1**) and vinyl acetate as the acyl donor, then the resulting mixture is stirred at 35°C ([**A**], [**B**] in Fig. 3.1). The lipase can recognize enantiomers and preferentially reacts with the (*R*)-isomer, thus converting it to the corresponding (*R*)-ester. While the (*S*)-alcohol remains in the reaction medium after the reaction, an ether or a mixed solvent of hexane and ether is added to the reaction mixture to form a biphasic layer, and the (*R*)-acetate **2** and unreacted (*S*)-alcohol **1** were extracted in the organic solvent layer. The organic layer was evaporated to dryness, then silica gel chromatography allows separation of the (*R*)-**2** and (*S*)-**1** ([**C**] in Fig. 3.1). We can obtain enantiomerically-pure alcohols and acetate through this process. Although the maximum yield of each enantiomer is 50%, the reaction provides both enantiomers at the same time, and no coenzyme is needed to achieve the reaction. Therefore, the lipase-catalyzed reaction is used as a sure way for obtaining a key intermediate of chiral medicinal compounds or chiral functional molecules. Since the enzyme remained into the IL phase, the addition of the next set of (±)-**1** and acyl donor ([**D**] in Fig. 3.1) allows the next cycle of the reaction. Therefore, the lipase could be repeatedly used, anchored in the IL as the reaction medium (Fig. 3.1).

Using the appropriate IL is the key to achieving the desired reaction. The Hofmeister ion interaction toward protein stabilization is well-known in protein sciences [17]. Zho et al., pointed the importance of the Hofmeister ion interaction toward protein stabilization as illustrated in Fig. 3.2 [18]. Since then, this interaction has been discussed for designing ILs of enzymatic reactions [18–21].

From the standpoint of realizing a simple workup process, hydrophobic ILs are preferable. Fortunately, hydrophobic imidazolium type ILs, such as 1-butyl-3-methylimidazolium hexafluorophosphate ([Bmim][PF_6]) or 1-butyl-3-methylimidazolium bis(trifluoromethylsulfonyl) amide ([Bmim][Tf_2N]) is a suitable solvent for lipase-catalyzed reactions; these ILs are consisted with combination of chaotropic-cations and anions, though PF_6 anion is unfavorable to the enzyme from the Hoffmeister interaction. Both ([Bmim][PF_6]) and [Bmim][Tf_2N] are considered as hydrophobic liquids. However, these liquids involve water (maximum ca. 10%(v/v)), while searching for pure liquids. As a trace amount of water significantly influences enzyme reactivity, water contents of the ILs should be carefully evaluated when use [Bmim][Tf_2N] or [Bmim][PF_6] as a solvent. Since [Bmim][PF_6] allowed easy separation of the extracting solvent like hexane or ether, this liquid has been used frequently in early days of biocatalytic reactions in ILs. However, it is now recommended to use [Bmim][Tf_2N] instead [Bmim][PF_6], because hydrolysis of [PF_6] anion by the moisture at high temperature took place to produce toxic hydrogen fluoride [22]. My group always recycle ILs after chemical or biocatalytic reactions and we have several ILs that have over 20 years recycling history. We had an experience that [Bmim][PF_6] was suddenly decomposed to generate HF during storing it at rt. Fortunately, we have no trouble when using [Bmim][Tf_2N] during two

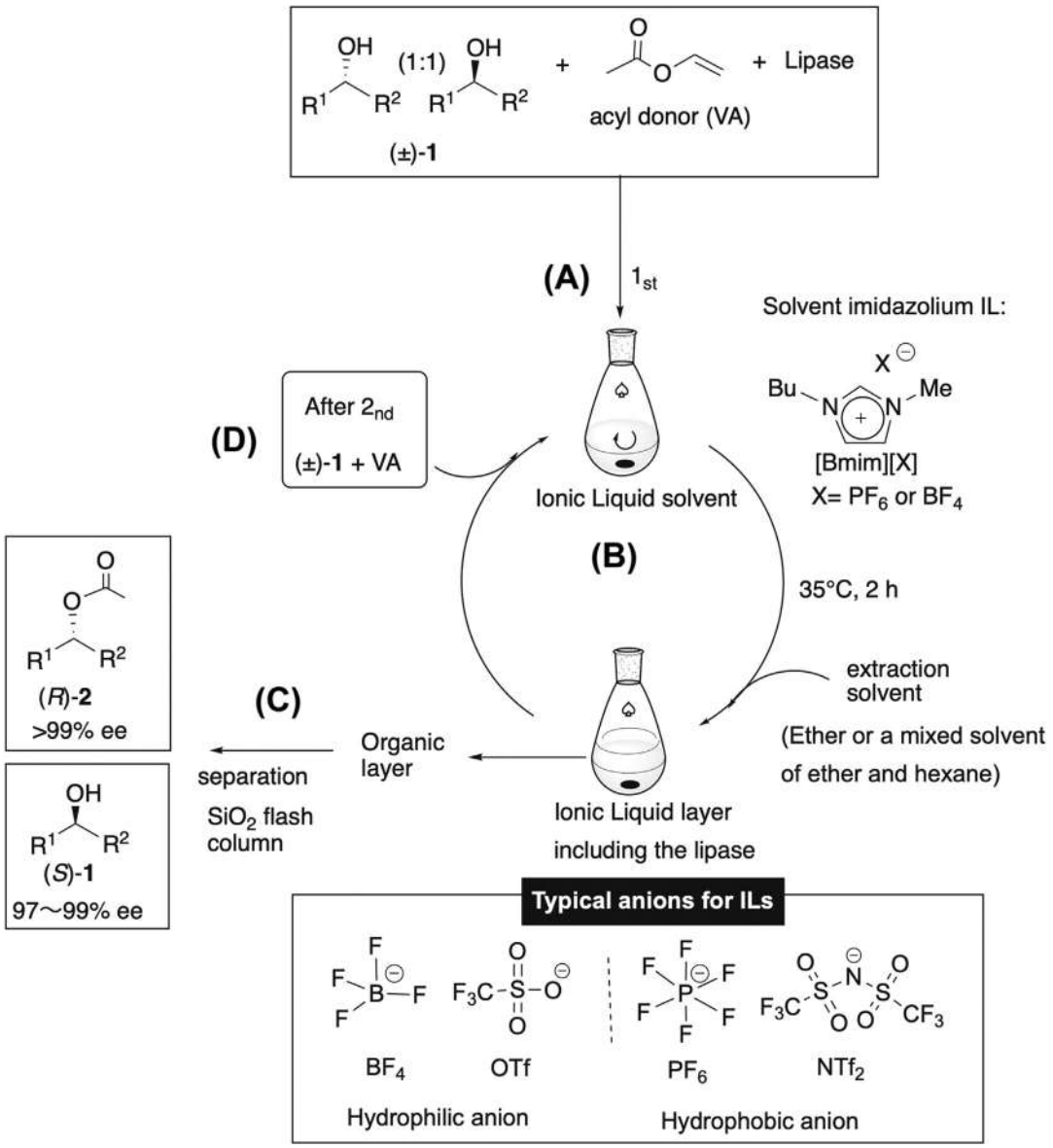


FIGURE 3.1

Typical lipase-catalyzed transesterification system for obtaining chiral alcohols using the IL solvent system.

decades in our laboratory. By this reason, we now use [Tf_2N] salts for biotransformation, though [Tf_2N] salts generally show inferior separation property against ether than [PF_6] salts. In addition, from the standpoint of cost effectiveness, [Tf_2N] salts may be a problem for large scale reactions.

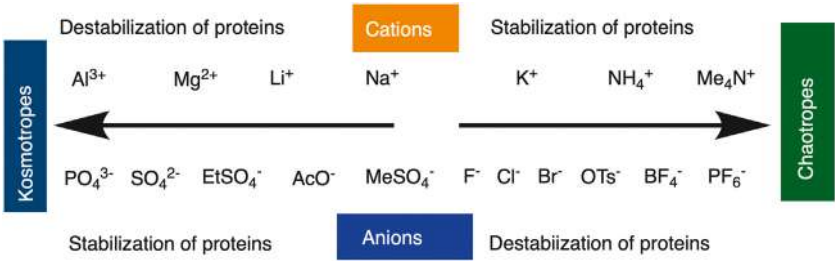


FIGURE 3.2

The Hoffmeister interaction of cations and anions against protein stabilization.

We succeeded in demonstrating the recycling use of lipase using the IL solvent system. However, we encountered an unexpected difficulty that the reaction rate gradually dropped with repetition of the reaction process when vinyl acetate was used as the acyl donor in [Bmim][PF_6] [23]. The reason why vinyl acetate is widely used as an acyl donor for the lipase-catalyzed transesterification is that this compound prevents any undesired reverse reaction during the transesterification; after the reaction of vinyl acetate with an alcohol, vinyl alcohol is produced, which is immediately tautomerized to acetaldehyde, then escapes from the reaction mixture due to its high volatility. However, the acetaldehyde trimer was gradually accumulated in the [Bmim][PF_6] solvent after repeating the reactions and the trimer indeed inhibited the lipase. As illustrated in Fig. 3.3E, we hypothesized that oligomerization of acetaldehyde might be caused by the IL solvent because of the high acidity of the 2-position of the imidazolium cation. Since the acidity of the 2-hydrogen seemed to be insufficient to directly act as a Brϕnsted acid, we anticipated that the 2-hydrogen might trap a water molecule and enhance its Brfnsted acidity, then catalyzed the trimerization of acetaldehyde. In fact, the pKa of the 2-hydrogen was reported to be 23.8 which is supposed to be an insufficient level for a Brϕensted acid but sufficient for activation of a water molecule [24].

We realized the recycling use of the enzyme without any drop of the reaction rate in an IL solvent system by two methods as illustrated in Fig. 3.3. One uses 1-butyl-2,3-dimethylimidazolium tetrafluoroborate ([Bdmim][BF_4]) as a solvent which has no 2-hydrogen at the imidazolium ring (Fig. 3.3F); no accumulation of an acetaldehyde oligomer was in fact observed in [Bdmim][BF_4] and we succeeded in demonstrating the recycling use of the lipase ten times while maintaining a perfect enantioselectivity and high reactivity [25].

The second solution that enables one to realize the efficient recycling use of a lipase in ILs is changing the reaction protocol; we conducted the lipase-catalyzed reaction under reduced pressure conditions (32°C at 133 hPa) and used methyl or ethyl esters as the acylating agents (Fig. 3.3G). It is well known that methyl esters are not suitable as acyl donors for the lipase-catalyzed transesterification because the reverse reaction takes place by the produced methanol. One of the important characteristics of an ionic liquid is its wide temperature range for the liquid phase and lack of vapor pressure; using this property, we developed the lipase-catalyzed reaction using a methyl ester as the acyl donor under reduced pressure conditions ([**G**] in Fig. 3.3) [26]. Although it is essential to choose both the substrate alcohols and an acyl donor ester, which have sufficiently higher boiling points compared to those of methanol or ethanol, the desired reaction smoothly proceeded under

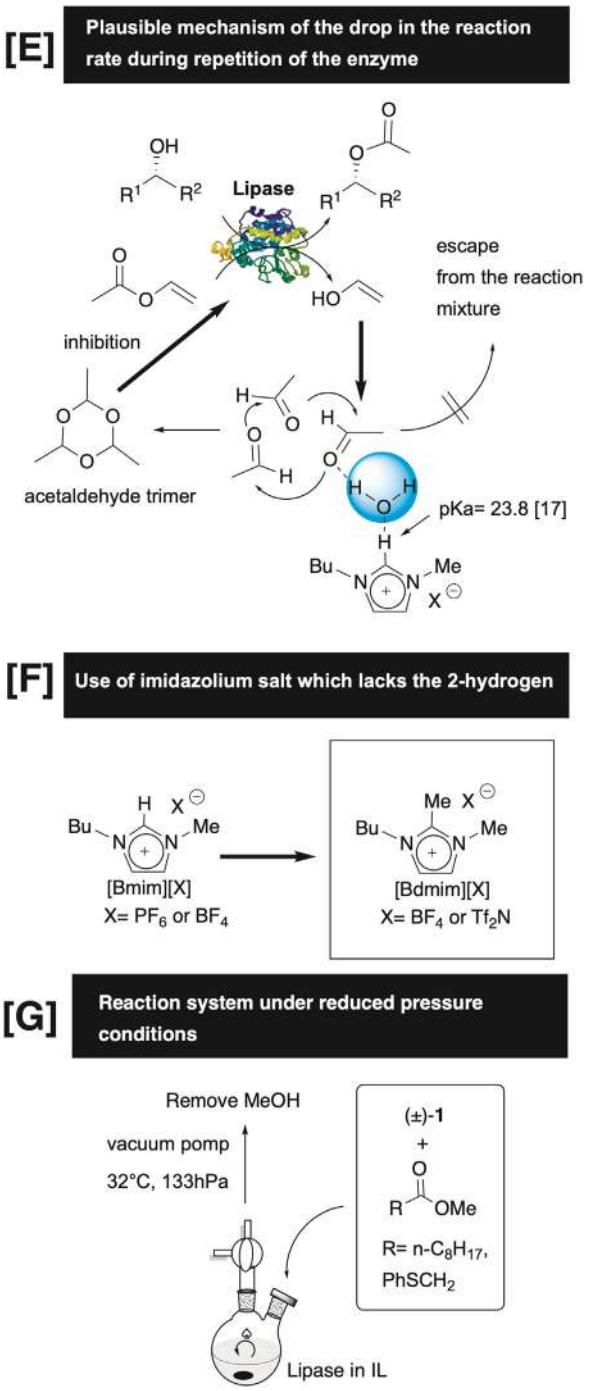


FIGURE 3.3

Two methods that prevent the drop in the reaction rate during repetition of the reaction cycles.

the stated conditions because the produced methanol or ethanol could be immediately removed from the reaction mixture and the reaction equilibrium occurred to produce the desired product; methyl pelargonate (RCO_2Me: R = n-C_8H_{17}) or methyl phenylthioacetate (RCO_2Me: R = $PhSCH_2$) was recommended as an acyl donor, thus an efficient optical resolution was accomplished and the reaction was repeated with no drop in the reaction rate while maintaining the perfect enantioselectivity [26].

Lourenco and Afonso reported an efficient transesterification system of the lipase-catalyzed reaction under reduced pressure conditions using the IL type acylating agent in an IL solvent (Fig. 3.4) [27]. In this system, the methyl ester **4**, which possessed the imidazolium salt moiety, was used as the acyl donor molecule and demonstrated that the ester was anchored in [Bmim][PF_6]. After extraction of (*S*)-1-phenylethanol (**1a**) (81% ee), the IL layer, which retained the imidazolium group substituted ester **5**, was treated with ethanol to release (*R*)-**1a** (99% ee). The system thus allowed a chromatography free kinetic resolution.

We further show two examples of the lipase-catalyzed reaction systems that apply the unique properties of the ILs for biotransformation.

An important property of the ILs is their unique solubility toward organic molecules. Sheldon et al. discovered that 1-butyl-3-methylimidazolium dicyanamide ([Bmim][$N(CN)_2$]) easily dissolved sucrose (195 g/L) and accomplished enzymatic esterification with dodecanoic acid by *Candida antarctica* lipase (Novozym435) [28]. Lee and Koo et al. accomplished the synthesis of a sugar fatty acid ester through the solubility-driven lipase-catalyzed reaction (Fig. 3.5) [29]. They

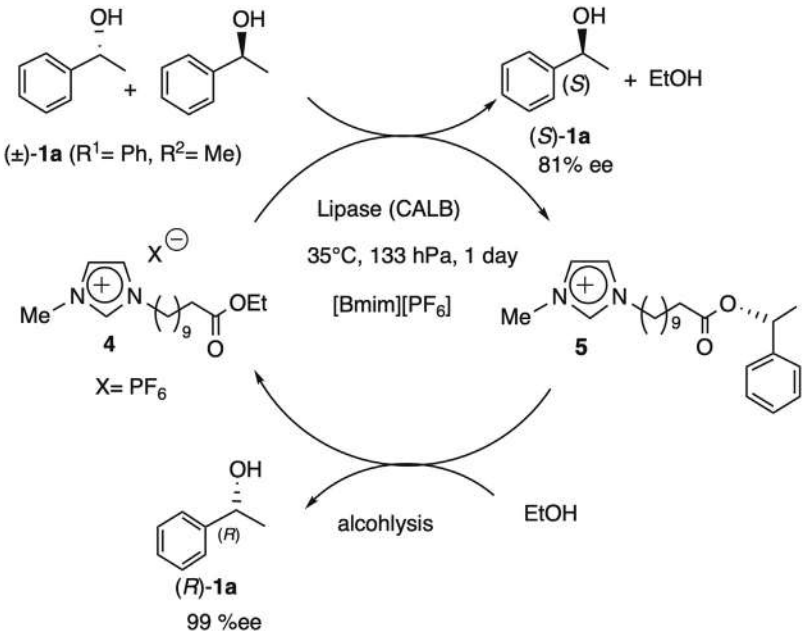


FIGURE 3.4

Lipase-catalyzed reaction using IL as the acylating agent in an IL solvent [27].

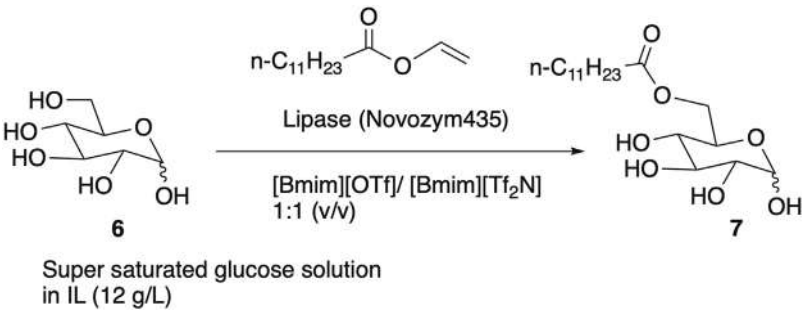


FIGURE 3.5

Synthesis of glucose ester using super-saturated glucose solution in an IL [29].

prepared a super-saturated glucose solution of 1-butyl-3-methylimidazolium trifluoromethanesulfonate ([Bmim][OTf]) and subjected this to the lipase-catalyzed transesterification in [Bmim][Tf_2N]. Since [Bmim][OTf] easily dissolved glucose (**6**), the authors prepared a super-saturated glucose solution of [Bmim][OTf] which was subjected to the lipase-catalyzed transesterification in the presence of methyl undecanoate to afford the corresponding fatty acid glucose ester **7** in high yield (Fig. 3.5) [29]. Since ILs generally easily dissolve sugar derivatives, this protocol might become a noteworthy one for sugar ester synthesis [13].

The enzymatic production of biodiesel oil has recently gained strong interest from the standpoint of sustainable energy production. Lozano et al. prepared *N*-cetyl-*N,N,N*-trimethylammonium bis(trifluoromethyl)sulfonylamide ([C_{16}tam][Tf_2N]), which was termed a "spongelike IL (SL-IL)." The authors used the SL-IL as a reaction medium of biodiesel oil production by the lipase-catalyzed transesterification (Fig. 3.6) [30]. Vegetable oil and methanol were mixed with SL-IL (solid at rt) and warmed at 60°C to afford a one-phase liquid solution. To this solution was added an immobilized lipase, transesterification occurred and afforded the fatty acid methyl ester (so-called biodiesel oil), glycerol, and water. After the reaction, the mixture was allowed to cool to rt then underwent centrifugation to provide the three layer-phased state, in which biodiesel oil was easily obtained from the top layer, the by-product glycerol and water were in the middle phase, and SL-IL was solidified under the bottom one. Since the lipase was retained by the SL-IL, the system allowed the recycling use of the lipase. The organic solvent-free separation of the biodiesel oil from the reaction mixture was thus accomplished using this reaction system [30]. The unique phase property of the ILs might be very attractive. SL-IL is also useful as reaction medium of production of flavor esters through lipase-catalyzed reaction [31]. Lozano's concept might be applicable to many biocatalysis systems.

From the standpoint of cost effectiveness, protic ILs (PILs) have gained strong interest [32]. Sheldon et al. reported *C. antarctica* lipase B-catalyzed enantioselective transesterification in protic ILs (Fig. 3.7A) [33]. Preparation of PILs is generally very simple and the resulting ILs have high biodegradability and lower toxicity than usual ILs. Since PILs display proton donating property, the ILs sometimes work as self-buffering ILs.

Zhu and Wang et al. reported the development of biocompatible protic ILs and use them as solvents for biodiesel oil production (Fig. 3.7B) [34]. The authors prepared choline hydrophosphate

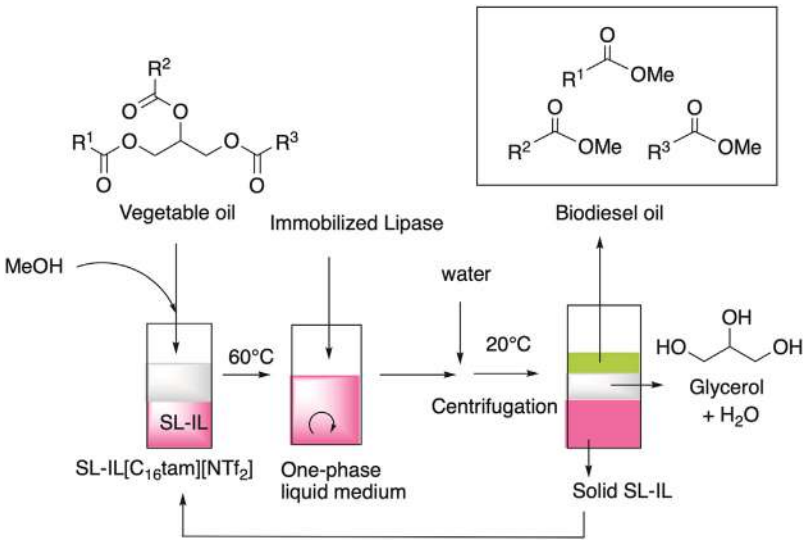


FIGURE 3.6

Biodiesel oil production using lipase-catalyzed reaction in the spongelike-IL (SL-IL) [30].

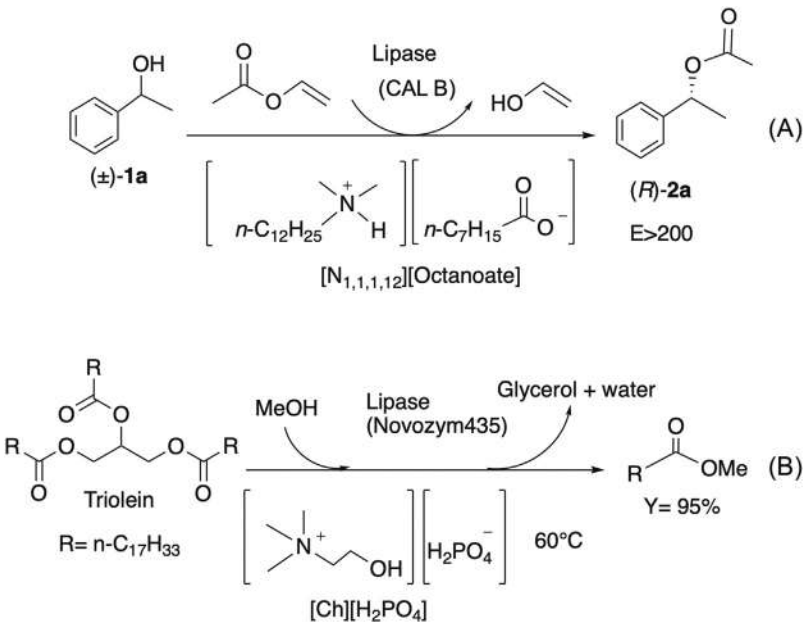


FIGURE 3.7

Lipase-catalyzed transesterification using protic ionic liquids (PILs) [33,34].

([Ch][H_2PO_4]) and accomplished easy production of biodiesel oil by the lipase-catalyzed reaction of triolein and methanol in this solvent. As the hydrophobic biodiesel oil phase was naturally separated from the reaction mixture due to hydrophilicity of the ILs, the authors succeeded in recycling the enzyme five times without a significant activity decrease [34].

These examples clearly indicate that the design of biocatalytic reactions from the chemistry side is very important to obtain the desired results. The ionic liquid engineering for activating lipase-catalyzed reactions has undergone great progress in this decade. I will focus on reviewing this topic in the next chapter.

3.3 Activation of lipase-catalyzed reactions using ionic liquid engineering

Immobilization is a common methodology for increasing the activity of enzymes [1]. Khmelnitsky et al. reported a unique immobilization method of activating enzymes in 1994 [35]. The authors reported that proteases *Subtilisin Carlsberg* and α-chymotrypsin could be activated by immobilization of a large amount of salts consisted of the combination of chaotropic cation and kosmotropic anion through the lyophilization process; the reaction rate of the KCl-treated protease mediated alcoholysis reaction was drastically improved and over a 3000-fold acceleration was accomplished compared to the native enzyme-catalyzed reaction [35]. However, the authors paid no attention to the influence on the enantioselectivity of their reactions. Kim and Lee first reported the ionic liquid mediated activation of an enzyme focusing on the modified enantioselectivity in 2002 [36]. They prepared the ionic liquid-coated lipase PS by mixing the enzyme powder with 1-methyl-3-(3-phenylpropyl)imidazolium hexafluorophosphate. Although the resulting enzyme showed a slightly enhanced enantioselectivity than that of the commercial lipase PSC in toluene, no acceleration was obtained [36]. Polyethylene glycol (PEG) treatment is known to cause a significant stabilization of an enzyme; Kaar et al. reported the improved activity of a lipase by PEG treatment in the ILs [37]. Inspired by these results, we synthesized ILs which consisted of the imidazolium cation and alkyl-PEG sulfate anion as immobilization material of a lipase; we found that IL immobilization through the lyophilization process became the key to realize the improved performance of lipases in 2004 [38].

As already mentioned, we accomplished the demonstration of the ten times recycling use of the lipase while maintaining perfect enantioselectivity and high reactivity in [Bdmim][BF_4] [25]. In this study, we noticed that lipase was very stable in this IL; lipase PS displayed an excellent reactivity after two months storage in this IL at rt, while it completely lost its activity in toluene or *i*-Pr_2O. With these results in mind, we synthesized various types of 3-methyl-1-butylimidazolium alkyl-PEG-sulfate ionic liquids as the immobilized agent and prepared ionic liquid coated *Burkholderia cepacia* lipase (IL-PS) through a lyophilization process and evaluated the IL-PS for the lipase-catalyzed transesterification of 3-hydroxypentanenitrile (**1b**) to acetate **2b** as a model reaction (Fig. 3.8).

Optimization of both the chain length of the alkyl group and PEG moiety of the anionic part of the IL was essential to achieve the desired activation; a 100-fold acceleration was accomplished when using IL1-PS compared to the native lipase-PS [39]. In addition, lyophilization is essential to

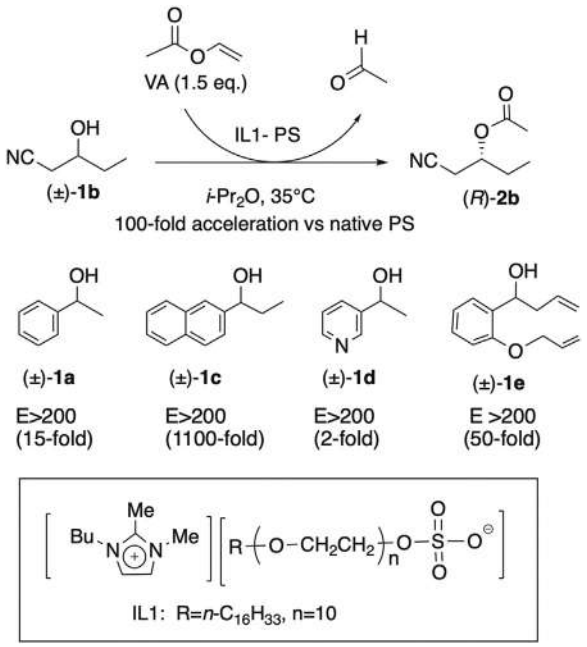


FIGURE 3.8

Activation of lipase PS immobilized by IL1 ([Bdmim][cetyl-PEG(10)SO_4]) [39].

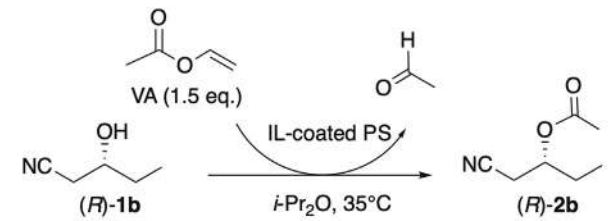
obtain the activated enzyme, though the enantioselectivity was modified simply by the addition of the IL to the transesterification reaction [38,39]. A notable result was also obtained when their IL-coated lipase (IL1-PS) consisted of only a small amount of IL1, ca. threefold (w/w) vs the enzyme protein and this is the most minimal amount of a supporting material that activated the enzyme [39]. It was also confirmed that IL1 effectively activated the *Candida rugosa* lipase [39]. Interestingly, the acceleration effect was dependent on the substrates and a significant acceleration was obtained for the bulky substrate like 2-naphthol (**1c**); a 1000-fold acceleration was recorded with an excellent enantioselectivity ($E > 200$). Although 15-fold or 50-fold acceleration was recorded for **1a** or **1e**, respectively, it was only double for the 3-pyridyl alcohol (**1d**) compared to those of the commercial lipase PS [39] (Fig. 3.8). It should also be noted that no leaching was observed when the IL1-PS-catalyzed reaction was carried out in a conventional organic solvent like toluene or *i*-Pr_2O. This indicated that IL1 was tightly bound to the enzyme by both coulomb and hydrogen bonding.

Abe et al. prepared chiral pyrrolidine-substituted imidazolium cetyl-PEG(10) sulfate ionic liquids (D-ProMe and L-ProMe) and revealed that the chirality of the pyrrolidine moiety significantly influenced the activation property of them against lipase PS; the D-ProMe immobilized lipase PS (D-ProMe-PS) recorded a 58-fold acceleration vs the commercial lipase PS and that was two-times higher than that of the L-ProMe-PS-catalyzed transesterification of 1-phenylethanol as a model substrate [40]. A more detailed investigation for differences in the activities of D-ProMe-PS and L-ProMe-PS was conducted in

the model transesterification reaction of 3-hydroxypenetanenitrile (**1b**) which revealed that the K_{cat} value of the D-ProMe-PS-catalyzed reaction was greater than that of L-ProMe-PS (Fig. 3.9). The k_m values of both enzyme-catalyzed reactions were slightly greater with a similar magnification for both isomers than the native lipase PS-catalyzed reaction, and the Vmax values of the D-ProMe-PS-catalyzed reaction of the favorable (*R*)-isomer were significantly enhanced; it reached a 17-fold vs the native lipase PS, and 1.four-fold higher than L-ProMe-PS as shown in Fig. 3.9 [40].

Yoshiyama et al. prepared the immobilized lipase PS by combining the amino acid with IL1 (1:1 molar ratio) and found an interesting synergetic activation of the lipase PS when the enzyme was immobilized by IL1 with an amino acid (Fig. 3.10) [41]. The activity of the immobilized enzyme was significantly dependent on the combination of the amino acid, and L-proline or L-tyrosine was found to be the best partner with IL1; the resulting immobilized lipases (L-Pro + IL1-PS and L-Tyr + IL1-PS) exhibited a greater reactivity for the transesterification of (±)-**1a** and 330-fold acceleration was accomplished for L-Pro + IL1-PS vs the native lipase PS with an excellent enantioselectivity ($E > 200$). On the contrary, neither the acceleration nor modified enantioselectivity was recorded when lipase PS was immobilized by amino acids, though the amino acids were utilized as a stabilizer of the enzymes during the purification process [41].

Furthermore, it was established that the chirality of the cationic part of ProMe-IL influenced the enzyme reactivity. Based on the results, we recognized that the cationic part played an important



	Enzyme	Km [M]	Kcat [min^{-1}]
(*R*)-**1b**	Native PS	0.23	0.29
	IL1-PS	0.30	1.8
	L-ProMe-PS	0.69	3.6
	D-ProMe-PS	0.51	5.0

FIGURE 3.9

Activation of lipase PS by the coating with chiral pyrrolidine-substituted imidazolium cetyl-PEG(10) sulfate [40].

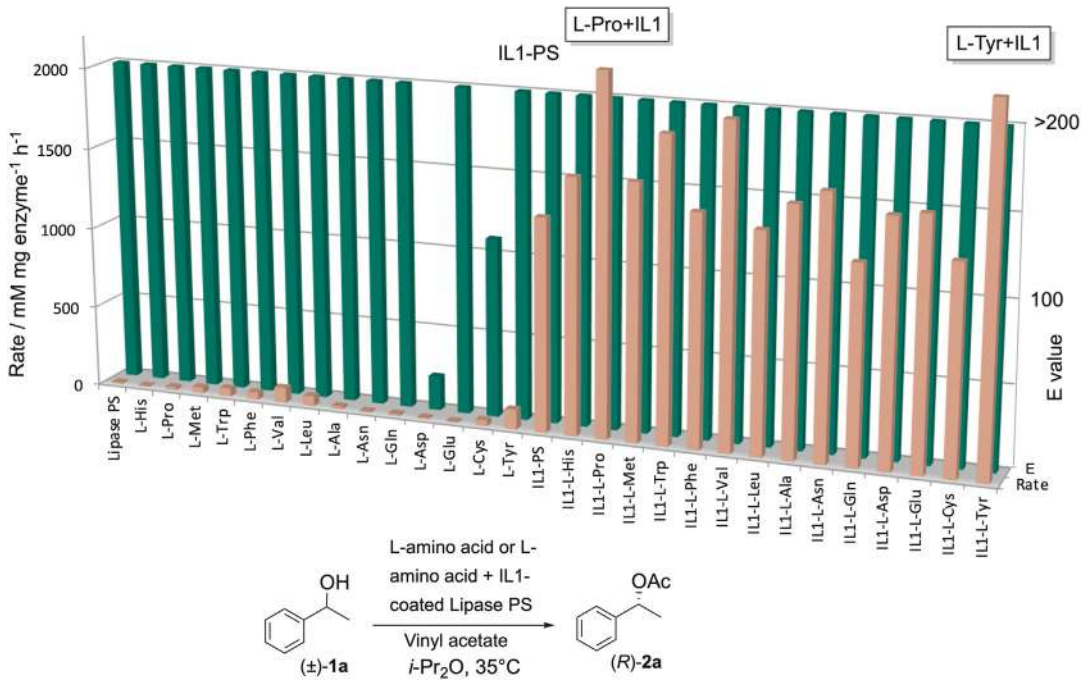


FIGURE 3.10

Synergetic activation of lipase PS with IL1 and amino acid [41].

role in the substrate specificity of the enzyme, and the anionic part mainly contributed to an improved stability of the lipase. On the other hand, Rahman et al. prepared the amino acid IL-coated *Candida rugose* lipase by lyophilization [42]. The resulting lipases displayed a superior reactivity versus the native enzyme for the esterification of oleyl alcohol with a carboxylic acid, and $[N_{2222}]$[His]-lipase showed a higher reactivity than that of $[N_{2222}]$[Asn] (Fig. 3.11) [42]. Xu et al. reported that the enhanced catalytic performance of lipase B from *C. antarctica* was obtained by the treatment with amino acid ILs [43]. The authors prepared the L- or D-N-acetyl proline salts with [Bmim] cations (Fig. 3.11) and lipase B was treated with these salts. The treatment caused a significant modification of the enzyme, in particular, the thermostability at 50°C−60°C, while no significant modified activity was obtained under the low temperature conditions. Interestingly, a slightly better modification was obtained for the L-isomer than that of the D-isomer [43]. Based on the MD simulation study of the amino acid-modified enzyme, the authors concluded that both the rigidity of the modified enzyme and the flexibility of the active center region were enhanced by the amino acid-modification process [43]. These results indicated that both the cation and anion of the ILs played important roles to activate the lipase-catalyzed reaction.

We investigated the design of the cationic part of the cetyl-PEG(10) sulfate ILs as an activating agent for a lipase PS (Fig. 3.12) [44−47]. All the ILs worked as strong enhancers of the *B. cepacia* lipase, though the substrate specificity of the enzyme was modified by the immobilized ILs. For

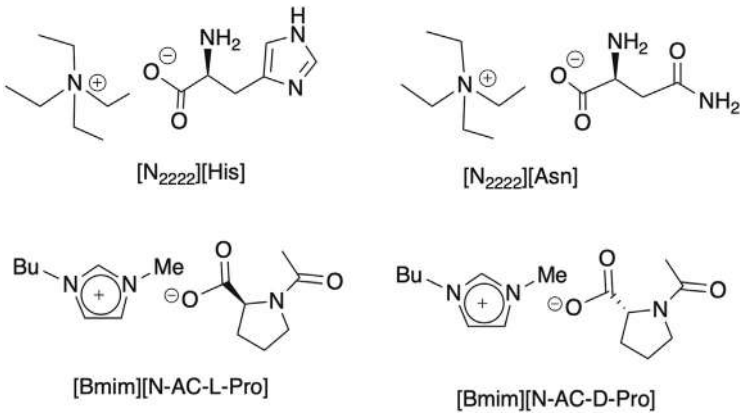


FIGURE 3.11

Activation of lipase by the amino acid ILs [42,43].

example, PL1-PS displayed an interesting solvent effect [44]. The immobilized lipase PS by tributyl(2-methoxyethyl)phosphonium cetyl-PEG(10) sulfate (PL1-PS) displayed an excellent enantioselectivity in the reaction of 2-chloro-1-phenylethanol with $E > 200$, though insufficient E values were recorded for lipase PS ($E = 12$) and IL1-PS ($E = 123$) with this alcohol [44]. On the other hand, tris(diethylamino)cyclopropenium cetyl-PEG(10) sulfate (TAC1)-PS also displayed a unique acceleration effect for the transesterification of several substrates, while no significantly modified enantioselectivity was obtained between the IL1-PS. TAC-1-PS showed an excellent reactivity for the pyridyl alcohols, while the acceleration effect of IL1-PS for this alcohol was moderate [45]. Interestingly, different synergetic activations of lipase PS with TAC1 were obtained. As already mentioned, the strongest cooperative activation of a lipase PS was obtained by the combination of L-proline with IL1 [41]. On the other hand, L-methionine (L-Met) was the best partner with TAC1, while L-asparagine (L-Asp) was the second one [45]. The amounts of the amino acids were also important; the 1:1 mixture of TAC1 with L-Met provided the highest activation of lipase PS and a significant drop in the reaction rate was recorded when the amount of the amino acids increased or decreased [45]. Activations of lipase PS were recorded for the pyridinium cetyl-PEG(10) sulfate immobilized enzymes, and the best reactivity was obtained for 1-ethylpyridin-1-ium cetylPEG(10) sulfate (PY_{ET}-PS) [46]. For the reaction of (±)-3-pyridyl alcohol (**1d**) as a substrate, the pyridinium salt (PY_{Et}, PY_{Bu}, or PY_{MP})-immobilized lipase PSs afforded better results than that of IL1-PS. Among these three immobilized enzymes, PY_{Et}-PS afforded the best results of many substrates [41]. Although the acceleration rates of the three types of triazolium cetyl-PEG(10) sulfate-immobilized lipase PSs (Tz1-PS, Tz2-PS, and Tz3-PS) were slightly inferior to that of IL1-PS, the stability of the enzyme was improved for these enzymes, in particular, in an IL solvent [47].

As already mentioned, selection of the appropriate ILs is essential for the desired lipase-catalyzed transesterification [10–16]. Lozano and coworkers first reported the enhanced stability of lipases by the ILs [48]. Qin evaluated ILs for the Novozyme435-catalyzed alcoholysis of monoolein with methanol based on the physical properties of the ILs, that is, log P, E^N_T, β-value, and viscosity [49]; the initial activity of the enzyme was increased with the increase in the log P value and reduced with the E^N_T value. On

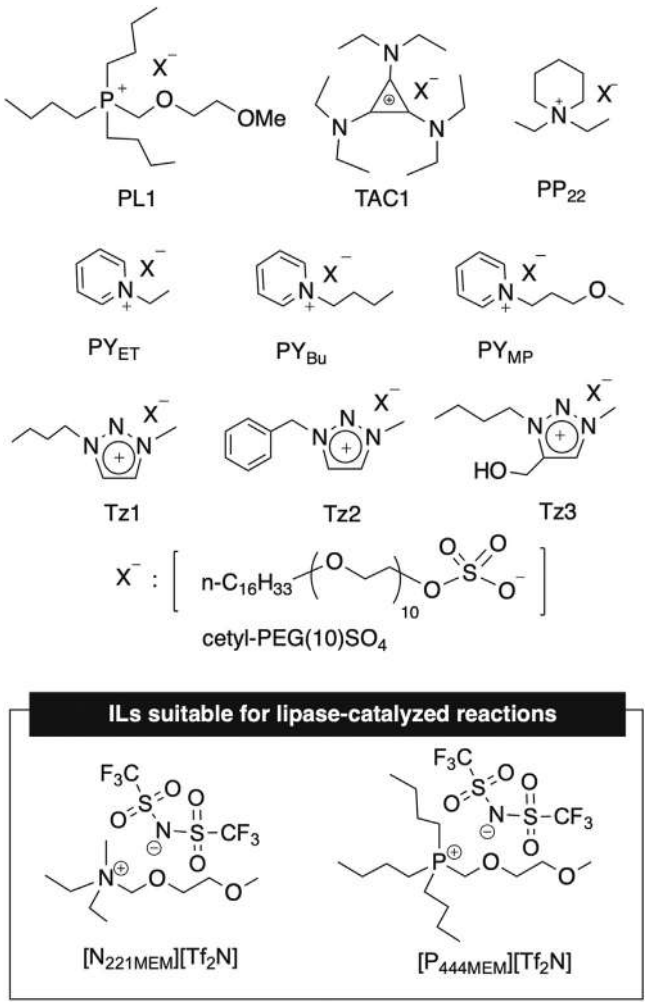


FIGURE 3.12

Design of cetyl-PEG(10) sulfate ILs as immobilization materials of lipase PS and two ILs suitable for these immobilized enzyme-catalyzed reactions [44–47].

the other hand, a high β-value and viscosity were not beneficial to the enhance enzyme activity. The authors reported that the enzyme stability was slightly dependent on the ratio of the α-helix content and the β-sheet content, and the increased β-sheet content of the enzyme in the ILs seemed to reflect deactivation [49]. For the solvent IL design, Abe et al. discovered that the introduction of the alkylether moiety in the ILs in the cationic- or anionic parts has generally provided good results, though it depends on the substrate; *N,N*-diethyl-*N*-methyl-*N*-(2-methoxyethoxymethyl)ammonium bis(trifluoromethyl)sulfonylamide ($[N_{221MEM}][Tf_2N]$) and tri-*n*-butyl(2-methoxyethoxyethyl)phosphonium bis(trifluoromethyl)sulfonylamide

$[P_{444MEM}][Tf_2N]$ were the best solvents for many of the IL-coated lipase PSs (Fig. 3.12) [50]. They revealed that increased K_{cat} values were suggested to be the most important factor for the IL1-PS working in these solvents [50]. It was well known that water molecules significantly affect the activity of a lipase in the ILs [51]. Ou et al. also reported that the introduction of the hydroxyalkyl moiety in the anion or cation of the ILs was effective to improve the lipase activity, though the resulting ILs have been generally hydrophilic and hydrophobic ILs that are preferable from the standpoint of an easy workup [52].

Optimization of the appropriate ILs is very important for the IL immobilized liapse-catalyzed reactions. We accomplished a very successful recycling of the Tz1-PS in $[N_{221MEM}][Tf_2N]$ and it exhibited a perfect activity after more than 200 recycles over a 2-year storage in $[N_{221MEM}][Tf_2N]$ at rt [47].

The structure of *B. cepacia* lipase based on an X-ray crystallographic analysis showed that there exist numerous water molecules, which were called essential water on the surface of the enzyme. It was reported that the water molecule plays an important role to maintain the motion flexibility of the enzyme protein (Fig. 3.13) [53]. The IL1 mainly binds with two parts of the enzyme, that is, the bottom part and the entrance part in the TOF-Ms experiment. Since the alkyl-PEG moiety was essential to cause an increased reaction rate, we assumed that the alkyl-PEG moiety, which binds with the bottom part of the enzyme, might prevent the removal of surface water and contribute to the improved flexibility of the enzyme. It is known that the lipase has a lid which consists of hydrophobic amino acid chains

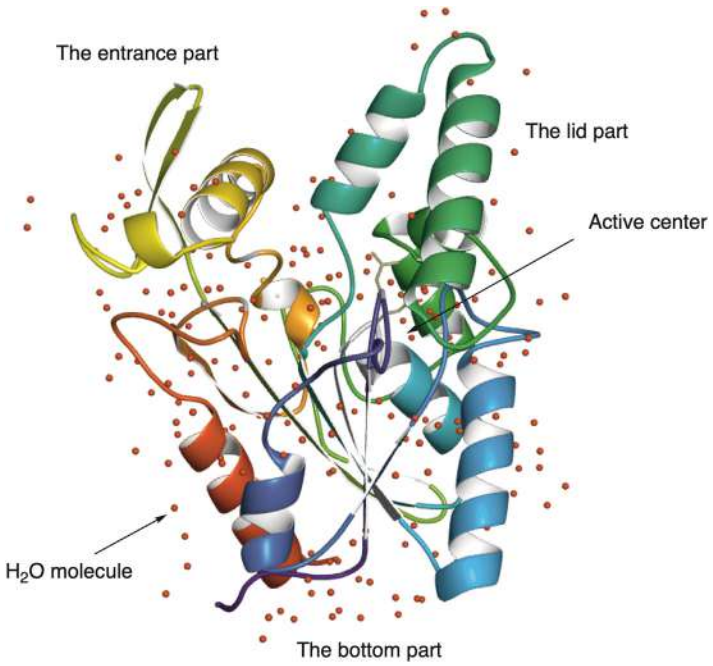


FIGURE 3.13

X-ray crystallographic structure of *Burkholderia cepacia* lipase with surface water (PDB ID: 3LIP, Entry DOI: 10.2210/pdb3lip/pdb) [53]. The red balls are water molecules.

and covers the entrance portion of the enzyme, and catalytic triads are buried beneath a helical lid segment. The substrate is then introduced into the catalytic site when the lid part has an open conformation [54,55]. It was reported that treatment of the *C. rugose* lipase by an alcohol, such as *i*-PrOH, by lyophilization altered the lid into an open form resulting in activation of the enzymatic reaction [54]. The substrate is then introduced into the catalytic site when the lid part has an open conformation [55]. Kim et al. reported that the large rigid movement of the lid part plays an important role in the catalytic activity of the lipases [56]. It was also suggested that both the k_m values and K_{cat} values might be increased in the open form [53]. For the reactions of the IL-immobilized PS-catalyzed reactions, significant increases for both the k_m and K_{cat} values were recorded [45,46] and the magnitude depended on the cations of the immobilized ILs.

Byrne et al. reported the results of an investigation of the effect of five ILs on the stability of *Thermomyces lanuginosus* lipase; both the nature of the ILs and pH of the reaction mixture had a significant impact on the hydrolytic activity [57]. The ion concentration of the ILs was correlated to the water activity χ_W, that is, five water molecules per IL, and $\chi_W = 0.6$ that is the threshold of water for an assured lipase activity. The authors also reported that ILs, which have a high hydrogen bonding basicity (β-value over 0.8), caused enzyme denaturation [57].

Based on these results, we believe that the origin of the activation effect caused by immobilization of our ILs might be two factors; the first is improved motion flexibility of the enzyme protein by the alkyl-PEG moiety that is bound to the bottom part of the enzyme and the second is binding the IL at the lid part which changed its structure to the open form of the enzyme and thus contributed to the increased reaction rate.

Although we didn't mention the stabilization or activation of an enzyme by the deep eutectic solvents (DESs) [58,59], it is well known that DESs having similar physical and chemical properties as the ILs and sometimes show even a better ability than the ILs from the standpoint of green chemistry [59]. In addition, we can prepare DESs very easily by just mixing the components and heating the mixture; they are readily available and relatively more inexpensive than ILs. Therefore, it should be expected that DESs might also become an important media of the lipase-catalyzed reaction [13,59].

3.4 Laccase-catalyzed reactions in ionic liquids

Laccase (EC 1.10.3.2) belong to the blue-copper family of oxidase that allows the oxidation of organic compounds using molecular oxygen under very mild reaction conditions. Laccase catalyzes oxidation of phenol derivatives such as **8** using molecular oxygen to produce a quinone derivative **9** as illustrated in Fig. 3.14 [60,61].

This enzyme mediates the oxidative degradation or polymerization of polyphenol formation, hence laccases are widely found in plants, fungi, bacteria, and insects [61–65]. This enzyme works as a catalyst for the environmentally benign degradation process of lignocellulosic biomass [66–68]. Lignin represents c. 30 wt.% of the plant biomass, since lignin consists of complicated polyphenol moieties. However, it is very difficult to convert high-value small molecules by chemical reactions. Laccase might be a key tool to achieve the selective oxidative degradation of lignin and afford high-value small products [67–70].

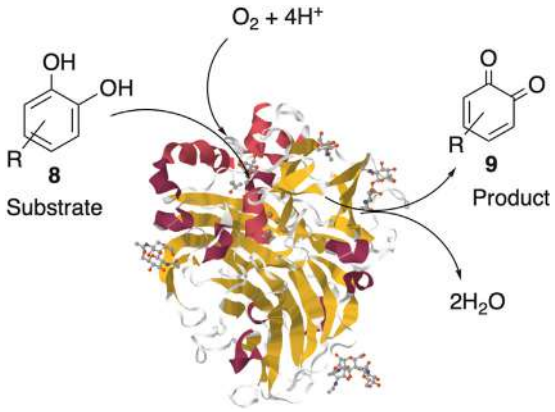


FIGURE 3.14

Typical laccase-catalyzed oxidation. Ribbon Structure of the laccase from *Trametes versicolor* was reproduced from the protein data bank (PDB ID: IGYC) [60].

The distance between the active site and the surface of the laccases is shorter than many enzymes [71], therefore, it is anticipated that the laccase's activity might be easily modified by the solvent and additives. In fact, ILs are successfully used as a co-solvent that can modify the laccase activity. Hinckley et al. reported the first example of the laccase-catalyzed reaction in ILs in 2002 [72]; the oxidation of several compounds was accomplished using laccase C which was isolated from Trametes sp. in a mixed solvent of ionic liquids with a citrate buffer solution [72]. Veratryl alcohol was thus converted to the corresponding veratryl aldehyde in 86% conversion in a mixed solvent of 25% (v/v) [4-MBP][BF_4] with citrate buffer (pH 5.6) using N-hydroxyphtalimide (**10**) as the mediator [72]. Anthracene was also converted to anthraquinonein 15% conv. in the same solvent system using 1-hydroxybenzotriazole (11) as the mediator (Fig. 3.15) [72].

Shipovskov et al. reported the additive effect of ionic liquids on the laccase-catalyzed oxidation of catechol to quinone derivatives (Fig. 3.16) [73]; ca. 1.ninefold increased activity was obtained when 15%(v/v) of [Bmim]Br was added to the sodium phosphate-citrate buffer (pH 6.0) for *Agaricus bisporus* laccase (ABL) and ca. 1.fivefold enhanced activity was recorded for the *Trametes versicolor* laccase (TVL)-catalyzed reaction in a 20% (v/v) of a [Bmim]Br mixed solvent. However, complete inhibition of these enzymes was recorded when the concentration of ILs was increased over 60% (v/v). On the other hand, a unique additive effect was found for 1-butyl-3-methylimidazolium dicyanamide ([Bmim][$N[CN]_2$]); although the relative activity of ABL was decreased to ca. half in the presence of this IL at 30% (v/v), it increased and reached a maximum at ca. 1.25-times at a 50% (v/v), then the activity was completely lost at over a 70% (v/v) concentration [73].

Although lignin is one of the most abundant biopolymers, depolymerization and valorization of lignin is a very challenging issue. ILs have excellent capability of solubilizing lignin during biomass pretreatment [74]. Stevens et al. reported the depolymerization of lignin by treatment of aqueous ionic liquids [75]. They found that the conversion yield of the alkaline lignin degradation by laccase was significantly improved when the reaction was carried out in 10 wt.% of [DEA][HSO_4] for 2 h; a 10-fold increased conversion yield was attained compared to the control reaction (no IL) (Fig. 3.17) [75].

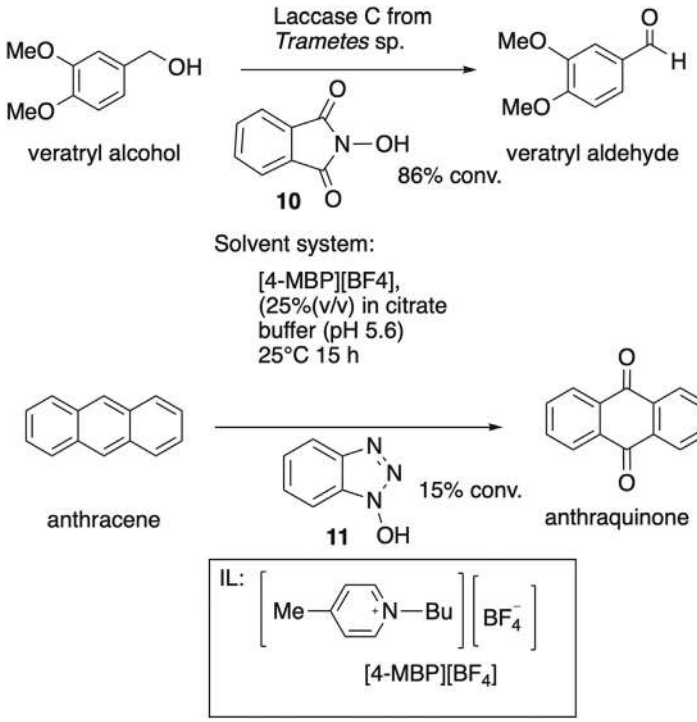


FIGURE 3.15

Laccase-catalyzed oxidation in a mixed solvent of [4-MBP][BF_4] (25% v/v) and citrate buffer (pH 5.6) [72].

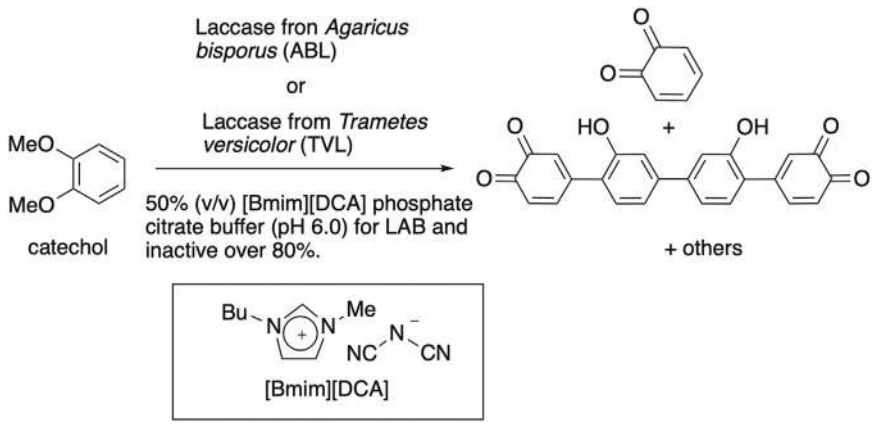


FIGURE 3.16

Laccase-catalyzed oxidation of catechol [73].

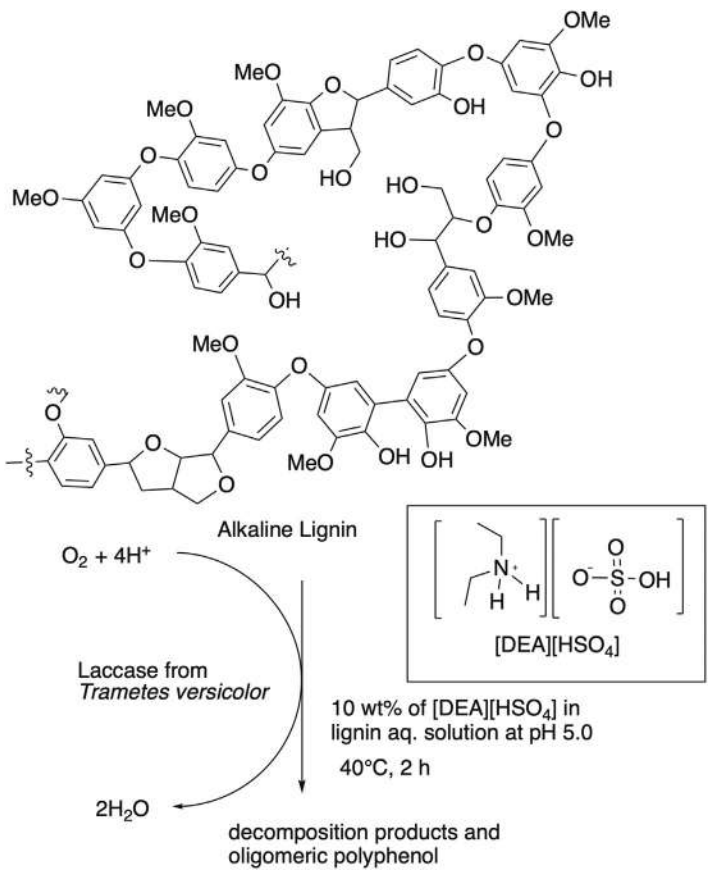


FIGURE 3.17

Conversion of lignin by laccase-mediated reaction in the presence of IL [75].

As previously mentioned, laccase can oxidize hydroquinone derivatives to the corresponding o-quinone derivatives. Since it is well known that a o-quinone derivative could be easily converted to the starting hydroquinone derivative by an electrochemical reduction, the reaction could be used as a biosensor. Franzoi et al. developed a biosensor system for polyphenol derivative detection in an IL solvent system using the laccase-mediated reaction; they prepared the electrode coated with a mixture of graphite powder, laccase and [Emim][Tf_2N] (50:20:15:15 (w/w/w/w)) and revealed that the resulting electrode worked well to convert rutin (quercetin-3-rutinoside) into the corresponding o-quinone derivative (Fig. 3.18) [76]. The author considered that the IL worked well as not only an effective binder but also improved stability of the sensor. Since then, numerous examples of the use of ILs for biosensor systems have been reported [77–81]. Brondani et al. accomplished to develop a biosensor which allowed selective detection of adrenaline among a mixture of dopamine, carbidopa, methyldopa, and levodopa (Fig. 3.19) [82].

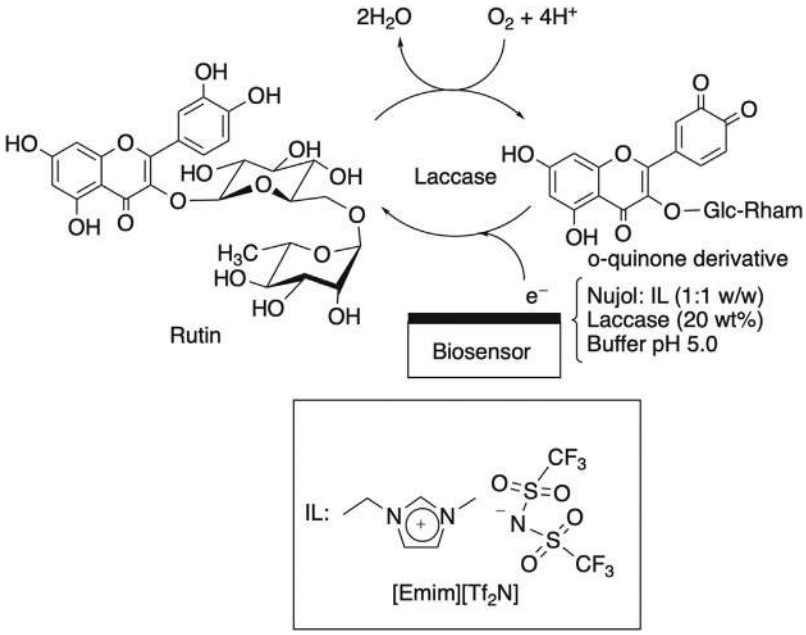


FIGURE 3.18

Laccase-catalyzed biosensor for the determination of rutin [76].

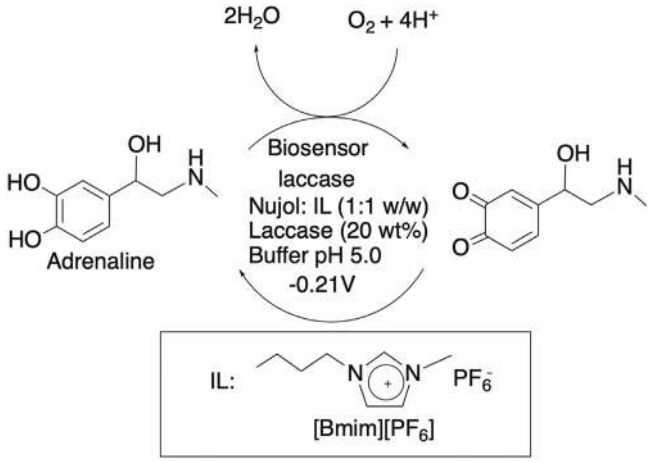


FIGURE 3.19

Reaction of the biosensor for selective detection of adrenalin [82].

The most popular model reaction of laccase is the single electron oxidation of 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) to the corresponding oxidized form $ABTS^{\bullet+}$ (Fig. 3.20) [62]; this caused clear color change of the reaction mixture from colorless (ABTS) to turquoise (ABTS• +), ABTS has been frequently employed as an indicator of the activity of the laccases [61,62]. Tavares and coworkers investigated the stability of laccase in a mixed solvent of buffer with water soluble ILs and organic solvents, such as CH_3CN or DMSO, under various pH conditions by the oxidation of ABTS; they revealed that the enzyme was active at pH 9.0 in the presence of all the tested ILs and, in particular, 1-ethyl-3-methylimidazolium 2-(2-methoxyethoxy) ethylsulfate ([Emim][$MEESO_4$]) effectively stabilized the enzyme (Fig. 3.20) [83].

Laccase accept various types of compounds as substates, such as indigo, benzyl alcohol, adlerol, or phenyl glycoside and convert them to the corresponding oxidized products, isatin, benzaldehyde, adlerone, or phenyl β-D-glucopyranosiduroic acid, respectively [84–88]. Recently laccase-catalyzed 1,4-addition of hydroquinone with coumarin has also been reported [89]. Although no one has not yet tested an IL as solvent for these reactions, we expect that interesting results might be obtained when using IL solvent system. As before mentioned, laccase is the only enzyme to achieve the oxidative degradation of lignin under hazardous chemical reagent-free conditions to

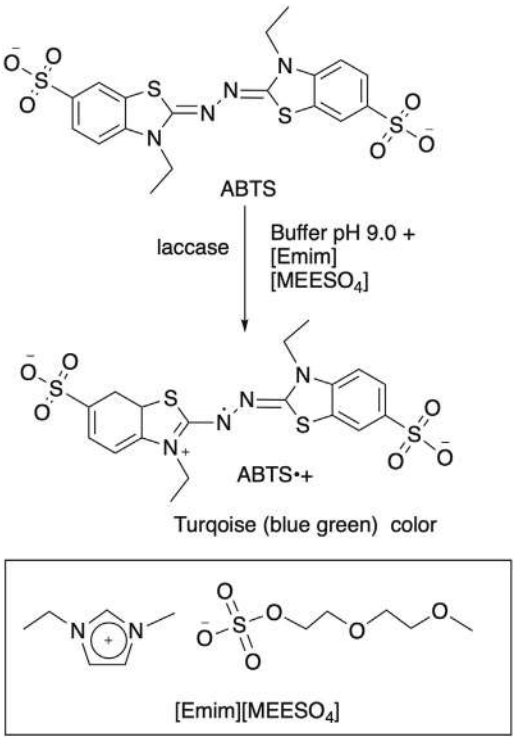


FIGURE 3.20

Laccase-mediated oxidation of ABTS [83].

date. Since the stability of laccase was successfully improved by immobilization of the enzyme or IL additives as examples, we expect that using ILs for laccase-catalyzed reactions might open the door of sustainable biomass engineering.

3.5 Conclusions and future perspective of enzymatic reaction using ionic liquids

ILs might become more popular in the future and provide a certain benefit in our life from the standpoint of green sustainable chemistry. We feel that oxidase-mediated reactions in the ILs might become more important in this field. Chemical oxidations generally require hazardous reagents and harsh reaction conditions, so that the resulting compounds are generally low-value molecules or complicated mixtures. Fortunately, enzymatic oxidation allows hazardous reagent-free oxidative chemical conversions. Since ILs generally dissolve oxygen gas, it is anticipated that the ILs might become an efficient media for the enzymatic oxidations. Among the enzymatic oxidations, we feel there might be a great possibility to improving efficiency of the laccase-catalyzed reaction using the IL engineering [13,66]. We are also interesting in the cytochrome c and P_{450}-mediated reactions [13,65,90–92] because enzymatic oxidations using these enzymes allows realizing the hazardous chemical reagent-free organic synthesis. Owing to the narrow substrate specificity of these enzymes, only limited application of these enzymes has been reported for organic synthesis to date [65,90–92]. Fortunately, Shoji and coworkers succeeded in broadening the substrate specificity of P_{450} [93,94]. Therefore, we expect that the combination of the IL engineering may provide a breakthrough in these enzyme-catalyzed reactions in the future.

Replacement of the reaction media from classical organic solvents or water to ILs in industry has still not yet been realized to date. Although successful examples are currently limited to only lipases, IL engineering might become more important in the future. Breakthroughs in chemistry occurred with the innovation of a reaction medium and this is true even in enzymatic reactions. We hope this chapter may provide a hint for the reader's research studies.

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Further reading

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CHAPTER

Refolding ability of ionic liquids against denatured proteins

4

Anamika Sindhu and Pannuru Venkatesu
Department of Chemistry, University of Delhi, Delhi, India

4.1 Introduction

Proteins are biopolymers involved in a multitude of processes in the living cell; they are located in an intracellular region, on the extracellular surface and also within the cell membrane. They are usually found in biological systems such as antibodies, hormones, enzymes, receptors, and transporters [1,2]. Owing to incredible biological functions, these biopolymers are being used in medical industries using explicit formulations and are also used as constituents in various industrial processes [3,4]. The regular local folds of polypeptide chains make up the proteins and for the biocatalytic functioning of enzymes; therefore, their native form must be preserved. The three-dimensional network of proteins is maintained by various interactions including hydrogen bonding, ionic, electrostatic and multiple hydrophobic interactions [5–8]. This structure emerges due to the sequence of amino acids in polypeptide chains in a unique pattern that further folds to form compact domains. These interactions get altered with even slight environmental stresses finally causing complete denaturation or unfolding leading to inactivation of protein functional groups [6,8].

Despite the numerous research works on protein folding, there is still inadequacy in outcomes that could suggest the conformation in which the protein will migrate after undergoing environmental changes like temperature, solvent conditions, and variation in pH. A protein native structure depends on the particular physiochemical environment, and the deviations in environmental conditions might point out the pattern of folding, misfolding and unfolding of a particular protein [9,10]. The protein folding process inside the cell is quite complex and follows multiple pathways [11]. Protein refolding and unfolding are vital biological events, yet they are still not completely understood. Protein misfolding can lead to series of fatal pathological diseases like Huntington's and Alzheimer's disease [12,13]. Extensive evidence suggests that numerous ways can protect the native structure of protein such as protein engineering, immobilization and solvation inappropriate cosolvent. Among, the optimization of cosolvent paves the way to preserve the stability and reusability of proteins. In virtue of this, low-melting-point organic salts known as ionic liquids (ILs) have emerged as a most promising class of green solvents for protein stability studies [14,15].

The ILs have established themselves as biocompatible cosolvents for proteins and offering an intriguing way to stabilize proteins for the long-term and hence protecting their enzymatic functioning. Their unique molecular architecture of various cations and anions leads to numerous

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potential IL species [16]. Because of the low vapor pressure, emerging from the strong electrostatic interactions among the constituent ions, they own a varied amount of useful features including lack of vaporization, minimal harmful vapor while handling, low flammability hence entitling them "greener solvents" [16,17]. Nowadays, ILs are being used in the fields of organic synthesis, catalysis, electrochemistry, biotechnology, as well as in nanotechnology [18]. The interaction of ILs with proteins offers protein refolding, enhancing thermal stability, improving enzymatic activity, increasing the shelf life and long-term protein packaging [19]. The study on folding patterns of proteins in presence of IL offers a new landscape for understanding various mechanism involved in protein aggregation, folding, unfolding and refolding. Summers and Flowers were the pioneers in employing ILs as protein refolding additives for denatured proteins [20]. Afterward, many families of ILs like imidazolium-based, cholinium-based, ammonium-based ILs were successfully discovered as refolding additives for chemically and thermally-induced denatured protein structures [21–23].

ILs being designer in nature can be synthesized as per the requisite characteristics, and it has been postulated that the polarity of IL alters with an alkyl chain length of cation of IL and the polarity of IL decreases with an increase in alkyl chain length of IL cation [24]. This effect of polarity change via lengthening of the alkyl chain proves negative for refolding of the protein [25]. Therefore, know the mechanism of IL–protein interaction; it has become quite obvious to get acquaintance on ILs families as well as structure of biomolecule under study. This article primarily focuses on studying the aspects of protein refolding in presence of ILs. The mechanism of protein refolding, various challenges faced while refolding the proteins and strategies to design new ILs as better refolding agents against denatured proteins will be discussed in this chapter.

4.2 Structural aspects of ionic liquids and their important applications in various bioscientific fields

ILs are organic salts solely composed of ions and possess a melting point below 100°C. They are governed by van der Waals, Coulombic, hydrogen bonding, dipole–dipole and solvophobic forces [26]. Typically, IL is composed of nitrogen-containing organic cation and an inorganic anion. The different combinations of constituent ions can lead to the generation of more than 10^6 ILs [2,27]. The first potent IL is ethylammonium nitrate (EAN) and was reported by Paul Walden in 1914, it was first recognized as an organic compound and later considered as IL [28]. Since then, the researchers have focused on generating ILs with critical biological properties, and hence these ILs are termed as task-specific ILs. The task-specificity in turn came from the designer nature of ILs, with wise selection of ions and then determining the toxicity; properties of ILs can be tuned as per the purpose [29,30]. ILs possesses the ability to act as host molecule and interacts with guest molecule via hydrogen bonding, van der Waals, electrostatic interactions. The noncovalent interactions in ILs can easily tweak and hence they are good candidates for dissolving recalcitrant materials [31]. Currently, ILs is used in green technologies, analytical chemistry, cellulose dissolution and regeneration, CO_2 capture and the field of biochemistry. Interestingly, ILs were significantly employed as biocompatible cosolvents, stabilizing agents for biomolecules and also engaged in drug carrier systems for poorly soluble drugs [32,33].

Intense research in this particular area has been conducted, which proved that ILs are promising solvents for optimizing the native form of ILs by hastening the kinetics of actual folding patterns, reverting the aggregation of denatured polypeptides and improving the in vitro refolding of denatured protein [20,34].

4.3 Protein folding/unfolding mechanism

Protein folding is a process of molecular self-assembly resulted via collapsing of distorted polypeptides into an active three-dimensional structure [2,35]. For a protein to carry out the evolved functions, understanding the molecular mechanism of protein folding is of prime importance and has been the area of intense research for more than 50 years [36,37]. Naturally, a folding process occurs within the cell and is assisted by chaperone proteins and the local environment [2]. Molecular interactions in native protein's structure depend on certain protein sequences, these interactions are both cooperative and supportive leading to the functional structure of a protein. These interactions encompass electrostatic interactions, hydrogen bonding, Van der Waals interactions and hydrophobic burial and occur between ligands, protein moieties, solvent molecules and cofactors [36,37]. Through these interactions, the functional groups of amino acids unite hence, facilitating the biological functioning of the protein.

Widespread research conducted in the field of protein folding has proposed several protein folding theories and models [38–40]. Anfinsen's experiment on ribonuclease A was the first hypothesis in the field of protein folding and was termed Anfinsen's dogma. The hypothesis determined that the primary structure of the protein directs its folding pathway which eventually leads to its native state. Furthermore, it added that in few cases, the protein folding is aided by small chaperone molecules [38]. The recent protein folding pathways are elucidated by the free energy funnel model which also explains the thermodynamics of the protein folding process [39]. According to which the native structure of the protein has the lowest energy in its whole conformational space and is represented as the narrow end of the funnel that is the bottom. Whereas, the unfolded state is symbolized by the broader end of the funnel and has high entropy and free energy. As the protein folding process advances down the funnel, the process faces a diminished number of protein conformations which includes molten globules, semistable and near-native state. The fate of escaping these protein conformations is considered as local free energy minimum and a protein reaches its native state which is both kinetically accessible and thermodynamically favorable.

Nevertheless, for the sake of simplicity, researchers focus on the reversible two-state equilibrium model [40]. Further, the Lumry-Eyring model explains the irreversible aggregation process resulted via reversible transformation of native structure to a partially unfolded/misfolded intermediate (T) [41]. Generally, the exposure of protein molecules to high temperatures perturbs the native conformation to an extent that creates intermediates promoting aggregation. The Gibbs free energy of protein unfolding is given by

$$\Delta G_u = \Delta H_u - T\,\Delta S_u$$

Here ΔG_u is Gibb's free energy of unfolding, ΔH_u is the enthalpy of unfolding and ΔS_u is the entropy of unfolding. The low value of ΔG depends on the mutual compensation of ΔH_u and ΔS_u,

which can be shifted by various solvents [41]. Furthermore, there exists limited knowledge about the mechanism of stabilization of protein in presence of ILs and further research needs to be elaborated to comprehend how ILs interacts with biomolecules.

4.4 Overview of protein stability in ionic liquids

The advantageous properties of ILs have made them potent solvents in enzymatic biocatalysis, media for protein preservation, protein purification, and are also the enhancers for the solubility of proteins [42]. The above-mentioned applications arise due to suitable physicochemical properties of ILs which are otherwise lacking in volatile organic compounds [43]. The various aspects of ILs in protein stability like refolding, crystallization, long-term protein packaging, enzymatic activity, protein solubility, thermal stability have been covered in the open literature [20,43–49]. For instance, a pioneering study conducted by Summers and Flowers [20] on lysozyme (Lys) revealed that EAN protected the protein against thermal denaturation. Afterward, Mann et al. [44] showed that ammonium-based ILs acted as excellent stabilizers for Lys and also protected it against thermal denaturation [44]. Meanwhile, the long-term protein packaging was studied by Fujita et al. [45], they developed series of cholinium-based ILs with different kosmotropicity investigated them for the long-term structural stability as well as the activity of cytochrome C (cyt C). Results revealed that cyt C dissolved in cholinium dihydrogenphosphate [Chn][DHP] remained in its native form and the enzymatic activity was maintained even after 18 months of storage at room temperature [45]. Similarly, Bisht et al. [46] demonstrated that cholinium-based ILs with dicarboxylate-based anions protected cyt C against multiple stresses and retained the long-term packaging of cyt C. Many studies were conducted on analyzing the behavior of ILs on the enzymatic activity of biologically relevant enzymes.

Remarkably, Raddadi et al. [47] scrutinized the enzymatic activity of the cellulose in imidazolium-based ILs. Outcomes suggested that in 20% of the 1-ethyl-3-methylimidazolium acetate [EMIM][Ac] and [BMIM][Cl], approximately 80% and 76% of the relative activity of the enzyme was preserved [47]. In the same way, researchers have successfully improved the solvation behavior of metalloprotein in presence of IL [2]. As illustrated by Fujita et al. [48], the interaction of hydrated [Chn][DHP] with several metalloproteins have dissolved the protein without any disturbance in the active sites of proteins [48]. Furthermore, the imidazolium-based ILs with varying cation such as 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]), 1-butyl-3-methylimidazolium tetrafluoroborate ([BMIM][BF_4]) and 1-butyl-3-methylimidazolium bromide ([BMIM][Br]) have protected the structure of Lys at extreme temperatures during crystallization [49]. Similarly, Sindhu et al. [50] reported the influence of varying concentrations of six cholinium-based ILs, [Chn][DHP], [Chn][Bit], [Chn][Ac] and [Chn] [Cl], [Chn][I] and [Chn][OH]. Through combined analysis of spectroscopy techniques, differential scanning calorimetry and molecular docking it is evident that except [Chn][I] and [Chn][OH] all the other four ILs supported the thermal and structural stability of β-Lactoglobulin (β-LG) [50]. Eventually, the same ILs were tested for their suitability toward stem bromelain (SB), it was demonstrated that [Chn][OH] completely destabilized the structure of SB while, [Chn] [Cl] have the highest ability to protect the native structure of the protein [51]. Furthermore, the effect of alkyl chain lengths in cation and concentration of imidazolium-based ILs on structural integrity

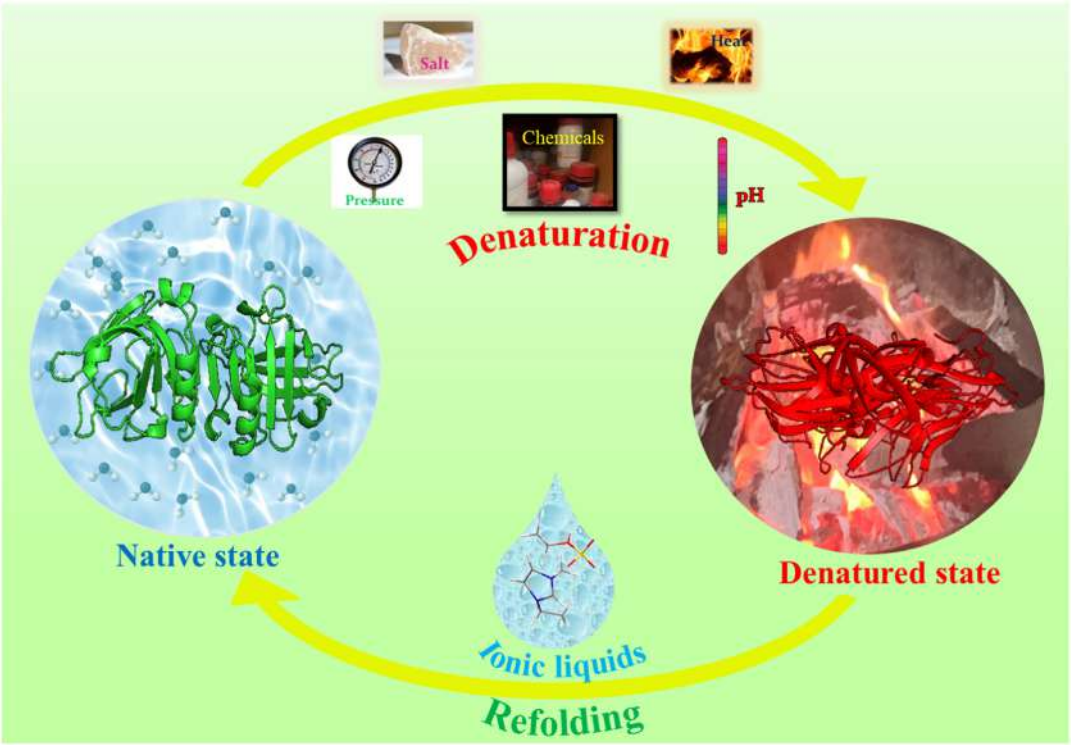
of SB. It was concluded that imidazolium-based ILs with lower concentrations and shorter cationic alkyl chain length suitably act as greener cosolvent and maintain the native structural features of protein [52]. Moving forward toward the refolding abilities of ILs, Byrne et al. [22] appraised the thermal refolding ability of EAN, the structure of Lys was refolded to its native form as well as stabilization was achieved for a prolonged period [22]. Although, for enlightening the concept of stability of proteins in the presence of ILs, there is a plethora of research in the field of IL−protein interactions still, the studies particularly related to protein refolding in presence of IL are not sufficiently understood and needs critical exploration.

4.5 Refolding ability of ionic liquids on the perturbed proteins

In vitro refolding of denatured protein is a subject of concern since for a protein to perform the appropriate functions; its native state must be preserved. The refolding of protein is a process through which the protein's native state is stabilized by accelerating the kinetics of the correct folding reaction and controlling the unspecific aggregation of unfolded polypeptide and the intermediates in its folding pathway [20,34,53]. Aggregation of protein is a serious challenge in the production, storage of industrial processes and hence causes major economic loss in pharmaceutical industries and biotechnology [54]. Protein misfolding leads to several fatal diseases like Parkinson, Alzheimer and Huntington diseases. These ailments share common features such as the occurrence of insoluble fibrillar protein aggregation rich in cross-β sheet structures [2]. To overcome such problems and to retain the biological activity of proteins, the unavoidable aggregates have to be detached from cell debris and then solubilized using chemical denaturants. Thereafter, reducing denaturant's concentration can begin refolding from unfolded to the native state of the protein [55−57].

As mentioned earlier, during the process of refolding, the aggregation of protein competes with the prescribed folding pathway. Examination of the environmental effects on protein folding suggests that solvents have fundamental importance in understanding the molecular interactions in determining protein structure [36]. Further, to improve the yield of refolding of denatured protein, additives are needed so that they can suppress the protein-denaturant interactions. Extensive research suggested that amino acids, low concentrations of chaotropes can control the intermolecular aggregation thus improving the yield of biologically active proteins [58,59]. Though these additives do not display the properties of universal refolding agents and further research needed to exploration.

Recently, ILs have been assigned to stabilize the protein activity, thereby inhibiting/reducing aggregation, and improving the in vitro refolding of denatured proteins. ILs are now being used for promoting protein refolding of the denatured proteins and thereby, acted as artificial chaperons [55,60]. Mechanistically, the additives with a tendency to interact with the protein surface more strongly than water will enrich in solvation sphere of the protein. The phenomenon is termed preferential bounding; these types of cosolvents increase the solubility of proteins, thereby favoring the denatured state ("salting in") over the native state. Whereas, the additives interact more strongly with water than with the protein and therefore, there will be preferential exclusion. Preferentially excluded cosolvents decrease the solubility of protein and increase the stability of the native form ("salting out") [54,61−64]. This simple view is directed by the Hofmeister series which provides a direct relationship between the type of constituent ions of ILs and protein activity in aqueous solutions. Based on the aforementioned



SCHEME 4.1

Schematic representation of the refolding ability of ionic liquids toward the denatured protein.

salting in and salting out effects, the ions can be ranked according to their capability to precipitate proteins. Accordingly, singly charged and large organic cations like N-alkylpyridinium having shorter alkyl chain lengths are chaotropic ("structure-breaker"), they tend to solubilize proteins by breaking the water structure. On the other hand, kosmotropes contributes to the stability of proteins and induce water-water interactions ("structure-maker") [53,62]. For a detailed understanding of refolding ability of a particular IL on perturbed protein, the structure of that IL must be well understood and for that purpose in the upcoming sections, we will separately discuss the refolding of proteins in various families of ILs such as ammonium-based, imidazolium-based, cholinium-based and pyridinium-based ILs. Scheme 4.1 represents the refolding ability of ILs toward the denatured protein.

4.6 Ammonium-based ionic liquids acted as refolding additives for denatured proteins

Ammonium-based ILs, due to their water-like properties, are considered to be good stabilizing solvents for proteins [21]. This effect arises due to the fascinating feature of ammonium-based ILs

that is to transfer the protons from acids to bases which lead to the formation of a three-dimensional hydrogen-bonded network [44,65,66]. Historically, EAN was the first protic ILs discovered in 1914 by Walden [28]. Along with the property of biocompatible cosolvent, EAN can improve the solubility of proteins, acts as precipitating agent. Under these unique properties, ammonium-based ILs becomes successful candidates for stabilizing the native form of protein and thus aiding in refolding of the denatured protein [34,67,68].

In 2000, Summers and Flowers [20] realized that EAN enhanced the recovery of denatured hen egg-white Lys. Results of the study explicitly conveyed that aggregation of denatured Lys have been suppressed with the help of EAN. The study also concluded that EAN has refolded the structure of denatured protein and there was regain in the enzymatic activity of protein [20]. Subsequently, Byrne et al. [22] discussed the thermal refolding and long-term stabilization of Lys (with protein concentration >200 mg/mL). For this purpose, the IL acquired was EAN; it has accomplished the property to protect the protein against aggregation up to 97% in a particular unfold−refold cycle [22]. Afterward, the enzymatic activity and stability of four ammonium-based ILs with varying cationic chain lengths toward Lys was obtained by Mann et al. [44]. Results obtained by circular dichroism (CD) study proposed that the refolding ability of ethylammonium formate (PAF) was similar to that of 2-methoxyethylammonium formate (MOEAF) whereas, the propylammonium formate (PAF) possesses refolding ability at higher concentrations. At a high concentration of PAF, Lys spontaneously denatures, as the adsorption of cation PA^+ at the hydrophobic core of protein protects it from the hydrophilic solvent. Now, the IL ethanolammonium formate (EtAF), uniquely stabilize the protein as chemically it is an alcohol containing IL and it interacts differently than IL without an alcoholic group. The alcoholic group of cation EtA^+ offers a complementary H-bonding acceptor/donor site thereby reducing the hydrophobic interactions of IL with protein. EtAF stabilizes the structure of Lys against thermal unfolding and the process of refolding achieves upon cooling. Lastly, the stability of Lys at higher temperatures is not recorded with MOEAF. From an activity point of view, EtAF reenforces protein structure to the maximum and has the highest ability to perform the designated activity. While, both EAF and MEOAF have the moderate ability to enhance Lys activity and comparatively an increase in activity is noted in PAF [44].

According to Attri et al. [69], the stability and enzymatic activity of α-chymotrypsin depend on the structure of constituent ions of ILs. The results obtained from biophysical studies revealed that ILs carrying longer alkyl chains are comparatively less stabilizing for enzymes than those with shorter alkyl chain lengths namely triethylammonium acetate (TEAA), triethylammonium phosphate (TEAP). Specifically, from CD and nuclear magnetic resonance (NMR) studies, it is highlighted that TEAA acted as refolding additive for thermally quenched α-chymotrypsin structure [69]. Later on, in a study performed by Mangialardo et al. [34], the fibrillar Lys was treated with four ILs from the ammonium family and their refolding efficacies were estimated. By the action of EAN, the IL with the shortest alkyl chain length; the fibrillar content was reduced to a significant extent. Further, the long alkyl chain IL that is PAN and butylammonium nitrate (BAN) partly revert the aggregated state to native conformation. These two ILs partly dissolved the Lys fibrils and hence only a partly unaggregated form was achieved. On the other hand, IL with an ether group that is 2-methoxy ethyl ammonium nitrate (MEOAN) completely failed to behave as a refolding medium for protein. With these long cationic chains ILs, the protein conformation can contain around 70% of actual protein structure that is in between fibrillar form and native form [34]. This study explicitly coneys that the refolding ability of IL depends on the molecular structure of cation;

as the alkyl chain length increases the refolding ability of IL decreases and vice-versa. A schematic representation of refolding ability of ILs (PAN, BAN, EAN and MEOAN) toward thermo-chemical induced denatured Lys has been presented in Fig. 4.1.

Meanwhile, Attri and Venkatesu [70] reported the biomolecular interactions of ILs with different kosmotropic anions and chaotropic ammonium cation with succinylated Con A. The ILs incorporated in the study was trimethylammonium hydrogen sulfate (TMAS), trimethylammonium dihydrogenphosphate (TMAP), trimethylammonium acetate (TMAA) and TEAP. Results explained that ammonium-based ILs acted as stabilizers for the native structure of succinylated Con A. Among all the four ILs, TEAP was the most compatible IL for native succinylated Con A and acted as refolding additive for thermal denatured succinylated Con A. The authors also confirmed that the ILs followed Hofmeister series and the chaotropic ammonium cation stabilizes the succinylated Con A structure by supporting the water structure around it [70]. Extending this area of protein refolding in presence of ILs, the same research group [71] comprehended the capability of TEAP to refold urea-induced denaturated α-chymotrypsin and succinylated Con A. Accordingly, TEAP has performed potent refolding of the urea-induced denatured state of α-chymotrypsin and succinylated Con A. TEAP has refolded up to 5 M urea-induced denatured succinylated Con A whereas; TEAP was able to refold up to a 3 M urea-induced structure of α-chymotrypsin [71]. Later in 2016

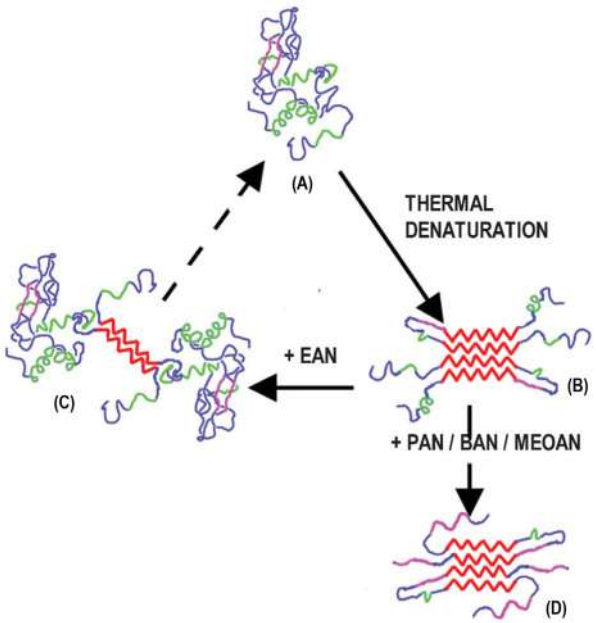


FIGURE 4.1

Conformations of (A) native Lys, (B) fibrillar Lys, (C) partially refolded Lys in presence of EAN and (D) average configuration of Lys in presence of PAN, BAN and MEOAN.

This figure has been reprinted from ref. S. Mangialardo, L. Gontrani, R. Caminiti, P. Postorino, Role of ionic liquids in protein refolding: native/fibrillar vs treated lysozyme, RSC Adv. 2 (2012) 12329–12336, with copyright permission from Royal Society of Chemistry.

Bisht et al. [65] demonstrated that the ammonium-based ILs has increased the activity of urea deactivated Lys over a Micrococcus lysodeikticus cell suspension up to 13% of the control value. The increase in activity directs toward the refolding ability of butyltrimethylammonium bis(trifluoromethylsulfonyl)imide and diethylmethyl(2-methoxyethyl) ammonium trifluromethylsulfonyl imide for urea-denatured Lys [65]. As a result, the shorter alkyl chain lengths of ammonium-based ILs are the best refolding additives for the denatured form of proteins.

4.7 Imidazolium-based ionic liquids acted as refolding additives for perturbed proteins

Another family of ILs that display stability for biomolecules and also the attention in terms of their refolding ability is imidazolium-based ILs. Reports confirmed that the imidazolium-based IL can act as a stabilizer or destabilize depending upon the nature of the constituent anion [72,73]. In a study by Lange et al. [62], a series of imidazolium-ILs with the same anion Cl^- and different cations like [Emim] + , [Bmim] + , [Hexmim] + was tested for their refolding ability for completely denatured Lys. As expected, the stability of the protein in these ILs decreased significantly as the length of the alkyl chain of cation increased from ethyl to butyl and then hexyl. Interestingly, all three ILs enhanced the refolding of guanidinium hydrochloride (GdnHCl)-induced denatured Lys in a concentration-dependent manner. At higher concentrations of ILs, there was a decrease in the refolding potential of all these ILs [62]. A trailblazing study was conducted by Yamaguchi et al. [74] in which the role of the chemical structure of IL on the refolding yield of protein was demonstrated. The authors scrutinized the properties range of N′-substituted N-methylimidazolium chlorides toward the oxidative refolding of Lys. Results proposed that the short length N'-alkyl chain cations were the great refolding agents for the distorted Lys structure. The study conveyed that as the hydrophobicity of IL increases, the refolding yield of protein diminishes [74]. Afterwards, Lange and coworkers [53] tested the protein aggregation suppressing ability and in vitro refolding ability of N-ethyl-N-methyl imidazolium (EMIM) salts with different anions. The anions chloride (Cl^-), 2(2-methoxyethoxy)-ethyl sulfate ($MDEGSO_4$), ethyl sulfate ($EtSO_4$), acetate (Ac^-), toluenesulfonylate (OTs), diethyl phosphate (Et_2SO_4), hexyl sulfate ($HexSO_4$). The model biomolecule in the study was a recombinant plasminogen activator. The authors concluded that the stability of a protein molecule in IL equally depends on the cation and anion of the IL. Though, the studies based on changing only one of the constituent ions (either fixed cation/anion) could generate an idea on how the individual ion promotes protein stability [53].

Afterwards, Mojumdar et al. [75] scrutinized the consequence of pentyl-3-ethylimidazolium bromide ([PMIM][Br]) on the innate state as well as on the unfolded state of the cytochrome c with the help of fluorescence correlation spectroscopy. Their results proposed that when the concentration of IL was increased in the protein solution, there was an increase in unfolded structure. The hydrodynamic radius of cytochrome c was increased up to 20.1, 23.2 and 25.2 Å in 0.3, 0.9 and 1.5 M of [PMIM][Br], respectively which was otherwise 18.6 Å in the control case that is in absence of IL. Importantly, [PMIM][Br] has also refolded the GdnHCl treated cytochrome c. The hydrodynamic radius of the cytochrome c decreased from 30.3 Å (i.e. in unfolded state by 6 M guanidinium hydrochloride) to 25.3 Å in of 1.5 M of [PMIM][Br] [75]. Eventually, Takekiyo et al. [8] utilized an aqueous solution of 1-butyl-3-methylimidazolium nitrate ([BMIM][NO_3]) for its suitability for Lys with

the help of CD, fourier-transform infrared spectra small-angle X-ray scattering analysis. Through the results obtained by employing these techniques, the authors illustrated that change in concentration and structure of IL controlled its suitability for protein. An increase in concentration up to 5 M of [BMIM][NO_3] disturbed the tertiary structure of the protein. Later, the secondary structure of Lys was partially refolded in presence of 6 − 10 M concentration of [BMIM][NO_3], though the tertiary structure was disrupted. Then, with an increase in the concentration of [BMIM][NO_3] (i.e. up to 10 M), the secondary structure was still perceived in a partially refolded state while the tertiary structure was completely disordered. The reason proposed behind the observation by Raman spectra analysis; the structural feature of aqueous IL directed that bulk-like water remained at the concentrations above 10 M and thus formed a water pool in the nanoheterogeneous structure. This structure consists of a polar domain with a high charge-density nonpolar region (the alkyl chain) [8].

Successively, in a study by Tischer et al. [76], the IL N-ethyl-N-methyl imidazolium chloride ([EMIM][Cl]) is an efficient refolding agent for the recombinant plasminogen activator. The reason proposed behind the observation is believed that IL favorably interacts with the amino acid side chain rather than with the peptide backbone. With a rise in concentration [EMIM][Cl] because of low water content strongly interacts with amino acid side chain hence denaturing the protein [76]. In due course, Reddy et al. [77] divulged the counteracting influence of 1-butyl-3-methylimidazolium bromide ([BMIM][Br]) on the perturbing effect of 1-butyl-3-methylimidazolium iodide ([BMIM][I]) of α-chymotrypsin. By applying various biophysical techniques, it was validated that at only low concentration [BMIM][Br] stabilized α-chymotrypsin, whereas the same acted as destabilizer with increased concentration. The IL [BMIM][I] destabilized α-chymotrypsin with all the studied concentrations. This structural destabilization was counteracted by [BMIM][Br] and the effect is prominent at low concentration [77]. Thereafter strikingly, in an analysis by Takekiyo et al. [78] 1-butyl-3-methylimidazolium chloride ([BMIM][Cl]) at low concentration have unfolded the native structure of myoglobin and cytochrome c while high concentration of the IL partially refolded the cytochrome c and induced aggregation to myoglobin [78]. A few years later this group Takekiyo et al. [79] discussed the three aspects of imidazolium-based ILs, they were their solubilization, cryopreservation and refolding ability. The model protein and ILs considered for this study was bovine heart cytochrome c and EAN and 1-butyl-3-methylimidazolium thiocyanate ([BMIM][SCN]) respectively. Both the ILs decreased the activity and unfolding of protein's secondary structure prior to and later after cooling at 77K.

Overall, the results directed that concentrated IL solution prospects the one-pot (solubilization, preservation and refolding) to proteins [79]. The effect of series of imidazolium-based ILs like [EMIM][Ac], 1-ethyl-3-methylimidazolium tetrafluoroborate ([EMIM][BF_4]), 1-ethyl-3-methylimidazolium ethylsulfate ([EMIM][ES]), 1-ethyl-3-methylimidazolium trifluorosulfonate ([EMIM][TfO]), [EMIM][Cl], [BMIM][Cl], 1-hexyl-3-methylimidazolium chloride ([HMIM][Cl]), 1-methyl-3-octylimidazolium chloride ([OMIM][Cl]) on heme protein horseradish peroxidase was corroborated by Bae et al. [80]. The metal cofactors of enzyme and ILs have a beneficial impact on refolding of the enzyme. For instance, upon addition of [EMIM][Cl] to the refolding buffer with calcium and hemin cofactors boosted the enzyme's refolding yield to 80% which was otherwise only 12% in refolding buffers. Hence it is assumed that metal cofactors when simultaneously used with ILs improve the refolding yield as well as the efficacy of metalloproteins [80]. Among all the studied ILs [EMIM][Cl] exhibited the highest HRP activity. The effect of the alkyl chain of cation of imidazolium chloride on its refolding ability for both GdnHCl and base induced denatured cytochrome c by Singh et al. [81]. The ILs utilized were 1-methyl-3-octylimidazolium chloride [C_8MIM][Cl], and 1-decyl-3-methylimidazolium chloride

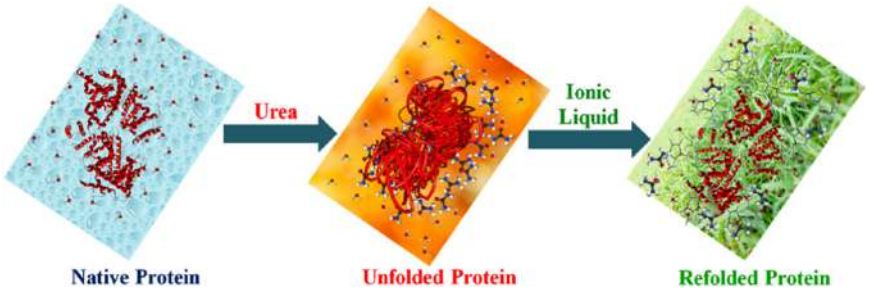


FIGURE 4.2

Refolding ability of imidazolium-based IL toward urea-denatured HSA structure.

This figure has been reprinted from ref. A. Sindhu, K. Bhakuni, K. Sankaranarayanan, P. Venkatesu, Implications of imidazolium-based ionic liquids as refolding additives for urea-induced denatured serum albumins, ACS Sustain. Chem. Eng. 8 (2020) 604 – 612, Copyright 2020, with permission from American Chemical Society.

$[C_{10}MIM][Cl]$. The outcomes of the study showed that IL with long alkyl chain length ($[C_{10}MIM][Cl]$) stimulated the refolding of base-denatured cytochrome c while short alkyl chain length IL ($[C_8MIM][Cl]$) showed only slight refolding. The mechanism proposed that $[C_8MIM]$ cation bounded the unfolded cytochrome c and triggered the refolding by the combination of hydrophobic and electrostatic interactions that stabilize the molten globule state [81].

Thereafter, Singh et al. [82] realized the temperature dependence on refolding of denatured cytochrome c in acidic conditions under the effect of anionic surface-active IL (SAIL) that is 1-butyl-3-methylimidazolium octyl sulfate ($[C_4MIM][C_8OSO_3]$). This SAIL has unfolded the cytochrome c under acidic conditions and stabilized its molten globule state. The effect of refolding by SAIL decreased with increased temperature. The SAIL can be efficiently employed in biomolecule studies and used as an additive to resolve problems related to aggregation and misfolding of proteins [82]. Very recently, in an investigation by Venkatesu and coworkers [83] the biocompatibility and refolding ability of two imidazolium-based ILs, 1-ethyl 3-methyl imidazolium ethyl sulfate $[EMIM][ESO_4]$ and [EMIM][Cl] against urea-denatured bovine and human serum albumin was monitored. The work was authenticated with several spectroscopic, docking and thermal studies. CD spectral data reflected the recurrence of α-helical content, indicated the refolding aptitude of both $[EMIM][ESO_4]$ and [EMIM][Cl]. Further, the thermal analysis predicted that these ILs have refolded the proteins even at higher temperatures up to 7 M urea concentration. The work was also sustained by dynamic light scattering (DLS) measurements and molecular docking studies [83]. Overall, the mechanistic intricacies of imidazolium-based ILs have been discussed in the open literature. Imidazolium-based ILs is an efficient recoiling medium for chemical, thermal and pH-induced denatured proteins and is portrayed in Fig. 4.2.

4.8 Cholinium-based ionic liquids acted as refolding additives for perturbed proteins

The cholinium-based ILs acquired immense attention from researchers in the field of protein stability analysis. They are cost-effective, organic, water-soluble and mainly they are biodegradable.

Choline (2-hydroxyethyltrimethyl ammonium chloride) is a part of quaternary ammonium salts and is associated with numerous anions [84]. Choline is acquired naturally and is largely classifies with B-complex vitamins [85]. The cholinium-based ILs has currently been expansively employed as a stabilizing additive for biomolecules, especially for proteins. Initially, Fujita et al. [86] investigated the dissolution of aggregated recombinant cellulase expressed in E. coli in presence of choilinium dihydrogenphosphate ([Chn][DHP]). The authors mentioned that controlling the water state of hydrated [Chn][DHP] influenced the process of renaturation of biomolecules. Results showed that hydrated [Chn][DHP] with controlled water content successfully refolded the aggregated recombinant protein from Escherichia coli without any further processing [86]. Later on, Jha et al. [87] delineated the counteracting ability of cholinium chloride ([Chn][Cl]) to offset the pH-induced denaturated state of Lys. The results procured from CD, UV − vis, fluorescence spectroscopy, and DLS indicated that the thermal stability and secondary structure of Lys increased considerably in [Chn][Cl] as compared to that in buffer at pH 2. In the concluding remark, the authors have elucidated that the high efficiency of [Chn][Cl] for counteracting the adverse effect of acidic conditions on Lys activity and conformation may help in resolving the problems encountered during the therapeutic application of Lys [87].

In open literature, it has been documented that when two ILs are mixed in certain fixed proportions, the negative effect of the first IL can abandon the effect caused by the second one, thus enhancing the advantageous characters of IL mixture [88]. Toward this, Sindhu et al. [89] mixed two cholinium-based ILs to counteract the effect of one IL by the other IL on β-LG structure. Surprisingly, all the four ILs that is [Chn][Ac], [Chn][DHP], [Chn][Bit] and [Chn][Cl] demonstrated counteracting ability against [Chn][I]-induced perturbed structure of β-LG [89]. This approach provides a good approach to offset the deleterious effect of one IL by the other and can be comprehended for its application in the field of protein folding/unfolding studies. Recently, Bhakuni et al. [90] have utilized five cholinium-based ILs with different anions and varying carbon chain lengths namely; cholinium citrate ([Chn][Cit]), cholinium malonate ([Chn][Mal]), ([Chn][Bit]), cholinium propionate ([Chn][Prop]), and [Chn][DHP] to study their refolding and counteracting behavior for perturbed human serum albumin by crowding effects caused by dextran-6. Among them, [Chn][DHP] and [Chn][Bit] were most capable of preserving the native structure of human serum albumin. Also, they have the higher ability to counteract the perturbed human serum albumin structure. Moreover, these ILs have positive effect to refold the structure of dextan-6 denatured protein and thermal stability of perturbed human serum albumin was also enhanced by both [Chn][Bit] and [Chn][DHP] [90]. Subsequently, the authors highlighted the application of ILs to provide stability to protein structure under a crowded cell-like environment and the schematic representation is provided in Fig. 4.3.

4.9 Pyridinium-based ionic liquids acted as refolding additives for perturbed proteins

Pyridinium-based ILs demonstrates a wide range of physicochemical features due to various combinations of pyridinium-cation and anions. In 2011, Yamamoto et al. [55] carried out an investigation on N-alkylpyridinium chlorides for their refolding potential for 30 mg/mL denatured Lys as a

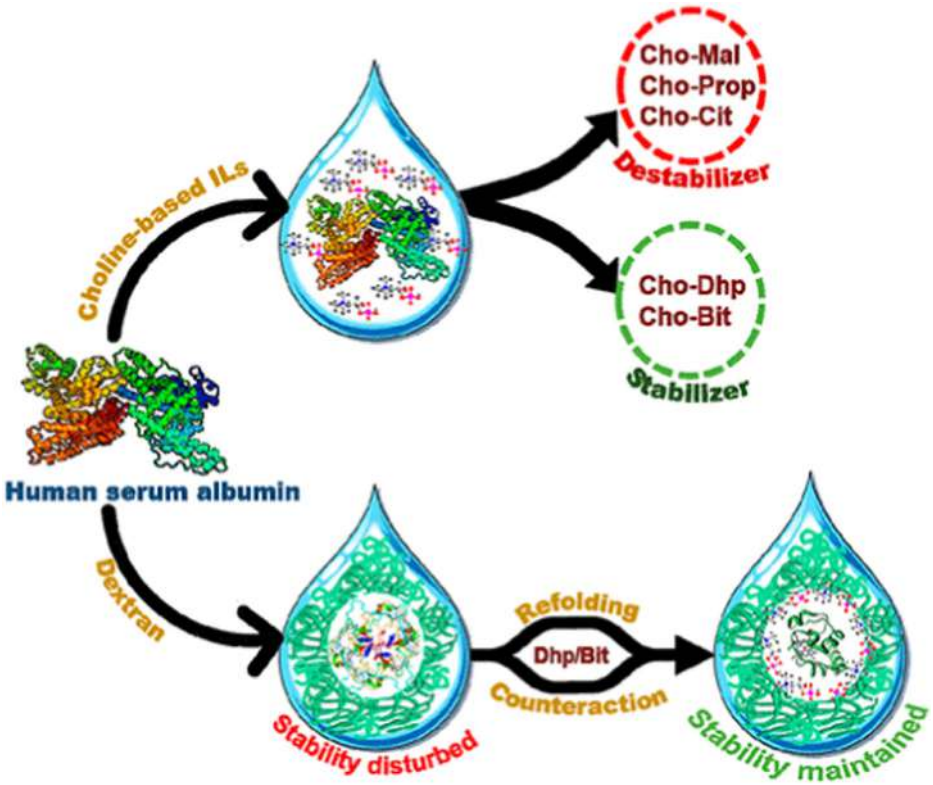


FIGURE 4.3

Refolding and counteracting ability of [Chn] ILs toward dextran-6 denatured HAS structure.

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model protein. The less hydrophobic ILs for instance N-ethyl, N-butyl and N-hexylpyridinium chlorides have effectively refolded the chemically denatured protein with a yield of 46%–69%. Primarily, these ILs suppressed the aggregation process of protein due to their chaotropic properties. The calculated partition coefficient (log P) values of cations computed the hydrophobicities of N-alkylpyridinium chlorides and the calculated log P supported the potential of ILs to prevent the aggregation even at very low concentrations. This study provides an array of ILs with high refolding yields and also provides knowledge regarding the influence of chemical structures of ILs on the procedure of protein refolding [55]. Recently, Patel and coworkers [60] have first synthesized the three pyridinium-based ILs namely; butyl pyridinium bromide, hexyl pyridinium bromide, octyl pyridinium bromide and then explored their refolding ability toward 2 M GdnHCl treated bovine serum albumin. Using fluorescence, CD spectroscopy, DLS and ANS (8-Anilinonaphthalene-1-sulfonic acid) binding measurements, it was corroborated that the ILs have increased the secondary

structure of the unfolded protein. Among all the studied ILs, 1-butyl pyridinium bromide found a prominent ability to refold the GdnHCl-induced denatured protein. As evident from % secondary structure analysis, in presence of 1-butyl pyridinium bromide with denatured protein, there is an increase in the α-helical content up to 47% which was otherwise 30% without IL. The hydrodynamic size analysis showed that in presence of these ILs the size of denatured protein decreased which may be ascribed to the enhancement in compactness thereby supporting the refolding of protein in ILs [60]. Hence, as all the three ILs assisted the folding of chemically unfolded bovine serum albumin structure they acted as artificial chaperons.

4.10 Pyrrolidinium-based ionic liquids acted as refolding additives for perturbed proteins

The refolding ability of N-butyl-N-methylpyrrolidinium chloride for Lys was examined by Yamamoto et al. [55]. It was demonstrated that due to the less hydrophobic and chaotropic nature of N-butyl-N-methylpyrrolidinium chloride, it has successfully suppressed the protein aggregation and hence effectively refolded the protein with a refolding yield of 46%−69%. The study proposed the nature of cations and anions of ILs governs their ability to provide biocompatibility, solvation, stability and refolding to the biomolecules [55].

4.11 Phosphonium-based ionic liquids acted as refolding additives for perturbed proteins

Recently, phosphonium and ammonium-based ILs have been employed for determining their dissolution and refolding properties for aggregated concanavalin A [91]. The results obtained from fluorescence emission intensity analysis and sugar-binding ability of dissolved concanavalin A presented that both the hydrated phosphonium and ammonium-based ILs were suitable for refolding the denatured protein. The solubility of aggregated concanavalin A in the studied ILs was decreased upon an increase in water content in IL [91]. In this study as phosphonium-based ILs are more hydrophobic than their ammonium-based counterparts and are expected to exhibit better solubility. However, there was no direct relationship between the solubility and hydrophobicity of IL [91]. Pictorial representation of relation between the fluorescence intensity of concanavalin A and total number of carbon atoms in alkylic chain of cation of IL is provided in Fig. 4.4.

4.12 Morpholinium-based ionic liquids acted as refolding additives for perturbed proteins

Lastly, we will discuss the impact of morpholinium-based ILs on the refolding of enzyme Lys. Pabbathi et al. [92] executed the near-UV CD and fluorescence correlation measurements to study the impact of morpholinium salts on native and chemically denatured chicken egg-white Lys

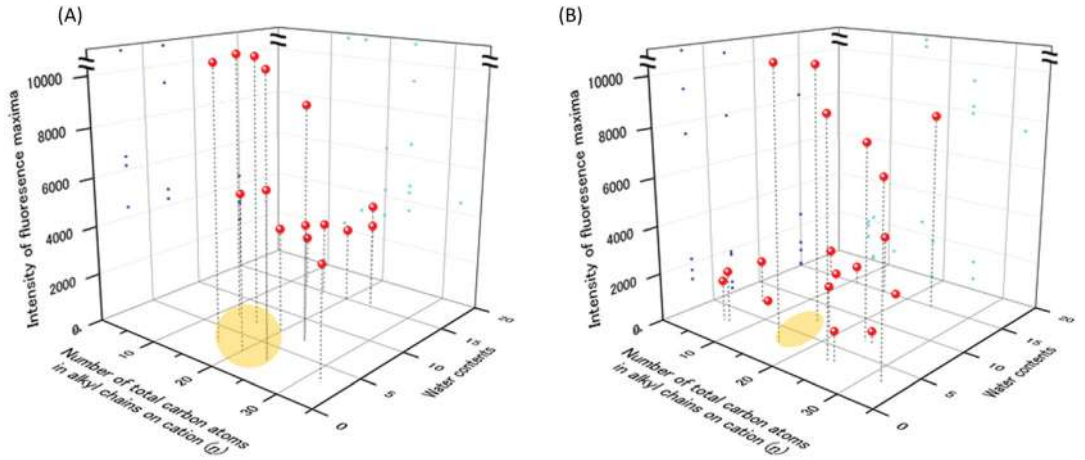

FIGURE 4.4

Relation between the fluorescence intensity of concanavalin A in aqueous IL solution, total number of carbon atoms in alkylic chain of cation (*n*), and water content for (A) phosphonium-based cation and (B) ammonium-based cation. The yellow colored areas display the excellent condition for greater solubility as compared to water content and *n*.

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structure. It was found that the two morpholinium salts varying in the alkyl chain lengths of cation stabilizes the native form of Lys. Moreover, the morpholinium salts showed the capability to refold the GdnHCl-induced unfolded Lys making it more compact [92].

The underlying reason for such stability was assigned to the biocompatibility of morpholinium salts. Hence, like other families of ILs such as imidazolium-, ammonium-, cholinium-, these morpholinium-based ILs are also efficient refolding enhancers for deleterious structures proteins. However, the information on this topic is limited and hence more research should be conducted.

4.13 Conclusions

The preservation of the native structure of a protein is of prime importance for its biological functioning. Toward this, the irreversible protein inactivation must be counteracted/renatured with suitable solvents. The prevailing strategy encompasses the preservation and enhancement in the native conformation of protein along with protecting the formation of unavoidable aggregates. ILs offers an interesting feature to promote the native form, provide refolding environment, and also suppress the aggregated form of protein. Among varied families of ILs, ammonium-based ILs has the pronounced ability to protect the biomolecules against various stresses like chemical and thermal denaturation. Along with the ammonium-based ILs cholinium-based ILs due to their

biocompatible nature also have the ability to promote the refolding of protein. Moreover, with appropriate concentration and smaller alkyl chain length cation of imidazolium-based ILs supports the refolding pathway of denatured protein and results in renaturation of the protein. Besides, cholinium-based and imidazolium-based ILs have the ability to counteract the deleterious action of denaturants like pH, high temperature, chemicals toward proteins. The governing factors of ILs for providing stability to protein include alkyl chain length of cation, type of anion, polarity, hydrophobicity and viscosity. Judicious selection of IL is the prerequisite step to optimize the relevant phenomena of stabilization of the native state of protein. Anions have a prominent role in deciding the characteristics of IL and are governed according to the Hofmeister series. Astonishingly, at the right concentration, an IL can provide stability to the protein as well as aids in biological activity irrespective of its type. Additionally, miscibility is also a determining factor in the protein folding/unfolding process, water-miscible ILs has been documented to be the strong stabilizers for proteins. Indeed, it may be concluded that the mechanism of protein folding depends on both the IL used and the protein under study. Overall, future endeavors in protein folding studies should focus on designing ILs to overcome the challenges faced due to diverse IL compositions and the complexity and variability in various proteins.

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CHAPTER

Stability and stabilization of biocatalysts by ionic liquids

5

Hua Zhao

Department of Chemistry, Howard University, Washington, DC, United States

5.1 Introduction

Ionic liquids (ILs) are made of ions and remain liquid at temperatures below 100°C. Those are liquids at room temperature, known as Room-Temperature ILs, and are favorable solvents for various applications. Compared with traditional volatile organic solvents, (most) ILs have a variety of desirable physiochemical properties such as very low volatility, low flammability, a wide liquid range, high ionic conductivity, high thermal conductivity, strong dissolution capability for many substrates, high thermal and chemical stability, and a wide electrochemical potential window. Because of their distinct properties, ILs gained wide applications in chemical, materials and engineering sectors such as organic catalysis [1–9], inorganic synthesis [10], biocatalysis [7,11–16], polymerization [17,18], and engineering fluids [19–21]. ILs usually consist of organic cations and organic/inorganic anions. Common cations are aromatic/nonaromatic nitrogen-containing (e.g., alkylammonium, *N, N′*-dialkylimidazolium, *N*-alkylpyridinium and pyrrolidinium), phosphorous containing (e.g., alkylphosphonium), or sulfur containing (e.g., alkylsulfonium). Some common anions are halides, BF_4^-, PF_6^-, OAc^- (acetate), $CF_3CO_2^-$, NO_3^-, Tf_2N^- [$(CF_3SO_2)_2N^-$], $[RSO_4]^-$, and $[R_2PO_4]^-$.

More importantly, structures and properties of ILs (e.g., polarity, hydrophobicity, and hydrogen-bond basicity) can be fully designed through a judicious section of cations and anions and proper functionalization of these ions. Especially when they are tailored for particular applications, these ionic solvents are known as task-specific ILs [22–24] or functionalized ILs [25–27]. Therefore, IL structures and properties can be individually tuned for particular biocatalytic systems, which allows many enzymatic reactions to be carried out in various types of ILs [12,13,16,28]. Although some ILs (such as those contain anions of PF_6^- and Tf_2N^-) tend to be more enzyme-stabilizing than organic solvents [16,29], many enzymes display similar activities in conventional ILs as in organic solvents, which are usually much lower than their activities in aqueous systems.

Many ILs could solubilize substrates that cannot be easily dissolved in common organic solvents, including cellulose (10–20 wt.%), other carbohydrates (such as >100 g/L β-D-glucose, sucrose, lactose, and β-cyclodextrin), ascorbic acid, amino acids, fatty acids, and triglycerides [30–33]. However, most of these ILs contain anions such as Cl^-, dicyanamide (dca^-), formate ($HCOO^-$), and acetate (OAc^-), which are enzyme-inactivating when used at high concentrations [34–36]. To convert these substrates via enzymatic transformations, it is important to understand what IL structures and properties ("controlling factors") dictate the enzyme activity and stability, and what methods could stabilize or even activate

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enzymes in these ionic media. Controlling factors to be discussed include IL network structure, IL polarity and hydrophobicity, hydrogen-bond basicity and nucleophilicity of anions, ion specificity and Hofmeister series, viscosity, and surfactant effect. Two categories of enzyme-stabilizing methods include modifying enzyme's microenvironment using ILs and designing enzyme-compatible functionalized ILs.

5.2 Enzyme stability in ionic liquids: controlling factors

The stability of enzymes in ILs is influenced by some common factors (e.g., the water activity, pH, excipients, and impurities) as in other nonaqueous solvents [37]. More importantly, enzyme stability in ILs is governed by unique structures and properties of these ionic solvents including IL network structure, polarity and hydrophobicity, hydrogen-bond basicity and nucleophilicity of anions, ion specificity and Hofmeister series, viscosity, and surfactant effect.

5.2.1 Ionic liquids network structure

1,3-Dialkylimidazolium cations can lose the acidic proton at C(2) of the imidazolium ring to form stable imidazol-2-yl carbenes; pK_a values of C(2) protons mostly fall in the range of 21−24 in water and DMSO [38,39]. Therefore, imidazolium-type ILs tend to develop polymeric supramolecular network through hydrogen bonds, also known as organized "nano-structures," which contain polar and nonpolar regions in solid, liquid and solutions of ILs, or sometimes in the gas phase; specifically, each imidazolium cation coordinates with at least three anions and each anion coordinates with three cations, leading to a hydrogen-bonded polymeric network in the form of $[(R_1R_2IM)_x(X)_{x-n}]^{n+}[(R_1R_2IM)_{x-n}(X)_x]^{n-}$ (where R_1R_2IM signifies 1,3-dialkylimidazolium cation, and X is the anion) [40,41]. In general, the supramolecular network of ILs can be demonstrated by Fig. 5.1, which evolves to various stages of structures upon dilution by other solvent molecules, including aggregates and inclusion compounds, charged and neutral clusters, triple ions, contact ion pairs, solvent-shared ion pairs and loose ion pairs [42]. When studying the structures of protic and aprotic ILs (i.e., [MMIM][Tf_2N], [MIM][Tf_2N] and [IM][Tf_2N]) by high-energy total scattering (HETS) experiments and MD simulations, the Umebayashi group [43] concluded that the closest cation−anion orientation varies without considerable longer range ordering of $r > 12$ Å by the *N*-methyl substitution to proton, yielding the second layer containing ions of the same sign configuration changes. Also, they reported that for the protic imidazolium, the O atoms of Tf_2N^- anions tend to form hydrogen bonds (O···HN) with the NH hydrogen; although this O···HN hydrogen bond is short and linear, the C_2H···O bond appears long and bent. Through far infrared spectra, Knorr and coworkers [44] found hydrogen bonding not only between cations and anion in [Cholinium][Tf_2N], but also between ions of the same charge such as forming cooperative hydrogen bonds as OH···OH···O═S (OH from two choliniums and O═S from Tf_2N^- anion). Therefore, [Cholinium][Tf_2N] has an enhanced hydrogen bond network than [Me_3NPr][Tf_2N], which explains a higher melting temperature, a higher viscosity, and a lower conductivity for the former IL. In summary, ILs comprise nanostructured segregates or microphases containing both polar region (high-charge density domain) and nonpolar region (low-charge density domain); the nonpolar region can expand from dispersed/isolated islands to a continuous phase with longer alkyl side chains [45−47].

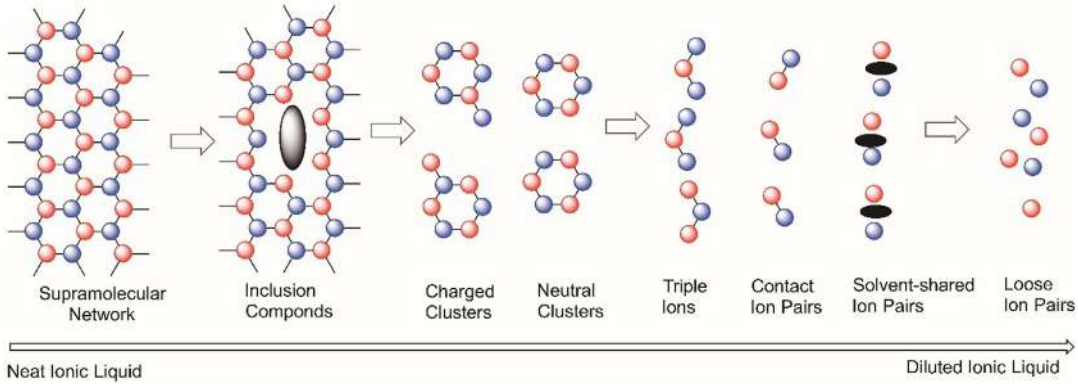

FIGURE 5.1

2D visualization of structural changes of an IL from neat state to its infinite dilution. Most of these structures have been verified by experimental and/or simulations data (red spheres = anions, blue spheres = cations, black spots = solvent molecules, and the lines represent hydrogen bonds and/or other weaker interactions).

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When solutes are mixed with ILs, these solute molecules could be solvated in different IL microphases depending on their polarity/compatibility with each domain; dipolar or quadrupolar solutes have strong interactions with polar nanophase and restructure the adjacent polar network [46]. The Dupont group [41] suggested that polymeric IL network could embed other molecules and macromolecules to form polar and nonpolar regions. For example, aqueous protein/enzyme molecules can be solvated by the IL network to maintain protein's native structures through preferential hydration and solvophobic interactions [48]. Due to the tight enclosure of protein molecules by highly ordered supramolecular IL structures, the thermal stability of biomolecules could be enhanced [49]. This IL network theory for enzyme stabilization (or destabilization) seems more applicable to hydrophilic or amphiphilic type of ILs than hydrophobic ILs (where enzymes or aqueous enzyme droplets are hardly dispersed in IL network).

5.2.2 Ionic liquids polarity and hydrophobicity

Solvent polarity can be described by different parameters such as dielectric constants (ε_r), Hildebrandt solubility (δ), dipole moments (μ), and solvatochromic polarity scales (e.g., E_T^N and Kamlet-Taft scales). E_T^N is a normalized solvatochromic polarity scale (in the range of 0.0−1.0), where 0.0 represents nonpolar tetramethylsilane and 1.0 represents highly polar water [50]. E_T^N values of some common organic solvents and ILs are listed in Table 5.1 [52]; based on this polarity scale, ILs have similar polarity with low alcohols [55,56] or formamide [50].

Biocatalytic reactions in ordinary organic solvents show a general rule of thumb that enzymes are usually more active and stable in less polar and more hydrophobic organic solvents [57] despite some exceptions such as solvent polarity and water-miscibility being not correlated with enzymatic

Table 5.1 E_T^N **Scale as a measure of solvent polarity [51].**

Solvent	E_T^N (25°C)	References
Formamide	0.775	[50]
Methanol	0.762	[50]
Ethanol	0.654	[50]
1-propanol	0.617	[50]
[EMIM][Tf_2N]	0.676	Selected value by Ref [52]
[BMIM][Tf_2N]	0.642	[53]
[HMIM][Tf_2N]	0.654	Selected value by Ref [52]
[BMIM][PF_6]	0.667	[53]
[EMIM][BF_4]	0.710	[54]
[BMIM][BF_4]	0.673	[53]
[BMIM][OTf]	0.667	Selected value by Ref [52]
[BMIM][NO_3]	0.651	Selected value by Ref [52]
[BMIM][OAc]	0.571	Selected value by Ref [52]
[BMIM][CF_3COO]	0.630	Selected value by Ref [52]
[EMIM][dca]	0.648	Selected value by Ref [52]
[BMIM]Cl	0.614	Selected value by Ref [52]

activities of three lipases and one protease in organic solvents [58]. In IL media, many studies came to a similar conclusion of higher enzyme activity and stability in more hydrophobic ILs (e.g., those carrying Tf_2N^- or PF_6^- anions) than in the hydrophilic type [containing halides, acetate or dicyanamide (dca^-) anions], although some hydrophilic ILs (such as those with BF_4^- and OTf^- anions) enabled relatively high enzyme activities and stabilities [16,57,59,60]. A few studies found high enzyme activities in more polar ILs [54,61,62]; however, a general correlation between IL polarity and enzyme activity/stability cannot always be established for most enzymatic reactions [37,63–65]. For example, Table 5.1 suggests similar E_T^N polarity values between enzyme-denaturing ILs (such as [EMIM][dca], [BMIM]Cl, and [BMIM][OAc] at high concentrations) and those enzyme-compatible ILs carrying anions of Tf_2N^- and PF_6^-. Therefore, a single polarity scale such as E_T^N may not be the controlling factor for enzyme activity and stability in ILs.

Hydrophobicity (or lipophilicity) is a function of solvent polarity and entropic effect on water [66]. In practice, it is valuable to differentiate "hydrophobicity" from "polarity" because the former concept is referred to as the water miscibility [15]. Hydrophobicity is often measured by the so called "log P scale," which is calculated from the partition coefficient of solute molecules distributed between 1-octanol and water. The partition coefficient (K_{OW} or P) is defined as the ratio of concentrations of unionized solute between two phases. The log P is derived from the partition coefficient at the unlimited dilution concentration of solute,

$$\log P = \lim_{c \to 0} K_{OW} = \lim_{c \to 0} \frac{C^o}{C^w} \tag{5.1}$$

where C^o is the solute concentration in octanol phase and C^w is the solute concentration in aqueous phase. Practically, very low IL concentrations are used in the experiment instead of extrapolating

the IL concentration to zero (Eq. 5.1). Since hydrophilic ILs dissociate into ions in water and K_{OW} values are typically determined as the ratio of concentrations of both undissociated and dissociated ILs between two phases, log P values of hydrophilic ILs in Table 5.2 should be called log D, where D is the *distribution coefficient*, the ratio of total concentrations of all forms of an IL (ionized and unionized) between two phases. Thus the intrinsic partition coefficients of ILs should be calculated from the apparent partition coefficients (D) [71].

As listed in Table 5.2, log P values of ILs (or log K_{OW} at low IL concentrations) compare the hydrophobicity between ILs and regular organic solvents. Overall, ILs are highly hydrophilic in nature because most ILs (even including some water-immiscible Tf_2N^- and PF_6^- ones) have negative log P values (or log K_{OW}). For the same ILs, there are different log P (or log K_{OW}) being reported possibly due to different initial concentrations of ILs (since high concentrations lead to higher K_{OW} values [69,71]), and different experimental methods.

A few studies have established some type of correlations between IL hydrophobicity and enzyme activity/stability. The Russell group [63] reported that free *Candida rugosa* lipase was only active in hydrophobic [BMIM][PF_6] (log $P = -2.39$), but not active in hydrophilic ILs such as [BMIM][CH_3COO] (log $P = -2.77$), [BMIM][NO_3] (log $P = -2.90$), and [BMIM][CF_3COO] [63]. Along the same lines, higher transesterification activities of lipases were observed in [BMIM][PF_6] versus in [BMIM][BF_4] by Nara et al. [76]; higher activities of PEG-modified lipase [77] and subtilisin [78] were found in more hydrophobic ILs such as [EMIM][Tf_2N]; and low penicillin acylase stabilities were seen in hydrophilic [BMIM][BF_4] and [BMIM][dca] [79]. When examining the enantioselective acylation of (*R*,*S*)-1-trimethylsilylethanol with vinyl acetate catalyzed by lipases in ILs, the Zong group [74] pointed out that the activity, enantioselectivity and thermostability of Novozym 435 increased with IL hydrophobicity in the order of [BMIM][PF_6] > [OMIM][BF_4] > [C_7MIM][BF_4] > [HMIM][BF_4] > [C_5MIM][BF_4] > [BMIM][BF_4]. A similar trend was also obtained for the cellulase activity decreasing with IL hydrophobicity as [BMIM][PF_6] > [BMIM][BF_4] > [BMIM]Cl [80], and for penicillin G acylase (PGA) with a lower stability in [BMIM][BF_4] than in hydrophobic ILs (with anions of Tf_2N^- and PF_6^-), especially in the absence of substrate [81]. Similarly, transesterification activities and/or stabilities of *Candida antarctica* lipase B (CALB) were significantly higher in hydrophobic ILs (carrying anions of PF_6^- or Tf_2N^-) than in water-miscible ILs (such as BF_4^-, dca^-, NO_3^-, OAc^-, and OTf^-, etc.), and lipase activities increased with the cation's hydrophobicity ($EMIM^+ < BMIM^+ < HMIM^+ < OMIM^+$) [36,82]; Novozym 435 was found more thermally stable in hydrophobic ILs than in the hydrophilic type following the order of [BMIM][Tf_2N] > [BMIM][PF_6] > [BMIM][OTf] > [BMIM][BF_4] > [BMIM][SbF_6] [83]. Other studies that reached similar conclusion include: a higher enantioselectivity (80% ee_p) of Amano lipase PS in hydrophobic [OMIM][PF_6] than in hydrophilic [HMIM][BF_4] and [HMIM]Cl (< 5% ee_p) during the kinetic resolution of racemic cyanohydrins [84]; the stability of CALB was in a decreasing order of [HMIM][PF_6] > [HMIM][Tf_2N] > [HMIM][BF_4], [BMIM][PF_6] > [BMIM][dca], and [OMIM][PF_6] > [HMIM][PF_6] > [BMIM][PF_6], and the stability of PGA was in a decreasing order of [BMIM][Tf_2N] > [BMIM][PF_6] > [BMIM][BF_4] although hydrophobic cations exhibited a reverse impact on the PGA stability ([EMIM][Tf_2N] > [BMIM][Tf_2N], and [BMIM][PF_6] > [OMIM][PF_6]) [85].

The Zhao group [70] reported that the transesterification activity of Novozym 435 in ILs increased with the log P value of ILs to a maximum value, and then decreased with a further increase in log P (a bell shape). Likewise, Lou et al. [86] observed initial rates of ammonolysis of

Table 5.2 Log *P* (or log K_{OW} at low concentrations[a]) values of ILs at 25°C [51].

	Solvent	log *P*/log K_{OW}	Reference
1	Dichloromethane	1.25	Selected value by Ref [67]
2	THF	0.46	Selected value by Ref [67]
3	*t*-butanol	0.35	Selected value by Ref [67]
4	Acetone	−0.24	Selected value by Ref [67]
5	Acetonitrile	−0.34	Selected value by Ref [67]
6	[EMIM][Tf_2N]	−1.18, log K_{OW} (−1.05 to −0.96) (at 0.28−2.8 mM)	[68], calculated from Ref [69]
7	[BMIM][Tf_2N]	0.11, log K_{OW} (−0.96 to −0.21) (at 0.15−2.2 mM), 0.33, −1.74	[70], calculated from Ref [69], [71], [72]
8	[HMIM][Tf_2N]	0.64, log K_{OW} (0.15 to 0.22) (at 0.32−0.38 mM), 0.65	[70], calculated from Ref [69], [72]
9	[OMIM][Tf_2N]	0.79, log K_{OW} (0.80−1.05) (at 0.099−0.21 mM)	[68], calculated from Ref [69]
10	[EMMIM][Tf_2N]	log K_{OW} (−1.15 to −0.92) (at 0.32−2.9 mM)	Calculated from Ref [69]
11	[PMMIM][Tf_2N]	log K_{OW} (−0.92 to −0.62) (at 1.4−2.8 mM)	Calculated from Ref [69]
12	[HMMIM][Tf_2N]	log K_{OW} (0.13 to 0.25) (at 0.36−0.49 mM)	Calculated from Ref [69]
13	[BMIM][PF_6]	−1.66 −2.39 −2.38 −2.06 −2.35	Calculated from Ref [69] [63,72] [71,73] [68] [74]
14	[HMIM][PF_6]	−1.86	[72]
15	[OMIM][PF_6]	−0.35 −1.33	[68] [72]
16	[ONIM][PF_6]	−2.19	[73]
17	[BMIM]Cl	−2.40	Calculated from Ref [69]
18	[BMIM]Br	−2.48	Calculated from Ref [69]
19	[EMIM][OAc]	−2.53	[70]
20	[BMIM][OAc]	−2.77	[63]
21	[EMIM][CF_3COO]	−2.75	[70]
22	[HMIM][CF_3COO]	−2.30	[70]
23	[BMIM][NO_3]	−2.90 −2.42	[63] Calculated from Ref [69]
24	[BMIM][dca]	−2.32	[70]
25	[EMIM][BF_4]	−2.57	[70]
26	[BMIM][BF_4]	−2.51 −2.44 −2.52	[70] [73,74] Calculated from Ref [69]
27	[OMIM][BF_4]	−1.34 −1.14	[70] [68]

Table 5.2 Log *P* (or log K_{OW} at low concentrations[a]) values of ILs at 25°C [51]. *Continued*

Solvent		log *P*/log K_{OW}	Reference
28	[EtPy][CF_3COO]	−2.57	[70]
29	[EtPy][Tf_2N]	−0.90	[70]
30	[BuPy][Tf_2N]	−0.26	[70]
31	[choline][Tf_2N]	log K_{OW} = −0.57 (calculated value)	[75][b]

Notes:
[a] *log* K_{OW} *values calculated from Ref [69] were converted from initial values of* K_{OW} *measured at room temperature (24°C ± 2°C), and the concentration range given for each log* K_{OW} *was the IL concentration range in water phase.*
[b] *This reference also provides* K_{OW} *values for a number of pyridinium and imidazolium ILs based on* Tf_2N^- *and* $B(CN)_4^-$.

(*R*,*S*)-p-hydroxyphenylglycine methyl ester catalyzed by Novozym 435 increased with the hydrophobicity of BF_4^- based ILs to a maximum, and then declined with an even higher IL hydrophobicity. To understand the higher enzyme activity/stability in more hydrophobic solvents, one explanation could be that polar organic solvents could strip off the "essential" water from enzyme molecules, causing their inactivation [87,88]. In addition, a lower log *P* value may be correlated with a higher hydrogen-bond basicity of an IL anion, which causes a lower enzyme activity [70]. The decreasing enzyme activity/stability in further more hydrophobic ILs could be explained by two factors: (a) hydrophobic solvents lead to a more thermodynamic ground-state stabilization of substrates [89], which reduces the conversion of substrates. (b) There are hydrophobic interactions between IL molecules and the enzyme. The Atkin group [90] evaluated the stability and activity of hen's egg white lysozyme in 25−75 wt.% aqueous ILs, and observed the catalytic activity decreased in the order of [$(EtOH)NH_3$][HCOO] > [$PrNH_3$][HCOO] > [$EtNH_3$][HCOO] and [$(MeOEt)NH_3$][HCOO]. Electrostatic interactions between $[(EtOH)NH_3]^+$ and lysozyme is similar to that of $[EtNH_3]^+$, therefore, the hydroxy group in $[(EtOH)NH_3]^+$ likely reduces hydrophobic interactions with the protein. It is also possbile that the hydroxy group interacts with formate via hydrogen bonds, minimizing the interaction of formate with lysozyme. Qin and coworkers [91] compared the activity and stability of Novozym 435 in different imidazolium-based ILs using the transesterificaiton of monoolein with methanol, and found the lipase was more active in Tf_2N^- and PF_6^--type ILs than in those with BF_4^-, dca^- and OAc^- anions; A general trend is that initial reaction rates increased with log *P* but decreased with the polarity (E_T^N). Additionally, the lipase stability was highly dependent on the type of IL anions: redisual lipase activities were 5%, 40%, and 100% after the enzyme incubation in BF_4^-, PF_6^-, and Tf_2N^--based ILs at 50°C for 24 h, respectively; the lipase destabilization was correlated with increased β-sheets and decreased α-hexical structures. In summary, *the hydrophobicity factor of ILs is a combination effect of anion's hydrogen-bond basicity and cation's hydrophobic effect*. All-atom MD simulations conducted by the Yingling group [92] provided some insights of CALB interactions with OTf^--based ILs paied with different cations ($EMIM^+$, $BMIM^+$, $HMIM^+$ and $OMIM^+$) to explain the highest activity and structural stability of CALB in [BMIM][OTf] and [HMIM][OTf]. In the case of [EMIM][OTf] (with a high ion coordination number), OTf^- anions interact with LYS-290 to interupt its hydrogen-bonding with IIL-285 causing a closed catalytic gate conformation with low substrate accessibility. In [OMIM][OTf], $OMIM^+$ cations interact strongly with LEU-278 via

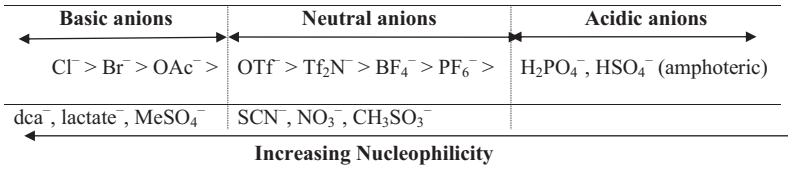


FIGURE 5.2

Comparison of hydrogen-bond basicity of selected anions in ILs [51].

hydrophobic interactions inducing the loss of α-10 helical structures and exposing the catalytic triad to the solvent.

As solvent hydrophobicity is one of the many factors influencing the enzyme activity and stability, there are examples that do not follow the hydrophobicity rule of thumb. For instance, Irimescu and Kato [93] observed lower reaction rates in ILs with longer alkyl chains in cations and noted that water miscibility of ILs was not a controlling factor for the reaction rate of lipase-catalyzed acylation of primary amines. A few studies also found relatively high enzyme activities in hydrophilic ILs (such as [BMIM][BF_4], [EMIM][BF_4], [BMIM][OTf] and [MMIM][$MeSO_4$]) [54,94–99]. Therefore, multiple factors must be evaluated for complex enzymatic reaction systems.

5.2.3 Hydrogen-bond basicity and nucleophilicity of anions

Hydrogen-bond basicity and nucleophilicity are two distinct but often related concepts.[‡] Generally, the stronger base is usually the stronger nucleophile in aprotic solvents for molecules containing the same nucleophilic atoms with the same charge. Based on the solvatochromic scales, the orders of IL anion's basicity (nucleophilicity) have been reported (in decreasing orders) by several studies:

Basicity series #1 [53]: OTf^- ($CF_3SO_3^-$) > Tf_2N^- > PF_6^-
Basicity series #2 [100]: Cl^- > Br^- > SCN^- > OAc^- > I^- > NO_3^- > OTf^- > ClO_4^- > BF_4^-
Basicity series #3 [101]: Cl^- > Br^- > OAc^- > OTf^- > ClO_4^- > BF_4^-
Basicity series #4 [102]: Cl^- > Br^- > $CH_3OSO_3^-$ > SCN^- > BF_4^- ~ OTf^- > PF_6^-

Following these sequence and literature discussions [103,104], the basicity of selected anions is categorized in Fig. 5.2. These anions fall into three categories (basic, neutral, and acidic). Basic anions include halides, acetate, dicyanamide (dca^-), lactate and methyl sulfate; these anions are strong hydrogen-bond acceptors and potentially form hydrogen-bonds with proteins resulting in enzyme denaturation and/or inactivation. Neutral anions include those species forming hydrophobic ILs (Tf_2N^- and PF_6^-) and others forming hydrophilic ILs (BF_4^-, OTf^-, SCN^-, NO_3^- and $CH_3SO_3^-$). These anions have weak abilities in forming hydrogen-bonds. Acidic anions include amphoteric $H_2PO_4^-$ and HSO_4^- and are not most common anions in ILs for biocatalysis. However, choline

[‡]Basicity refers to the ability of a base to accept a proton and is a matter of equilibrium. Nucleophilicity of a Lewis base refers to the relative reaction rate of different nucleophilic reagents toward a common substrate, most usually involving the formation of a bond to carbon; nucleophilicity is a matter of kinetics (rate).

dihydrogen phosphate (m.p. 119°C) with 20%−30% (wt.) water could solubilize and stabilize proteins such as cytochrome *c* (cyt *c*), peroxidase, ascorbate oxidase, azurin, pseudoazurin, fructose dehydrogenase, lysozyme and interleukin-2 [105−108].

In addition to the solvatochromic scale, there are other methods to compare hydrogen-bond basicity of anions. Bernson and Lindgren [109] studied the IR band shifts of −OH stretching for solutions of lithium salts LiX dissolved in poly(propylene glycol) (MW = 3000) with hydroxy end-groups, and thus derived the order of hydrogen-bond basicity of anions in LiX salts (**Basicity series #5**). Dupont [41] obtained the strength of hydrogen-bond basicity based on experimental data of IR and ESI-MS (**Basicity series #6**). Generally, these two basicity series are consistent with solvatochromic measurements shown in Fig. 5.2.

Basicity series #5 [109]: $\mathbf{PF_6^- < BF_4^- < ClO_4^- < OTf^- < I^- < Br^- < Cl^-}$
Basicity series #6 [41]: $\mathbf{BPh_4^- < PF_6^- < BF_4^- < CF_3COO^-}$

Alternatively, the *ionic association strength* of anions in aprotic solvents can be estimated from interactions between LiX salts and aprotic solvents such as glymes (see a brief summary in *Supporting Information* of Ref [110]) in an increasing order [110,111].

$\mathbf{beti^-, Tf_2N^- < PF_6^- < ClO_4^-,}$
$\mathbf{I^- < SCN^- < BF_4^- < CF_3SO_3^- < Br^- < NO_3^- < CF_3COO^- < Cl^-}$

This series reflects the strength of an anion to interact with solvated cations through ionic attraction, which can be implied to understand the interactions of anions with charged regions of macromolecules (e.g., proteins). It is also interesting to point out the similarity between this ionic association strength series and the anion's hydrogen-bond basicity sequence in Fig. 5.2.

Built upon the beforementioned background on nucleophilicity and hydrogen-bond basicity of anions, two groups of enzymatic reactions are described in the following sections to elucidate the impact of nucleophilicity and basicity of IL anions on enzyme activity and stability. In these biocatalytic systems, the dominating factor for enzyme stabilization is the direct interaction between the protein and IL molecules (due to anion's nucleophilicity and basicity, and cation's hydrophobicity); for example, when the positive-to-negative charge ratio on enzyme's surface is reduced, the anion interaction with the protein decreases and the enzyme stability in ILs increases [112,113]. Another example by Manna and Ghosh [114] performed MD simulations of hyperthermophilic endoglucanase Cel12A from *Rhodothermus marinus*, which is known to tolerate up to 40% (v/v) [EMIM][OAc] [115]. They observed that $EMIM^+$ cations gather near the enzyme's active sites (average radial distance about 5.62 Å) due to negatively charged active site tunnel and thus interact with catalytic residues (via hydrophobic interaction, π-cation, π-π stacking, and salt bridges) especially at a higher IL concentration (e.g., 60 v%), while acetate anions stay a far distance from active sites (average radial distance about 11.58 Å). Although endoglucanase appears to maintain its stability in 20−60 v% [EMIM][OAc], the loss of enzyme activity can be correlated with the reduction in essential dynamic motions.

In the first group of examples, the effect of anion's nucleophilicity was analyzed. Kaar and coworkers [63] noted that three hydrophilic anions (NO_3^-, OAc^- and CF_3COO^-) are more nucleophilic than PF_6^-, and could cause the protein conformation changes. As a result, they found that free *C. rugosa* lipase was only active in hydrophobic [BMIM][PF_6], but inactive in all hydrophilic ILs during the transesterification of methylmethacrylate with 2-ethyl-1-hexanol. Hernández-Fernández et al. [85]

established the stability of CALB in ILs in a decreasing order of [HMIM][PF_6] > [HMIM][Tf_2N] > [HMIM][BF_4], and [BMIM][PF_6] > [BMIM][dca], and the stability of PGA in a decreasing order of [BMIM][Tf_2N] > [BMIM][PF_6] > [BMIM][BF_4]. The decreasing enzyme stability was explained due to the increasing nucleophilicity of anions (PF_6^- < BF_4^- < Tf_2N^- < dca^-) as shown in Fig. 5.2, which increases the anion's interaction with positively charged regions of enzymes to change their conformations. Meanwhile, it is apparent that a higher enzyme stability was observed in more hydrophobic ILs. Lee et al. [83] found that the transesterification activities of three lipases (Novozym 435, *Rhizomucor miehei* lipase, and *C. rugosa* lipase) were dependent on IL anions in a decreasing order of Tf_2N^- > PF_6^- > OTf^- > SbF_6^- ~ BF_4^-. This group rationalized that OTf^- and BF_4^- are more nucleophilic than PF_6^-. Another factor was the hydrophobicity since hydrophobic ILs enabled higher lipase activities than hydrophilic ILs.

In the second group of examples, the anion's hydrogen-bond basicity was focused. Chloride anion has a high hydrogen-bond basicity (Fig. 5.2), thus [BMIM]Cl can form hydrogen bonds with hydroxy groups in cellulose to disrupt its hydrogen-bonding network to dissolve it [116–118]. Anderson and coworkers [119] employed multiple salvation interactions to conclude that [BMIM]Cl has the largest hydrogen-bond basicity among ILs evaluated; thus [BMIM]Cl could dissolve complex polar molecules like cyclodextrins and antibiotics [120]. For the same reason, halides form hydrogen bonds with the protein, and thus high concentrations of [BMIM]Cl led to the deactivation of *Trichoderma reesei* cellulase [34]. Also, Lee et al. [121] observed a considerable drop in lipase activity in [OMIM][Tf_2N] with an increasing addition of [OMIM]Cl. Similarly, dicyanamide (dca^-) has a high hydrogen-bond basicity and thus [BMIM][dca] can dissolve some carbohydrates [30,122], but [BMIM][dca] is an enzyme-denaturing IL [31,35,70]. Low stabilities of cytochrome *c* (cyt *c*) was observed by Fujita and coworkers [106] in [BMIM][$MeSO_4$], [BMIM][lactate] and [BMIM][OAc] (all containing 20 wt.% water), suggesting high hydrogen-bond basicity and enzyme-denaturing nature of $MeSO_4^-$, lactate and OAc^-. Bermejo et al. [123] reported that free CALB lost 35% of its initial activity in [HOPMIm][NO_3], but preserved 80% of the remaining activity after 3 months of incubation in this IL. The CALB activity loss in [HOPMIm][NO_3] could be explained by the denaturing nature of NO_3^-. However, when compared with [BMIM][NO_3], this hydroxy-functionalized IL is less enzyme-denaturing due to two reasons: (1) [HOPMIm][NO_3] carries the hydroxy group, which interacts with NO_3^- to minimize its interaction with the lipase; (2) $HOPMIm^+$ cation is bigger than $BMIM^+$, and thus the molar concentration of NO_3^- in [HOPMIm][NO_3] is lower than that in [BMIM][NO_3].

5.2.4 Ion specificity and Hofmeister series

In *diluted* aqueous solutions, hydrophilic ILs dissociate into individual solvated ion pairs just like inorganic salts (see Fig. 5.1). Therefore, the specific ion effect on the enzyme's microenvironment becomes an important factor in solutions. As early as in 1888, Franz Hofmeister noticed different ions having different capabilities in precipitating proteins such as globulins from blood serum and hen's egg, and constructed the sequence of cations and anions based on their ability in salting out proteins known as the "Hofmeister series" (Fig. 5.3) [132,133]. Over the years, many theories have been developed to explain the ion specificity on protein stability, which include salt-in and salt-out interactions [134,135], water-structure changes (low/high density water) and protein preferential hydration [124,136–141], hydrophobic interactions [141–143], excluded volume [144–146],

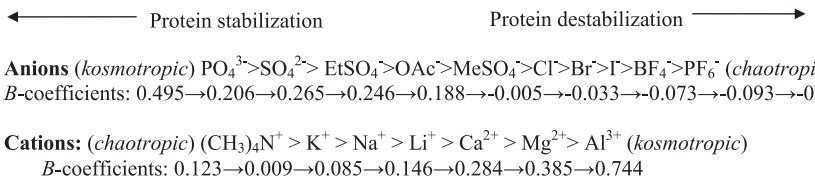


FIGURE 5.3

The Hofmeister series in an order of ion effect on protein stability [51,124,125] (The viscosity *B*-coefficients in dm^3/mol at 25°C are taken from the Marcus collection [126] except those of $EtSO_4^-$ and $MeSO_4^-$ were from Ref [127]; the positions of $EtSO_4^-$ and $MeSO_4^-$ are based on the consideration of *B*-coefficients, NMR *B'*-coefficients [128] and enzyme stability data [106,129–131]).

preferential interactions/binding [147–150], and electrostatic interactions [151,152], etc. There have been debates over these theories, but there is no individual theory that could completely interpret the Hofmeister series.

Regardless of the debates over different theories, protein stability is often correlated with the hydration properties of ions [134,153]. Highly hydrated cations and anions (e.g., Mg^{2+}, Ca^{2+}, Li^+, CH_3COO^-, SO_4^{2-}, and HPO_4^{2-}) have strong interactions with water molecules and possibly strengthen the water structure, yielding a lower fluidity (or a higher viscosity) of the solution when compared with pure water. Therefore, these ions are called "structure-makers" or "kosmotropes" (see Fig. 5.3). In contrast, weakly hydrated ions in aqueous solutions, such as SCN^-, I^-, NO_3^-, BF_4^-, Cs^+, $(NH_2)_3C^+$ (guanidinium), and $(CH_3)_4N^+$ (tetramethylammonium), have loose interactions with water and disrupt the water structure, leading to a higher fluidity of the solution. Such hydration behavior is known as "negative hydration" [154,155], and these ions are called as "structure-breakers" or "chaotropes" (see Fig. 5.3).

The ability of an ion in strengthening the water structure, known as kosmotropicity (*vs.* chaotropicity), therefore, is directly correlated with the extent of ion hydration. As detailed in a review paper [156], ion kosmotropicity can be measured by various parameters such as viscosity *B*-coefficients, structural entropies, structural volumes, structural heat capacities, NMR *B'*-coefficients, and ion mobility, etc. Jones-Dole viscosity *B*-coefficients are the most commonly used parameter for quantifying ion kosmotropicity. *B*-coefficients can be derived from the Jones-Dole empirical equation (Eq. 5.2) of relative viscosities of electrolyte solutions as the functions of their concentrations [157],

$$\eta/\eta_0 = 1 + Ac^{1/2} + Bc + Dc^2 + \ldots \tag{5.2}$$

where η is the solution viscosity, and η_0 is the solvent viscosity (both of them use the same unit such as mPa · s), and c is the molar concentration (mol/cm^3). The *A*-coefficient (also called the Falkenhagen coefficient [158]), signifying the solute-solute or electrostatic interactions, is calculated theoretically. However, *A*-coefficients are typically small and negligible for nonelectrolytes [126]; thus, they are often neglected during the calculation. *B*-coefficients correspond to short-range dispersion forces for solute-solvent interactions, and *D*-coefficients represent both solute-solute interactions and solute-solvent interactions [159]. For typical salts at low concentrations [(< 0.5 M) [126] or (< 0.1 M for binary strong electrolytes) [160]], the *D* or higher coefficients can be ignored but they are important at higher concentrations [126]. Positive *B*-coefficients usually

imply ions being kosmotropes because highly hydrated ions impose a bigger change in viscosity with the concentration, while negative *B*-coefficients suggest chaotropes for weakly hydrated ions [126]. An exception to this rule is that hydrophobic solutes often have positive *B*-coefficients owing to their so called "hydrophobic hydration" [156]. As an example, tetramethylammonium cation (Me_4N^+) is considered as a structure-breaker (chaotrope) [138,161–165], but has a positive *B*-coefficient of 0.123 [126]. Alternatively, first derivatives of *B*-coefficients over temperature are believed to be more indicative in assessing the structure-making or breaking capability of ions over *B*-coefficients [126,166–168]. The positive value of d*B*/d*T* indicates chaotropic (structure-breaking) while the negative sign implies kosmotropic (structure-making).

In the presence of inorganic salts in aqueous solutions, ion specificity on enzyme activity and stability can be summarized by the following rule of thumb: ***kosmotropic anions and chaotropic cations stabilize the enzyme, while chaotropic anions and kosmotropic cations destabilize it*** [169]. The Zhao group [65,128,129,156,170–174] pioneered the work of extending this rule to the enzyme activation and stabilization in IL solutions. In their first study, the hydrolytic activity of Amano protease P6 (from *Aspergillus melleus*) in 0.7 M aqueous IL solutions decreased with IL anions in the order of $\mathbf{CH_3COO^-}$, $\mathbf{CF_3COO^- > Cl^-}$, $\mathbf{Br^- > OTs^- > BF_4^-}$ (decreasing anion kosmotropicity), and decreased with IL cations in the order of $\mathbf{EMIM^+}$, $\mathbf{BuPy^+ > BMIM^+ > EtPy^+}$ [65]. In a later study [129], they performed the enantiomeric hydrolysis of phenylalanine methyl ester catalyzed by *Bacillus licheniformis* protease in aqueous hydrophilic ILs (0.5 M). The enzyme enantioselectivity decreased with IL anions in the order of $\mathbf{PO_4^{3-} > citrate^{3-}}$, $\mathbf{OAc^-}$, $\mathbf{EtSO_4^-}$, $\mathbf{CF_3COO^- > Br^- > OTs^-}$, $\mathbf{BF_4^-}$ (decreasing kosmotropicity), and decreased with IL cations in the order of $\mathbf{EMIM^+ > BMIM^+ > HMIM^+}$ (increasing kosmotropicity). The overall kosmotropicity of an IL can be quantified by the δ value (the difference of viscosity *B*-coefficients between anion and cation). Typically, a high δ value corresponds to a high enzyme enantioselectivity in IL solutions. The Zhao group [128] further determined NMR *B'*-coefficients of several ions (see Fig. 5.4), and identified a linear correlation between enzyme enantioselectivity in aqueous solution and the δ' parameter of ILs (the difference in NMR *B'*-coefficients between anion and cation), implying that high enzyme enantiomeric ratios (*E*) were obtained in IL solutions with high δ' values. Several other groups [11,175,176] also found low/no activities of β-glycosidase in aqueous solutions of [BMIM][BF_4], which could be related to the chaotropic and denaturing property of BF_4^- in solutions [176]. However, in neat or highly concentrated BF_4^--containing ILs, the chaotropic nature of this anion becomes minimized and thus reasonable enzyme activities were found in these low-water ILs. Furthermore, the Zhao group [170] examined the enzymatic hydrolysis of

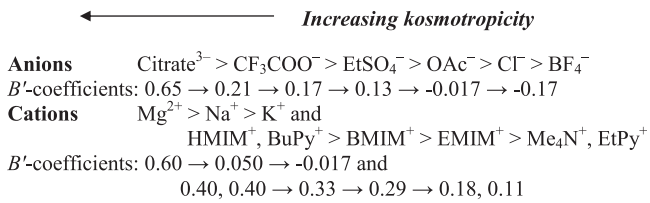


FIGURE 5.4

NMR *B'*-coefficients of several ions [51,128].

DL-phenylalanine methyl ester in aqueous IL solutions (0.5 M) carrying anions of chiral- or ω-amino acids, and discovered higher enantiomeric excess (*ee*) and yields in aqueous ILs carrying anions of D-amino acids *vs* in those ILs with L-amino acid anions. A possible reason is that D-amino acid anions appears more kosmotropic than L-amino acid anions [172]. Based on the same concept, kosmotropic IL anions (OAc^- and CF_3COO^-) could activate hydrolases in aqueous solutions [173,174].

Both Ohno and MacFarlane groups [105,106,177] suggested that the stability of cytochrome *c* in ILs with 20% (wt.) water was highly dependent on specific ions and decreased in the order of (for cation) **Cholinium$^+$ > BMPyrr$^+$ > BMIM$^+$** (an increasing cation kosmotropicity), and (for anion) **$H_2PO_4^-$ > $Bu_2PO_4^-$ > OAc^- > lactate$^-$ > $MeSO_4^-$** (a decreasing anion kosmotropicity: *B*-coefficients at 25°C: $H_2PO_4^-$ = 0.340 [126], OAc^- = 0.246 [126], $MeSO_4^-$ = 0.188 [127]; lactate could be regarded as a kosmotropic anion [178]). Furthermore, the Ohno group [107] looked into different metallo proteins (cytochrome *c*, ascorbate oxidase, peroxidase, azurin, pseudoazurin and D-fructose dehydrogenase) solubilized in hydrated [Cholinium][H_2PO_4] (containing 30 wt.% water), and confirmed the preservation of proteins' active sites and secondary structures in this biocompatible medium. As a result, some of these proteins maintained their activities in hydrated [Cholinium][H_2PO_4] and D-fructose dehydrogenase even exhibited an enhanced thermal stability. In another study, Bisht and Venkatesu [179] examined the activity and stability of α-chymotrypsin in 0.05−1.5 M aqueous cholinium ILs based on different anions (OAc^-, Cl^-, $H_2PO_4^-$, dihydrogencitrate, and OH^-), and observed that the protease maintained high caseinolytic activities in up to 1.5 M of ILs carrying anions of OAc^-, Cl^-, and $H_2PO_4^-$ but inactivated by the other two ILs. In addition, the stability of α-chymotrypsin decreases in the order of IL anions as OAc^- > Cl^- > $H_2PO_4^-$ > dihydrogencitrate > OH^-, which could be partially explained by the Hofmeister series and also by the direct ion interaction with the protein (such as hydrogen bonding of dihydrogencitrate, and OH^- with α-chymotrypsin). In another study, Bisht and coworkers [180] investigated the activity and stability of cytochrome *c* in aqueous solutions of cholinium-based ILs with anions derived from dicarboxylic acids, and noted that the enzyme activity decreased in the order of [cholinium][hydrogenglutarate] > [cholinium][hydrogensuccinate] > [cholinium][dihydrogencitrate] > [cholinium][hydrogenmalonate] > [cholinium][bitartrate] > [cholinium][H_2PO_4]. Compared with cytochrome *c* in phosphate buffer (pH 7.2, 10 mM), there was >50-fold activity boost in 50 wt.% [cholinium][hydrogenglutarate] and >25-fold activity increase in 33 wt.% [cholinium][H_2PO_4]. Aqueous solutions of these ILs also enabled a high thermal stability (up to 120°C) and storage stability (>21 weeks at room temperature) for cytochrome *c*, and protected cytochrome *c* from denaturants (guanidine hydrochloride and H_2O_2) and protease (α-chymotrypsin) digestion. In a related study [181], cytochrome *c* was found to preserve its native structure in aqueous [Et_3NH][H_2PO_4] (0.1−0.5 M) but lost its tertiary structure in 0.1−0.5 M [Bu_3NH][H_2PO_4] as indicated by CD spectra, which was due to stronger hydrophobic interaction of latter IL with the protein. Interestingly, the native state of cytochrome *c* exhibited faster conformational dynamics in IL solutions based on the single molecule-based fluorescence correlation spectroscopy.

The Weingärtner group [130,182] indicated that the Hofmeister series could also be applied to the thermal stability of ribonuclease A (RNase A) in aqueous ILs (up to 2.0 M) using differential scanning calorimetry (DSC). The effect of IL cation on RNase A stability is in a decreasing order of **K^+ > Na^+ ~ Me_4N^+ > Li^+ > Et_4N^+ ~ EMIM$^+$ > BMPyrr$^+$ > BMIM$^+$ ~ Pr_4N^+ > HMIM$^+$ ~ Bu_4N^+** and **K^+ > Na^+ ~ Me_4N^+ > Cholinium$^+$ > EMIM$^+$ ~ Guanidinium$^+$ > BMIM$^+$**

while the effect of anions can be summarized in a decreasing order of $\mathbf{SO_4^{2-} > HPO_4^{2-} > Cl^- > EtSO_4^- > BF_4^- \sim Br^- > MeSO_4^- > OTf^- > SCN^- \sim dca^- > Tf_2N^-}$. The cation series generally agrees with earlier discussions of a higher cation's hydrophobicity, a higher cation's kosmotropicity, and a lower protein stability. The anion series confirms the higher anion's kosmotropicity, the higher the protein stability (with small variations in anion ranking from earlier discussions). This group [182] further pointed out that ILs could lead to an improvement in native state stability, an enhancement in refolding, and a suppression in irreversible aggregation. Yang and coworkers [131] observed a higher mushroom tyrosinase activity in aqueous [BMIM][BF_4] than in aqueous [BMIM][$MeSO_4$], but enzyme stability decreased in the order of $KMeSO_4 > NaBF_4 > KPF_6$. The Yang group [183] evaluated the enzymatic activity and stability of alkaline phosphatase in aqueous inorganic salts (up to 1.0 M), and identified that the initial reaction rate or V_{max}/k_m followed a bell-shaped curve with ($B_- - B_+$) values of these salts (where B_- and B_+ are Jones–Dole viscosity B-coefficients of anions and cations respectively). They concluded that salts (e.g., NaCl, KCl, and KNO_3) with similar anion and cation kosmotropic/chaotropic properties usually induced highest activities, which could be explained by the effect of ions on enzyme's surface pH, active site, and catalytic mechanism. Additionally, they observed a higher enzyme thermal stability in the presence of salts with higher (B_-) or ($B_- - B_+$) values, and anions play a more critical role to the enzyme stability.

Through using continuous wave electron paramagnetic resonance (EPR) spectroscopy and nanoscale distance measurements with double electron–electron resonance (DEER) spectroscopy, the Hinderberger group [184] studied the effect of ILs on tertiary structures of human serum albumin (HSA). This group detected protein unfolding in 15% (v/v) [BMIM][BF_4] and found more hydrophobic alkyl chains caused stronger IL-protein interactions; On the other hand, 25% (v/v) aqueous [Cholinium][H_2PO_4] was able to sustain the binding capacity and tertiary structures of HSA. This finding could be justified by the Hofmeister series: [BMIM][BF_4] consists of a kosmotropic cation and a chaotropic anion but [Cholinium][H_2PO_4] comprises a chaotropic cation and a kosmotropic anion. Attri and coworkers [185] noted that [Et_3NH][OAc] minimized the denaturing ability of urea (a known nonionic chaotrope [186]) on α-chymotrypsin in aqueous solutions through circular dichroism (CD), fluorescence, and NMR methods; a possible explanation is that acetate as a kosmotropic and basic anion forms hydrogen bonds with urea and water to reduce urea-enzyme interactions. The Yang group [187] correlated activities of *Penicillium expansum* lipase in 4.14% (w/v) ILs with the Hofmeister series: (for the cation series) **[MMIM][$MeSO_4$] > [EMIM][$MeSO_4$] > [BMIM][$MeSO_4$], [Me_4N][OAc] > [Bu_4N][OAc], [Me_3NH][$MeSO_3$] > [Bu_4N][$MeSO_3$]**, and **[Me_3NH][H_2PO_4] > [Et_3NH][H_2PO_4] > [Bu_3NH][H_2PO_4]**, and (for the anion series) **[Cholinium][OAc] > [Cholinium][$MeSO_3$] > [Cholinium][NO_3], [Bu_4N][OAc] > [Bu_4N][$MeSO_3$]**. Meanwhile, they established a similar Hofmeister cation effect on mushroom tyrosinase: (for the activity in 5.85%, (w/v) ILs) **[MMIM][$MeSO_4$] > [EMIM][$MeSO_4$] > [BMIM][$MeSO_4$], [Me_4N][OAc] > [Bu_4N][OAc], and [Me_3NH][H_2PO_4] > [Et_3NH][H_2PO_4]**, and (for the stability in 5% (w/v) ILs) **[MMIM][$MeSO_4$] > [EMIM][$MeSO_4$] > [BMIM][$MeSO_4$]**.

Attri and Venkatesu [188] calculated transfer free energies ($\Delta G'_{tr}$) of a homologous series of cyclic dipeptides from water to aqueous protic ILs (30%, 50%, and 70%, v/v) based on their solubilities at 25°C under atmospheric pressure. All $\Delta G'_{tr}$ values are positive and decreased in the order of different ILs: **[Et_3NH][HSO_4] > [Et_2NH_2][HSO_4] > [Et_3NH][OAc] > [Et_2NH_2][OAc] > [Et_3NH][H_2PO_4] > [Et_2NH_2][H_2PO_4]**. Since a higher $\Delta G'_{tr}$ implies more undesirable interaction of cyclic dipeptide with IL molecules, the reverse order of the above $\Delta G'_{tr}$ sequence indicates the

decreasing compatibility of ILs with the dipeptide, which coincides with the Hofmeister series: kosmotropic anions and chaotropic cations stabilize proteins [viscosity *B*-coefficients (dm^3/mol at 25°C) [126]: $H_2PO_4^-$ (0.340) > OAc^- (0.246) > HSO_4^- (0.127), and Et_3NH^+ (0.385) > $Et_2NH_2^+$ (0.293)]. Lu and coworkers [189] correlated anodic peak current of horseradish peroxidase (HRP) at bare glassy carbon electrode with the catalytic activity and secondary structures of HRP. The HRP structural stability depended on IL cations and anions (up to 1.0 M) following the Hofmeister series:

(Cations) Me_4N^+ > Cholinium$^+$ > $EMIM^+$ > $BMPip^+$ > $BMPyrr^+$ > $BMIM^+$ > $BuPy^+$ > $HMIM^+$,
(Anions) Cl^- > Br^- > NO_3^- > BF_4^- > OTf^- > SCN^- > dca^-

Weibels et al. [190] reported that enzymatic efficiency k_{cat}/K_M of yeast alcohol dehydrogenase in 0.5 M ILs followed the Hofmeister series below, which can be explained by the hydrophobic interactions as a controlling factor.

(Anion) Cl^- > Br^- > $EtSO_4^-$ > OTf^- > BF_4^- > dca^- > SCN^- (same $EMIM^+$ cation)
(Cation) Na^+ > Me_4N^+ > Cholinium$^+$ > $EMIM^+$ > Et_4N^+ > Bu_4N^+ > Guanidinium$^+$ > $BMIM^+$ (same Cl^- anion)

It is important to point out that ion specificity of ILs on enzyme activity and stability exists mostly in *diluted* aqueous ILs [65,129,169], and sometimes in concentrated ILs (such as with 20 wt.% water [106]). However, ion specificity may not be applicable to neat or highly concentrated ILs. As an example, PF_6^- is a known chaotropic anion [156] and deactivates enzymes in aqueous solutions as Na^+ or K^+ salt (more denaturing than BF_4^- and $MeSO_4^-$ in the case of mushroom tyrosinase [131]). However, ILs carrying PF_6^- anion (e.g., [BMIM][PF_6]) are usually rather hydrophobic and are enzyme stabilizing [16]. Therefore, the Hofmeister effect could not explain the enzyme's performance in hydrophobic ILs (with or without water). On the other hand, in the absence of adequate water to hydrate them, kosmotropic or borderline anions (e.g., acetate, lactate and chloride) of ILs become enzyme-deactivating because they possess high hydrogen-bond basicity to bind strongly with the enzyme. It has been reported that low-concentrations of chloride or acetate-based ILs in water are enzyme activating but high concentrations are enzyme deactivating [34,65,129,173,174,191]. There are a few studies that the enzyme activity and stability in aqueous ILs do not follow ion specificity or follow a reversed order (see more examples in a review paper [191]). For example, the activities and conformational changes of formate dehydrogenase failed to follow the exact Hofmeister series because direct interactions between ILs and the enzyme were neglected by the series [192]. The Venkatesu group [193] screened the stability and structure of α-chymotrypsin in aqueous solutions (0.01−2.0 M) of sodium salts and $BMIM^+$ based ILs using fluorescence, thermal fluorescence analysis, and CD spectroscopy, and suggested that the effect of anions (sodium salts and ILs) on protein stability is concentration-dependent and does not necessarily follow the same Hofmeister series. They noted that α-chymotrypsin structure is stabilized by OAc^- Cl^- and Br^-, but destabilized by SCN^- HSO_4^- and I^- when they are paired with $BMIM^+$ cations.

5.2.5 Viscosity

ILs are more viscous than water and conventional organic solvents typically by several hundred or thousand-fold (see viscosity data of ILs in Ref [9]). When enzymes are solubilized in ILs, they tend

to be inactivated although there are some exceptions [60]. In most scenarios, enzyme powder or immobilized enzymes are practically suspended in ILs as heterogeneous systems and thus internal and external mass-transfer limitations could be an important factor [12]. In addition to IL polarity, Lozano and coworkers [61] noticed that a higher α-chymotrypsin activity in [EMIM][Tf_2N] than in [MTOA][Tf_2N] (MTOA = methyl trioctylammonium) could be correlated with their viscosities: [EMIM][Tf_2N] (34 mPa s) is much less viscous that the latter IL (574 mPa s). Eckstein et al. [194] reported that lipase from *Pseudomonas* sp. lipase showed a higher enantioselectivity in [BMIM][Tf_2N] at low water activities ($a_w < 0.53$) than in methyl *tert*-butyl ether (MTBE), and rationalized two possible reasons: (1) a higher IL viscosity (52 mPa s) (vs MTBE with 0.34 mPa s) reduced the reaction rate; (2) a lower substrate solubility in [BMIM][Tf_2N] (than in MTBE) induced a lower activation energy in the ionic solvent. van Rantwijk and Sheldon [16] suggested that a high IL viscosity could decrease the conformation changes of proteins, enabling the preservation of their native structures. In contrast, Basso and coworkers [195] observed no effect of IL viscosity (*e.g.*, [BMIM][PF_6] and [BMIM][BF_4] vs much less viscous toluene) on the initial reaction rates for the immobilized penicillin G amidase-catalyzed amide synthesis. The Zhao group [70] found no apparent correlation of IL viscosity with the transesterification activity of CALB in more than 20 ILs, implying that viscosity is not always the primary factor for enzyme stabilization. Qin et al. [91] noticed that the impact of IL viscosity on transesterification activity of Novozym 435 was highly dependent on the type of ILs: the lipase activity increased with viscosity for Tf_2N^--based ILs while decreased with viscosity for ILs containing BF_4^- and PF_6^- anions.

5.2.6 Surfactant effect

ILs with long alkyl chains have both lipophilic and hydrophilic regions and thus usually behave like amphiphilic compounds. These amphiphilic ILs form aggregates and micelles in solutions and behave like surfactants, which have been evidenced by both experimental and simulations works [196–199]. Sodium *n*-dodecyl sulfate and *n*-dodecyltrimethylammonium bromide are classical examples of conventional ionic surfactants; they bind to enzymes through two types of interactions: (a) electrostatic attraction of surfactant's head group and charged amino acid residues of the enzyme, and (b) hydrophobic interactions between the hydrophobic region of the surfactant and hydrophobic amino acid residues [200]. Ionic surfactants are known as nonspecific denaturants of enzymes although they have mixed effects on enzymes (either no effect, or some activating/stabilizing effects) [200–202]. The prior knowledge of surfactant-protein interactions allows us to further understand the IL effect on enzyme activity and stability in some reaction systems. The Banerjee group [203] evaluated the surfactant effect of 1-hexadecyl-3-methyl imidazolium bromide on the hydrolytic activity and stability of *Rhizopus oryzae* lipase, and noted that a low IL concentration (100 μM) could increase the lipase activity by 80% and improve thermal deactivation temperature by 2.5°C although higher IL concentrations led to enzyme inactivation. Through UV-visible and CD spectroscopy, they suggested the improvement in enzyme activity and stability was due to a reduction in α-helix (opening of active site and exposure of hydrophobic domains) and an increase in β-sheets (higher protein rigidity) induced by noncovalent interactions between the lipase and IL surfactant. Bharmoria et al. [204] examined the surfactant effect of 1-methyl-3-octylimidazolium dodecylsulfate, [OMIM][$C_{12}H_{25}OSO_3$], in aqueous buffer at pH 4.8, and reported concentration-dependent binding due to both electrostatic and hydrophobic interactions (see Fig. 5.5): (a) slight unfolding and disturbance of

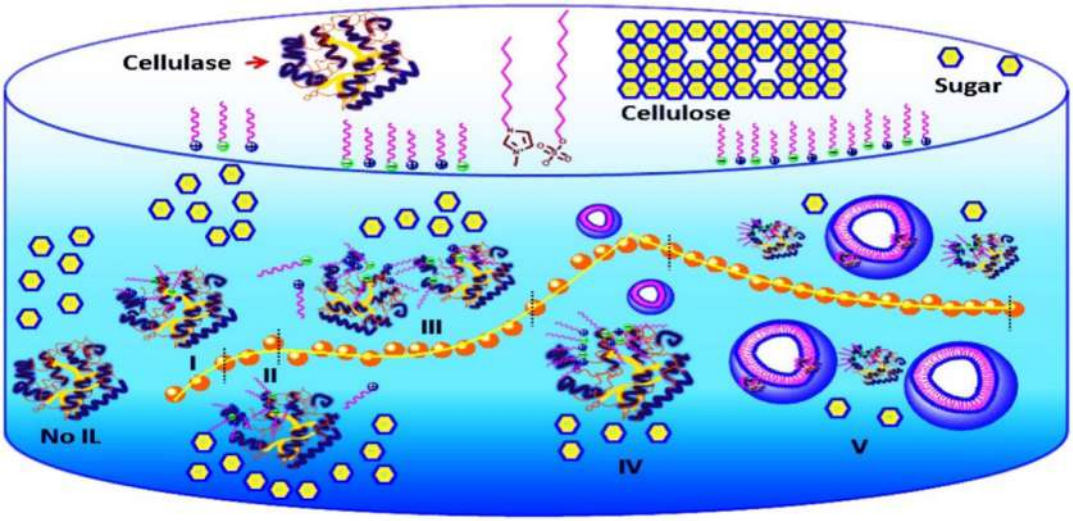


FIGURE 5.5

Concentration-dependent cellulase binding with IL surfactant in different regions.

Reproduced by permission from Ref P. Bharmoria, M.J. Mehta, I. Pancha, A. Kumar, Structural and functional stability of cellulase in aqueous-biamphiphilic ionic liquid surfactant solution, J. Phys. Chem. B 118 (2014) 9890 – 9899, © 2016 The Royal Society of Chemistry.

secondary and tertiary structures of cellulase in the monomeric region (up to the aggregation concentration of 0.026 mM), (b) protein refolding in the aggregation region (up to the saturation concentration of 0.08 mM), and (c) unfolding in the shared aggregation region (below vesicular concentration of 0.39 mM). They also found that cellulase has more binding sites for the cation than the anion, and cellulase was most active in the aggregation concentration region. Bharmoria and Kumar [205] dispersed an IL surfactant (known as choline dioctylsulfosuccinate) in [EMIM][$EtSO_4$] to form vesicular structures above the critical vesicular concentration of 13.52 mM, and found cytochrome *c* was soluble in this colloidal medium (with 100 mM IL surfactant) affording high peroxidase activities that were two-fold of that in neat IL and four-fold of that in aqueous buffer. High activity and stability of cytochrome *c* were seen in this colloidal solution at up to 180°C.

Depending on specific biocatalytic reaction conditions, one or more of these factors could become controlling parameters of that process. The following section focuses on how ILs could be used to improve the enzyme stability following two major routes: modifying the microenvironment of enzymes using ILs and designing enzyme-compatible functionalized ILs as benign media.

5.3 Enzyme stabilization by ionic liquids

Generally, there are three types of methods to attain enzyme stabilization: the modification of enzymes, the modification of enzyme's microenvironment, and the modification of bulk solvent

environments. *The first type* includes physical/chemical modifications of proteins [206] (e.g., physical or covalent attachment to PEG [77,207–209]), genetic engineering or gene cloning of enzymes [210–212], protein engineering [213–217], and various enzyme immobilizations such as through the solid support [218–221], sol-gel and organogel using ILs (so called "ionogel") [68,222–225], cross-linked enzyme aggregates [35,226–230], or coaggregation and cross-linking of enzymes with amyloid fibrils (which were induced by using ILs) [231]. *The second type* of methods includes rinsing with *n*-propanol methods (PREP and EPRP) [226,232], lyophilization with cyclodextrins [233], salt hydrates to control water activity and pH [234–237], enzyme-amphiphile/surfactant complexes/ion-pairing [238–242], water-in-oil microemulsion or reverse micelles [243,244] and water-in-IL microemulsion [245,246], enzyme coating with ILs [247–250], molecular imprinting [251], and ligand-induced enzyme memory [252]. *The third type* is the design of enzyme-compatible solvents such as functionalized ILs and deep eutectic solvents [57,60,253]. This Chapter primarily focuses on how ILs have been utilized to stabilize enzymes through (1) modifying the microenvironment of enzymes and (2) modifying the bulk solvent media through functionalized ILs.

5.3.1 Modifying enzyme's microenvironment using ionic liquids

ILs have been used to modify the microenvironment of enzymes including three common methods discussed below: water-in-IL microemulsions, coating enzymes with ILs, and chemical modification of enzymes with ILs.

5.3.1.1 Water-in-ionic liquids microemulsions

Instead of conventional water-in-oil microemulsions, water-in-IL microemulsions are gaining attractive applications in enzymatic reactions. Goto and coworkers [245] dissolved anionic surfactant known as sodium bis(2-ethyl-1-hexyl)sulfosuccinate (AOT) in hydrophobic [OMIM][Tf_2N] containing 10% (v/v) 1-hexanol (as the cosurfactant), which was mixed with aqueous buffer to form water-in-IL microemulsions. This new microemulsion medium could solubilize several enzymes and proteins (including lipase PS, *C. antarctica* lipase B, α-chymotrypsin, HRP, and enhanced green fluorescent protein). In particular, they observed a higher hydrolytic activity of lipase PS in water-in-IL microemulsions than in water-saturated IL or in conventional water-in-isooctane microemulsions [245]. This group further evaluated HRP-catalyzed oxidation of pyrogallol in water-in-IL microemulsions, resulting in higher enzyme activity and stability than the AOT/water/isooctane microemulsion [254].

Through using a nonionic surfactant Triton X-100, water-in-[BMIM][PF_6] microemulsion was prepared as a new reaction medium leading to higher lignin peroxidase and laccase activities than neat [BMIM][PF_6] or water-saturated [BMIM][PF_6] [255], as well as a high catalytic activity for alcohol dehydrogenase from yeast [256]. The Stamatis group [246] employed nonionic surfactants (Tween 20 and Triton X-100) in [BMIM][PF_6] to yield water-in-IL microemulsions, which was demonstrated as a suitable medium for the esterification of natural fatty acids with various aliphatic alcohols and the hydrolysis of *p*-nitrophenyl butyrate catalyzed by various lipases from *C. rugosa, Chromobacterium viscosum*, and *Thermomyces lanuginose*, resulting in much higher operational stability than other microheterogeneous media. Native protein conformation or a more rigid structure was observed in water-in-IL microemulsions as confirmed by FT-IR and CD spectroscopy.

Pandey and coworkers [257] reported a facile procedure to probe the formation of water-in-IL microemulsions. After the microemulsions were formed by using [BMIM][PF_6] and nonionic surfactant TX-100, the $CoCl_2 \cdot H_2O$ salt was added to show different colors of hexa-coordinated and tetra-coordinated complexes of the cation (depending on the solvating environment) by UV-visible absorbance spectroscopy.

5.3.1.2 Coating enzymes with ionic liquids

Enzymes can be coated with a layer of ILs to provide the protective microenvironment for enzyme stabilization. Lee and Kim [247] coated *Pseudomonas cepacia* lipase with [PPMIM][PF_6] (PPMIM = 1-(3'-phenylpropyl)-3-methylimidazolium) to allow a high enantioselectivity and activity. The Itoh group [248,258] coated lipases with an imidazolium IL carrying anions of cetyl-PEG10-sulfate (Fig. 5.6) and then used the coated enzymes in organic solvents (such as diisopropyl ether), achieving a high enantioselectivity and high reaction rate in enzymatic resolution reactions (up to 500- to 1000-fold increase for some substrates). Later, this group [259] synthesized triazolium alkyl-PEG sulfate ILs (e.g., 1-butyl-3-methyl-1,2,3-triazolium cetyl-PEG10 sulfate), and used them to coat *Burkholderia cepacia* lipase leading to higher enzyme activity and enantioselectivity in diisopropyl ether and ether-functionalized ILs for the transesterification of vinyl acetate with various secondary alcohols. In addition, the IL-coated lipase displayed excellent storage stability in [$CH_3OCH_2CH_2OCH_2$-Et_2N-Me][Tf_2N] (for 2 years at 4°C). Various ILs were used to coat *Mucor javanicus* lipase to activate and stabilize the lipase (vs untreated lipase in aqueous solution), and these ILs (i.e., [BMIM][PF_6], [EMIM][Tf_2N], [BMIM][BF_4] and [EMIM][BF_4]) enabled different degrees of enzyme activation (by 1.81, 1.66, 1.56 and 1.60-fold respectively) [260]. Itoh and coworkers [261] conducted the acylation of secondary alcohols with vinyl acetate catalyzed by lipase PS coated with [$CH_3OCH_2CH_2OCH_2PBu_4$][Tf_2N], leading to high enzymatic activities and selectivities. Lozano and coworkers [249] performed Novozym 435-catalyzed synthesis of citronellyl esters, and found that the lipase coated with ILs could be activated by up to two folds while the activation factor decreased with a lower IL hydrophobicity ([OMIM][PF_6] > [HMIM][PF_6] > [BMIM][PF_6]). Similarly, Mutschler et al. [62] obtained higher conversions of the esterification of methyl-α-D-glucopyranoside with fatty acids catalyzed by IL-coated Novozym 435 beads than by uncoated lipase.

5.3.1.3 Chemical modification of enzymes with ionic liquids

Amino acid residues in enzymes can be covalently modified with functional groups in ILs to improve the enzyme stability. Through carbamate linkages, the Doumèche group [262] covalently attached hydroxy groups of three ILs [i.e.,1-(2-hydroxyethyl)-3-methylimidazolium chloride, 1-(3-hydroxypropyl)-3-methylimidazolium chloride, and choline chloride] to lysine residues of *Candida*

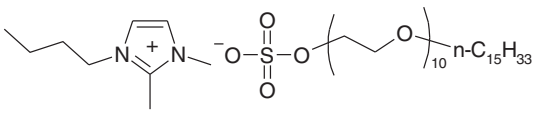


FIGURE 5.6

Structure of 1-butyl-2,3-dimethylimidazolium cetyl-PEG10-sulfate.

boidinii formate dehydrogenase (FDH) (Fig. 5.7). Despite the native FDH was inactive in 70% (v/v) [MMIM][Me_2PO_4], IL-modified FDH retained 30%-45% of its activity. More importantly, IL-modified FDH showed 3-6-fold higher stability in aqueous buffer; and FDH exhibited higher half-time $t_{1/2}$ values in 37.5% (v/v) [MMIM][Me_2PO_4] than in carbonate buffer. Furthermore, this group [263] expanded hydroxylated cations to a series of imidazoliums and pyrrolidiniums with different alkyl chain lengths, and discovered that FDH grated to larger cations (kosmotropic) cause the enzyme inactivation, but FDH modified by smaller cations (chaotropic) retained high activities even in 30% (v/v) [MMIM][Me_2PO_4] although the wild-type enzyme was deactivated by 2.8 folds in this IL solution than in buffer. The Huang group covalently linked *C. rugosa* lipase (CRL) to dual-functionalized ILs containing carboxylic acid and ether groups (via *N,N'*-carbodiimide) [264] (see Fig. 5.8) or to choline dihydrogen phosphate (via 1,1′-carbonyldiimidazole) [265]. After the IL modification, CRL in the enzymatic hydrolysis of olive oil displayed an enhancement in catalytic activity, thermal stability, organic solvent tolerance, and adaptability to temperature and pH fluctuations. Their CD spectra suggested that the lipase modification by ether-functionalized ILs resulted in an increase in β-sheet structure and a decrease in α-helix content. In a similar approach, CALB and porcine pancreatic lipase (PPL) were functionalized with carboxylic acid-functionalized imidazolium and cholinium cations separately, enabling a higher lipase thermal stability and/or enantioselectivity [266,267]. A more recent development is using functionalized ILs as coupling agents to link enzymes with solid supports [e.g., chitosan-mesoporous silica hybrid nanomaterials [268] and multiwalled carbon nanotubes [269,270]], leading to improved thermal/storage stability and better reusability of enzymes.

Following the steps described in Fig. 5.9, the Huang and Hu group [271] synthesized magnetic carboxymethyl cellulose first, followed by the covalent attachment of a carboxyl-containing hydrophobic IL, which was linked to lysine residues of PPL or PGA. These immobilized enzymes

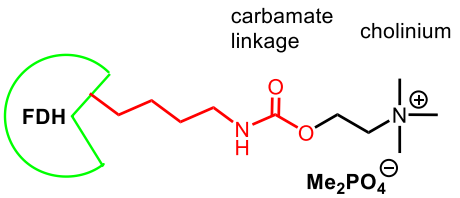


FIGURE 5.7

Covalent attachment of a cholinium salt to the lysine residue of formate dehydrogenase.

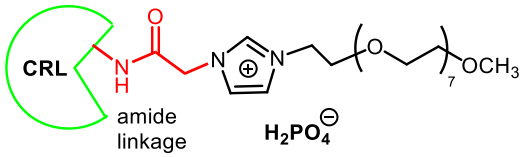


FIGURE 5.8

Covalent modification of *Candida rugosa* lipase (CRL) by an ether-functionalized IL.

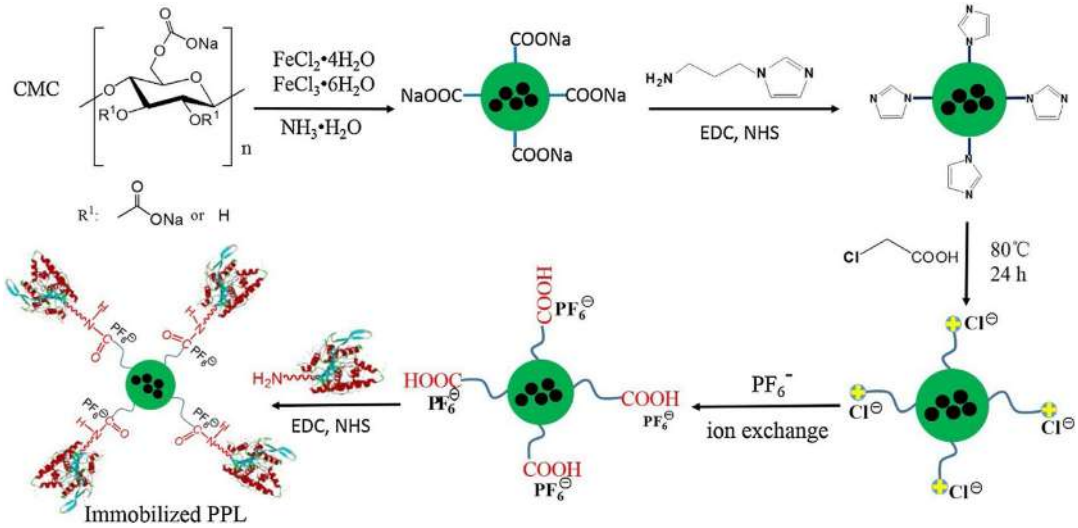


FIGURE 5.9

Lipase immobilization onto IL-modified magnetic carboxymethyl cellulose.

Reproduced by permission from Ref H. Suo, L. Xu, Y. Xue, X. Qiu, H. Huang, Y. Hu, Ionic liquids-modified cellulose coated magnetic nanoparticles for enzyme immobilization: Improvement of catalytic performance, Carbohydr. Polym. 234 (2020) 115914, © 2020 Elsevier.

showed greater thermal stability and higher tolerance to denaturant urea and pH changes than free enzymes or immobilized analogs without using ILs. This improvement was correlated with a lower α-helix content and a higher β-sheets after the immobilization.

5.3.2 Designing enzyme-compatible functionalized ionic liquids

Many solute or solvent molecules carrying hydroxy (e.g., polyols and sugars) and ether groups (e.g., diisopropyl ether) are highly compatible with enzymes affording high enzyme activity and stability, possibly due to favorable "water-like" hydrogen-bond donating and accepting environment generated by these functional groups [57,169]. Therefore, this section mainly focuses on how the incorporation of hydroxy- and/or ether-groups onto IL structures leads to enzyme activation and stabilization.

Park and Kazlauskas [54] discovered that an ether-functionalized IL [$CH_3OCH_2CH_2$-MIM][BF_4] could solubilize about 5 mg/mL D-glucose at 55°C, which afforded a faster reaction (99% conversion) of CALB-catalyzed acylation of D-glucose with vinyl acetate than nonfunctionalized imidazolium and pyridinium ILs. Kim and coworkers [272] conducted enzymatic acylation of monoprotected glycosides with vinyl acetate catalyzed by *C. rugosa* lipase, and reported ILs (i.e., [BMIM][PF_6] and [$CH_3OCH_2CH_2$-MIM][PF_6]) enabled faster and more selective reactions than organic solvents such as THF and chloroform. They explained that this could be due to a higher substrate solubility in ILs and a better lipase compatibility of polar ILs. The Itoh group [248,258] synthesized anion-functionalized ILs containing polyoxyethylene(10) cetyl sulfate (Fig. 5.10) paired

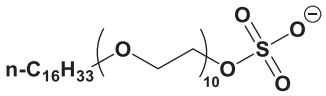


FIGURE 5.10

Structure of polyoxyethylene(10) cetyl sulfate.

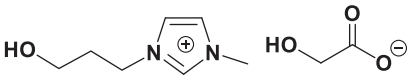
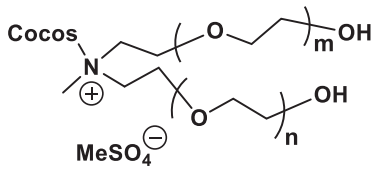


FIGURE 5.11

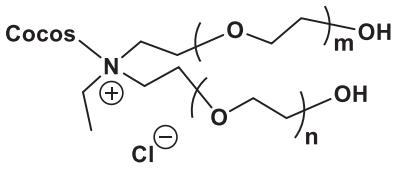
Structure of 1-(3-hydroxypropyl)-3-methylimidazolium glycolate.

with imidazolium cations, which were used as either additives or lipase-coating-agents for lipase-catalyzed transesterifications of secondary alcohols (such as 1-phenylethanol) and vinyl acetate in diisopropyl ether or hexane. Both approaches improved the enantioselectivity while the IL-coating method also enhanced the reaction rate. The Stephens group [273] found that laccase maintained its activity in aqueous 20% (v/v) hydrophilic ILs carrying alkyl sulfate anions with long alkyl chains or alkoxy chains, and in 20% (v/v) hydrophobic ILs containing anions of Tf_2N^-, AOT^- (= 1,4-bis (2-ethylhexyl)sulfosuccinate), or PF_6^-. In this case, water-immiscibility is not necessarily correlated with high enzyme activity; as an example, laccase was not active in 20% (v/v) hydrophobic [C_{10}MIM][SCN] and [C_{10}MIM][saccharin]. Walker and Bruce [274,275] functionalized both cation and anion by hydroxy groups of a hydrophilic IL, 1-(3-hydroxypropyl)-3-methylimidazolium glycolate (Fig. 5.11), and dissolved both morphine dehydrogenase and its cofactor nicotinamide in this IL and recorded a high enzymatic activity for the oxidation of codeine to codeinone.

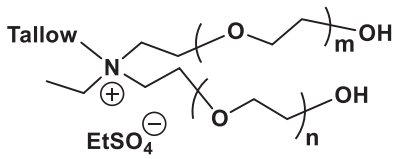
A series of ionic mixtures comprising multiple alkoxy and/or hydroxy groups are known as Ammoeng ILs (Fig. 5.12). This family of ionic solvents shares similar structures and properties with polyglycols. The Xu group [276–281] noted that triglycerides are soluble in Ammoeng 100 (i.e., [CPMA][$MeSO_4$], CPMA = cocosalkyl pentaethoxy methylammonium methylsulfate) and Ammoeng 102 (see Fig. 5.12), and obtained high lipase activities during glycerolysis reactions in these ionic solvents [277,278]. Trioctylmethylammonium bis(trifluoromethylsulfonyl)imide ([TOMA][Tf_2N]) and its mixture with Ammoeng 102 were also ideal solvents for enzymatic glycerolysis [280–282]. De Diego and coworkers [99] reported that [CPMA][$MeSO_4$] enabled high CALB transesterification activities but much lower activities for two other lipases from *Thermomyces lanuginosus* and *R. miehei* when compared with ILs containing PF_6^- and BF_4^- anions. The Kroutil group [283] reported higher alcohol dehydrogenase activities in 50%-90% (v/v) hydroxy-functionalized ILs than nonfunctionalized ILs, and ranked the enzyme activity in a decreasing order with different ILs as [$(HOCH_2CH_2)_3MeN$][$MeSO_4$] > Ammoeng 101 > Ammoeng 100 > Ammoeng 102. The Kragl group [284] purified two different alcohol dehydrogenases using aqueous two-phase based on Ammoeng 110 (Fig. 5.12), and observed this functionalized ionic mixture could dissolve enzymes and hydrophobic substrates. Wallert et al. [285] performed enantioselective hydrolysis of diester malonates in 10% aqueous isopropanol catalyzed by pig liver esterase using 1% functionalized ILs (as additives) such as Ammoeng 100, 101 and



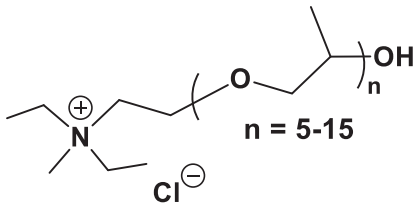
(A) Ammoeng 100
Cocos = C_{14} alkyl group; m + n = 4-14



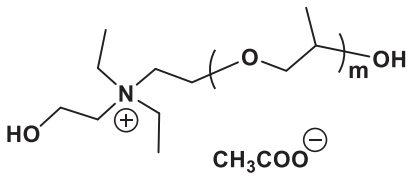
(B) Ammoeng 101
m + n = 14-25



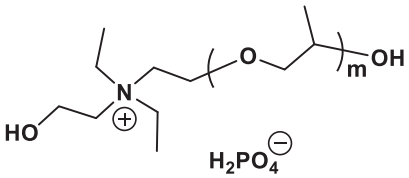
(C) Ammoeng 102
Tallow = C_{18} acyl group; m + n = 14-25



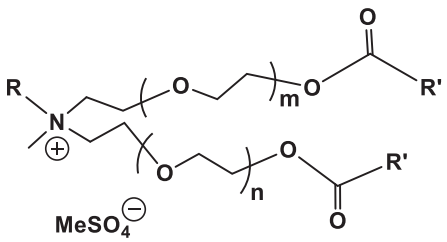
(D) Ammoeng 110



(E) Ammoeng 111
m = 50-60



(F) Ammoeng 112
m = 50-60



(G) Ammoeng 120
R, R' = C_{18} acyl group; m, n, unavailable

FIGURE 5.12

Structures of functionalized Ammoeng ILs.

112, and $[HOCH_2CH_2\text{-MIM}]Cl$, and reported up to four-fold increase in enzyme activity and high enantioselectivities (up to 97% *ee*).

Tris(hydroxymethyl)aminomethane (Tris) is often used in buffer preparation (pH mostly in the range of 7-9). In addition, Tris acted as a lyoprotectant during lyophilization of HRP [286]. To mimic the Tris structure, Das and coworkers [287] prepared tetrakis(2-hydroxyethyl)ammonium triflouromethanesulfonate {$[(HOCH_2CH_2)_4N][CF_3SO_3]$, Fig. 5.13}, and obtained the activation of HRP in this new medium for up to 10-fold (vs in methanol) and for 30−240 times (vs in nonfunctionalized ILs). Another approach reported by Lee and coworkers [288] combined several Good's buffer anions (MOPSO, BES, TAPSO, and CAPSO, see Fig. 5.14) with Bu_4N^+, Bu_4P^+ and cholinium cations respectively to form self-buffering ILs (retaining most temperature-independent buffering capacity as Good's buffers themselves). Although these ILs are too viscous (or solid at room temperature) to be used as solvents alone, their aqueous solutions have found applications in biocatalytic processes. Lipase from *P. cepacia* showed higher stability in some of these self-buffering ILs especially at a higher concentration (i.e., 1.0 M). More interestingly, these self-buffering ILs could form aqueous biphasic systems with potassium citrate to facilitate the enzyme partition into IL-rich layer and the partition of reaction product into citrate salt-rich layer. Vafiadi et al. [289] conducted the esterification of glycerol with sinapic acid catalyzed by feruloyl esterase-catalyzed in $[HOCH_2CH_2\text{-MIM}][PF_6]$ and $[CH_3(OCH_2CH_2)_2\text{-MIM}][PF_6]$, resulting in conversion yields up to 72.5% and 76.7% (respectively) under optimized conditions. The Itoh group [29] prepared an ether-functionalized phosphonium $[CH_3OCH_2CH_2\text{-}Bu_3P][Tf_2N]$, and observed a faster reaction rate in this IL than in diisopropyl ether for lipase PS-catalyzed transesterification of secondary alcohols.

The Zhao group [31,32,290−292] systematically evaluated glycol-functionalized ILs with the combinations of imidazolium or alkylammonium cations and acetate (or formate) anions (Fig. 5.15), and discovered these tailored ionic solvents could solubilize many unique substrates including cellulose, xylan, lignin, D-glucose, 3,4-dihydroxy-DL-phenylalanine (DOPA), betulinic acid, and Miglyol oil (a mixture of triglycerides of caprylic acid and capric acid). The high solubilization ability of these ILs is primarily due to high hydrogen-bond basicity of anions (e.g., acetate and formate), which is likely the mechanism that induces enzyme deactivation. However, when a longer glycol chain is grafted onto ILs, the overall molar concentration of deactivating anions is decreased.Therefore, the deactivating nature of these ILs is minimized [32]. This rationale was indirectly demonstrated by another group [293] that [BMIM][dca] deactivated Novozym 435 while [aliq][dca] [$aliq^+$ = trioctylmethylammonium (Aliquat 336® is a mixture of C_8 and C_{10} chains with C_8 predominating)] retained a relatively high activity and enantioselectivity. A logical explanation

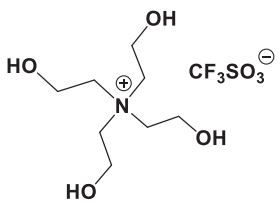


FIGURE 5.13

Structure of tetrakis(2-hydroxyethyl)ammonium triflouromethanesulfonate.

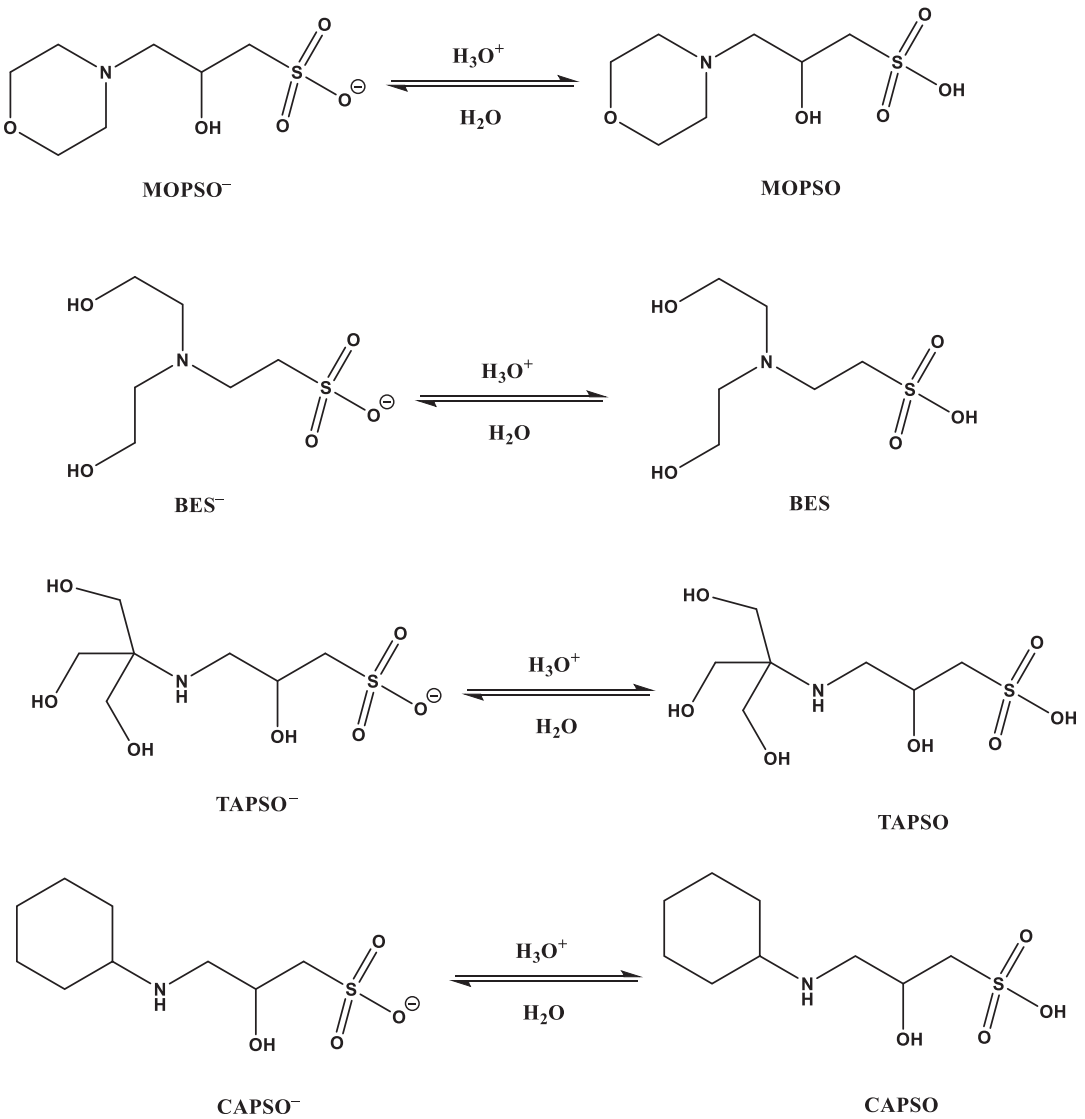


FIGURE 5.14

Equilibria of Good's buffer anions with acid compounds (**MOPSO**: 2-hydroxy-3-morpholinopropanesulfonic acid; **BES**: 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; **TAPSO**: *N*-[tris(hydroxymethyl)methyl]-3-amino-2-hydroxypropanesulfonic acid; **CAPSO**: 3-(cyclohexylamino)-2-hydroxy-1-propanesulfonic acid).

is that $aliq^+$ is a much larger cation than $BMIM^+$ and thus the molar concentration of denaturing dca^- in [aliq][dca] is significantly lower than in [BMIM][dca]. Based on the above rationale, trans-esterification activities of free and immobilized CALB in most acetate- and formate-based ILs are

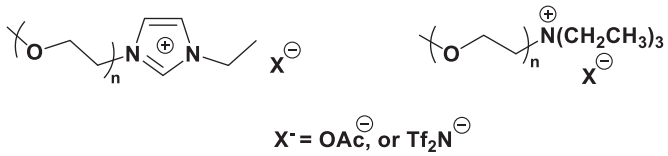


FIGURE 5.15

Ether-functionalized imidazolium (IM) and ammonium ILs {[$CH_3(OCH_2CH_2)_n$-Et-IM]X and [$CH_3(OCH_2CH_2)_n$-Et_3N]X, respectively} ($n = 1, 2, 3,\ldots$).

higher than or comparable with those in *tert*-butanol and [BMIM][Tf_2N] [32]. Because of their unique capability in dissolving D-glucose and cellulose, these ILs were suitable for accomplishing the regioselective transesterification of carbohydrates catalyzed by Novozym 435 [31]. Additionally, the enzymatic transesterification of Miglyol oil with methanol was evaluated in these ILs to produce biodiesel, leading to up to 70% triglyceride conversion in 1 h and 85% conversion in 48 h [292]. Also, because of their dissolution ability toward lignocelluloses, these solvents have been used to pretreat biomass before their enzymatic hydrolysis [291,294,295]. Following a similar approach, the Tian group [296] synthesized acetate-based imidazoliums functionalized with both glycol and allyl groups, and found that these ILs could dissolve 10−12 wt.% corn starch at 100°C; the dissolved starch was esterified with methyl stearate or methyl palmitate catalyzed by Novozym 435 at 50°C, achieving the degree substitution up to 0.138 and 0.153 respectively. Unfortunately, proteases show a higher sensitivity to ILs and are easily deactivated by acetate- or formate-based ILs, so the Zhao group [227] converted these functionalized to their hydrophobic analogs pairing with Tf_2N^- anions (e.g., [$CH_3(OCH_2CH_2)_3$-Et_3N][Tf_2N], [$CH_3(OCH_2CH_2)_3$-Et-IM][Tf_2N] and [$CH_3(OCH_2CH_2)_3$-Me-Et-IM][Tf_2N] in Fig. 5.15), and reported high synthetic activities (1−3 μmol/min/g) and selectivity (97%−99%) of immobilized subtilisin and α-chymotrypsin in these hydrophobic ILs containing 10%−15% (v/v) water. The same reaction in *tert*-butanol or [BMIM][Tf_2N] showed poor transesterification activities (e.g., 0.2−1 μmol/min/g in *tert*-butanol) and/or poor selectivity (40%) when the water concentration was greater than 2% (v/v). Meanwhile, a lipase (Novozym 435) also displayed comparable or higher transesterification activities in these functionalized hydrophobic ILs than in [BMIM][Tf_2N] and *tert*-butanol by using the assay reaction of ethyl sorbate and 1-propanol [297].

Zhou et al. [298] studied lipase-catalyzed kinetic resolution of secondary alcohols in ether-functionalized imidazolium ILs carrying Tf_2N^- or PF_6^- anions, and observed higher enantioselectivities (95%−99% *ee*) in several ether-functionalized ILs than in nonfunctionalized ILs (i.e., [BMIM][BF_4] and [BMIM][PF_6]) and diisopropyl ether. Ståhlberg and coworkers [299] obtained high glucose isomerase activities in the isomerization of glucose to fructose in *N*,*N*-dibutylethanolammonium octanoate with 20 wt.% water, but lost its activity in ILs containing smaller alkanoate anions (such as formate, acetate, and propionate). Ou et al. [300] found that CALB lost its transesterification activity in deactivating [BMIM][NO_3], but retained certain activities in hydroxy-functionalized ILs (such as [$HOCH_2CH_2$-MIM][NO_3] and [$HOCH_2CH_2$-MIM][BF_4]) and these activities were further improved by 2−5 folds when adding so called "IL buffer" ([BMIM][H_2PO_4]). Fluorescence spectra indicated the native compact structure of CALB in hydroxy-functionalized ILs. The same group [301] pointed out that enzyme-benign ILs should have similar

properties as water molecules for having high dielectric constants and both solvent donor and acceptor properties to adequately ionize ionizable groups in protein molecules to allow enzyme molecules to be solubilized and stabilized. Following this logic, this group functionalized both cations (imidazoliums and ammoniums) and anions with hydroxy groups (Fig. 5.16), and observed high transesterification activities for two lipases (CALB and *P. cepacia* lipase) in these ILs. One drawback of hydroxy-functionalized ILs is their relatively high viscosities attributed to hydrogen-bonding [26,301]. Kundu and coworkers [302] formed reverse micelles along with cyclohexane and water by using a surface-active protic IL, known as L-proline propyl ester lauryl sulfate ($[ProC_3][LS]$). The inclusion of bovine serum albumin (BSA) in these micelles resulted in a higher percentage of secondary structures without any buffer than the native BSA in the droplet core. The Feder-Kubis group [303] grafted (1*R*,2*S*,5*R*)-(−)-menthol to imidazolium, pyridinium and alkylammonium cations to produce ether-functionalized chiral ILs (see Fig. 5.17), and reported that laccase maintained similar activities in buffer saturated with these hydrophobic ILs [except 20% activity loss in IL (**B**) shown in Fig. 5.17] as in buffer alone. The laccase stability in buffer saturated with ILs (**A**, **D**, and **E**) was higher than that in buffer itself.

The Zhao group [304,305] grafted glycols with different chain lengths to various IL cations (such as phosphonium, imidazolium, pyridinium, alkylammonium, piperidinium, and sulfonium) (see Fig. 5.18), and observed these functionalized ILs having low dynamic viscosities (33−123 mPa s at 30°C), and high thermal stability with decomposition temperatures (T_{dcp}) between 318 and 403°C (except the sulfonium IL **9** in Fig. 5.18 with $T_{dcp} = 254$°C). Among these functionalized ILs (all containing 0.02 wt.% water), $[CH_3OCH_2CH_2\text{-}Et_3N][Tf_2N]$ (**7**) and $[CH_3OCH_2CH_2\text{-}Py][Tf_2N]$ (**6**) afforded the highest enzymatic transesterification activities (6.57 and 6.08 μmol/min/g CALB, respectively) at 50°C, which are higher than $[BMIM][Tf_2N]$ (5.12 μmol/min/g CALB) but are lower than *tert*-butanol (7.38 μmol/min/g CALB). More excitingly, the thermal stability of Novozym 435 was significantly improved in $[CH_3OCH_2CH_2\text{-}Et_3N][Tf_2N]$ (**7**) than in *tert*-butanol at 50°C and 70°C. Due to their high enzyme-compatibility and high thermal stability, these functionalized ILs were investigated as cosolvents in enzymatic ring-opening polymerization (ROP) of L-lactide (130°C for 7 days) and ε-caprolactone (70°C for 2 days) to form polyesters with high molecular mass (M_w ∼20−25 kDa) and modest yields (30%−65%). Shi and coworkers [306] synthesized glycol-functionalized ammonium ILs including $[Me_2N(Et)(CH_2CH_2O)_2H][PF_6]$, which was blended with 1,2-dimethoxyethane at the 5:18 (v/v) ratio to produce a homogeneous

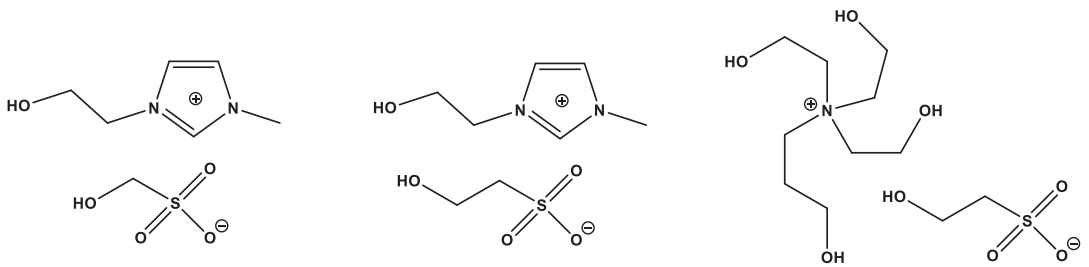


FIGURE 5.16

Structures of hydroxy-grafted imidazolium and ammonium ILs.

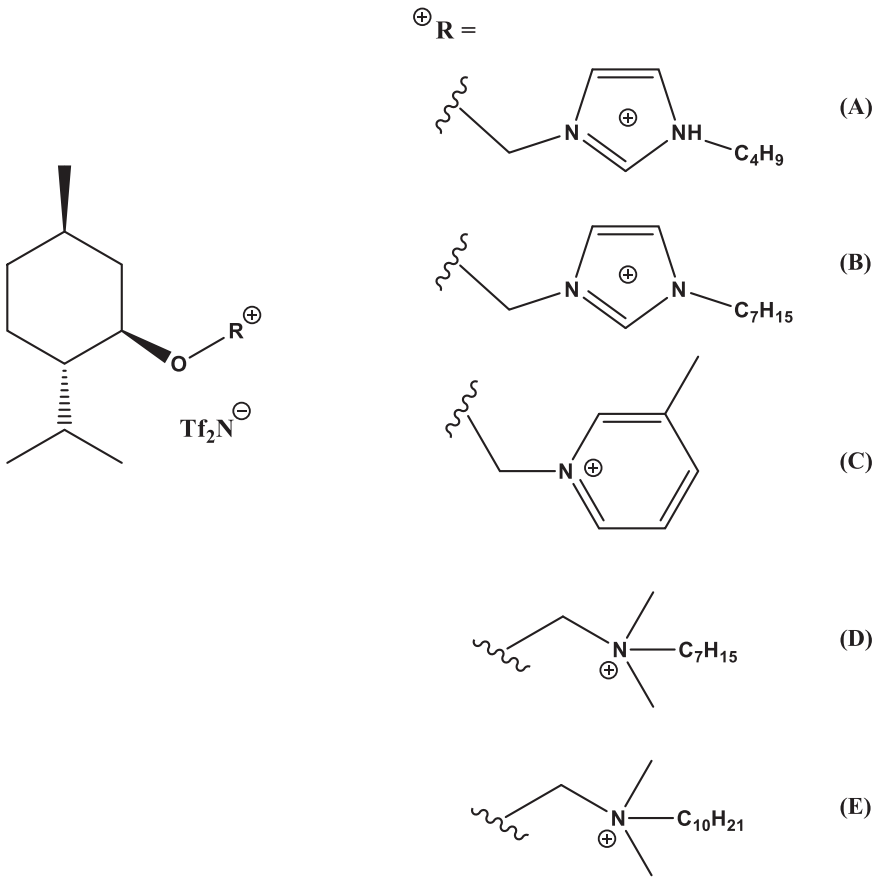


FIGURE 5.17

(1*R*,2*S*,5*R*)-(−)-Menthol-grafted chiral ILs.

solution below 33°C but two layers above this temperature. This temperature-controlled phase-separation solvent system found its application in a biocatalytic process for the enantioselective reduction of ethyl 2-oxo-4-phenylbutyrate to an alcohol using baker's yeast at 30°C (one phase), and after the reaction was finished, the temperature was increased to form two phases (IL layer and organic layer) to allow simple product separation. This new enzymatic process enhanced the product yield by 35% and *ee* by 25%−30% (vs that in 1,2-dimethoxyethane).

Most of the previous functionalized ILs are grafted with only ether or hydroxy group ("monofunctionalization"). The Ammoeng type of ILs (Fig. 5.12) have both functional groups, but their disadvantages are ionic mixtures without exact structural composition and properties, relatively high viscosities, reactive primary/secondary hydroxy groups, and discontinued commercial availability. It has been reported that *tert*-alcohol groups cause less inhibition to enzymes and are considerably less reactive substrates than primary or secondary alcohols in nonaqueous biocatalytic processes

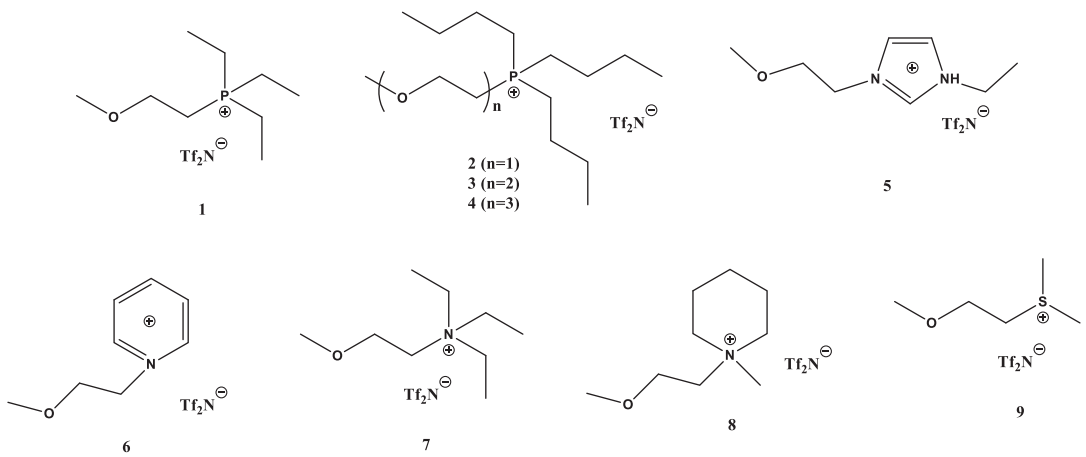
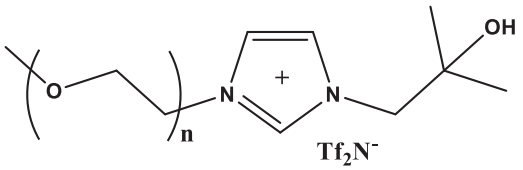


FIGURE 5.18

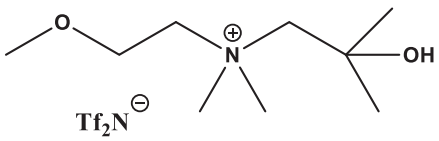
Structures of ether-functionalized ILs.

[307,308]. Based on this consideration, the Zhao group [309] constructed dual-functionalized imidazolium-type ILs carrying both *tert*-alcohol and ether groups (e.g., ILs **10**-**12** in Fig. 5.19), which mimics the water molecule that has both hydrogen-bond donating (−OH) and accepting (R-O-R) structures. Consequently, these "water-like" ILs significantly improved Novozym 435's transesterification activity, which was up to 2-4-fold higher than nonfunctionalized ILs such as [BMIM][Tf_2N], and up to 40-100% higher than *tert*-butanol and diisopropyl ether. The thermal stability of lipase in these dual-functionalized ILs was considerably higher than in *tert*-butanol, and was similar to that in diisopropyl ether. To address the issue of these imidazolium-ILs having relatively high dynamic viscosities (~300 mPa s at 30°C), the Zhao group [310] switched the cation core to alkylammonium and prepared dual-functionalized ammonium ILs (**13**-**15** in Fig. 5.19) with lower viscosities (as low as 129 mPa s at 30°C); transesterification activities of Novozym 435 in these ammonium-based ILs was about 1.5-fold higher than in *tert*-butanol, and slightly higher than in diisopropyl ether. Enzymatic ROP reactions of ε-caprolactone in these ammonium-based ILs enabled polyesters with high molecular mass M_w (up to 18,000 Da) and high yields (up to 74%). The latest report by the Zhao group [311] revealed a systematic design of water-mimicking IL structures incorporating glycol ether, *tert*-butanol, and/or trimethylsilyl groups, which displayed high compatibility with hydrolases. In addition to IL structures, transesterification activities of hydrolases were found highly dependent on the water content; in the presence of 2%−3% water, several top-performing ILs (such as ILs **10**, **13**, **16** and **17** in Table 5.3) improved Novozym 435 activity by up to 1.8-fold than *tert*-butanol and by up to 1.6-fold than diisopropyl ether. The subtilisin activity in these water-mimicking solvents could be very high but the selectivity dropped quickly when the water content was above 2% (v/v). To maintain the high selectivity (100%), IL **16** (Table 5.3) activated the protease by 1.2-fold than diisopropyl ether. Fluorescence emission spectra confirmed the preservation of characteristic emission maximum peaks in "water-like" ILs in most cases.

In several enzymatic reactions, functionalized ILs underperformed than nonfunctionalized analogs. Lozano and coworkers [312] carried out enzymatic enantioselective transesterification of



10 (n=1)
11 (n=2)
12 (n=3)



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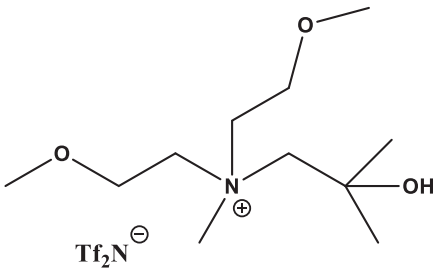

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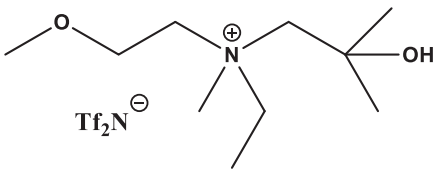

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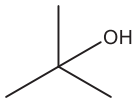
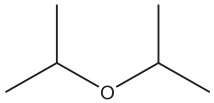
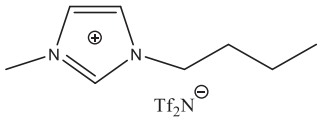
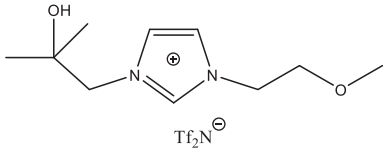
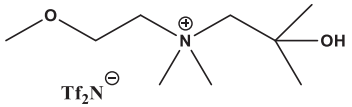
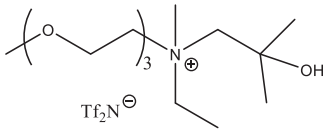
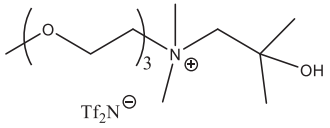
FIGURE 5.19

Structures of "water-like" dual-functionalized imidazolium and ammonium ILs.

1-phenylethanol and vinyl propionate in ILs, and noticed a higher selectivity but lower CALB activity and stability in functionalized [$HOCH_2CH_2CH_2$-Me_3N][Tf_2N] than in nonfunctionalized ammonium ILs. The Goto group immobilized enzymes using comb-shaped PEG (so called PM_{13}) to form PM_{13}-lipase [77] and PM_{13}-subtilisin [78,313], and found these enzyme preparation being more active in [EMIM][Tf_2N] than in functionalized ILs such as [$CH_3OCH_2CH_2$-MIM][Tf_2N] and [$HOCH_2CH_2$-MIM][Tf_2N].

The underlying reasons of how ether-/hydroxy-functionalization enables higher enzyme compatibility have been discussed in several studies. The IL lipophilicity usually decreases when appending hydroxy, ether or nitrile group to the side chain of ILs, which minimizes the IL inhibition of acetylcholinesterase (used as a broad toxicity screening assay) [314–317] and reduces IL acute toxicity toward *Daphnia magna* and *Vibrio fischeri* [318]. Luo and coworkers [319] observed that imidazolium and pyridinium cations formed complexes with PEG-800 or PEG-1000 via ion-dipole interaction where cations were surrounded by PEG chains. It is known that ether chains are more flexible than rigid alkyl chains in ILs [320], and thus the incorporation of ether chain decreases intermolecular correlation (particularly tail-tail segregation) and cation-anion specific interactions [321,322]. Therefore, glycol-functionalized cations favorably interact with ether chains through intramolecular and/or intermolecular attractions, reducing the cation-protein interaction. When IL cations are grafted with long alkyl chains (such as $P_{666(14)}^+$), there exists hydrophobic interactions between the cation and Leu17 residue of *B. cepacia* lipase

Table 5.3 Optimized water contents for highest transesterification activities [311].

	Solvent	Novozym 435			Subtilisin		
		Water (v%)	Lipase activity[a]	Lipase selectivity	Water (v%)	Protease activity[b]	Protease selectivity
		0.017	6.66	97%	5.0	12.65	25
		0.02	7.83	>99%	1.0 2.0	11.76 44.47	100 41
		1.0	8.80	>99%	2.0 10.0	1.31 10.45	97 20
10		2.0	11.41	>99%	2.0 10.0	3.52 21.79	21 12
13		2.0	11.37	>99%	2.0 10.0	7.04 34.44	18 16
16		2.0	11.94	>99%	2.0 10.0	14.28 55.55	100 28
17		3.0	12.21	>99%	2.0 10.0	9.77 24.62	100 20

Notes

[a] *Lipase (Novozym 435) activity in the unit of μmol/min/g free CALB.*

[b] *Immobilized* Bacillus licheniformis *protease (known as subtilisin A) activity in the unit of μmol/min/g free protease. All lipase and protease activities were within the margin of error of 5%.*

evidenced by molecular docking, causing lower enzymatic/hydrolytic activities [323,324]. Spectroscopic and computational studies allow us to further understand the enzyme structures and conformation changes in functionalized ILs. The Rogers group [325] observed less denaturation of HSA in aqueous hydroxy- and ether-functionalized imidazolium ILs than in [BMIM]Cl as visualized by fluorescence and CD spectroscopy, which could be attributed to preferential hydrogen bonding between functionalized ILs and HSA. Using near-UV CD spectroscopy, Mann et al. [90] studied the thermal stability and refolding patterns of lysozyme in aqueous ILs and observed that ethanolammonium formate prevented lysozyme from thermal unfolding and enabled its renaturation upon cooling. Also, lysozyme was found six times more active in aqueous ethanolammonium formate than in aqueous buffer, which was explained by that IL hydroxy groups provided hydrogen-bond donating and accepting sites to reduce hydrophobic interactions between IL and lysozyme. In contrast, aqueous 2-methoxyethylammonium formate was unable to improve the thermal stability of lysozyme, suggesting the important role of hydrogen-bond donor (−OH group) in lysozyme stabilization. The Bruce group [326] dissolved subtilisin in diethanolammonium chloride and found that the protease retained its activity; however, they found no enzyme activity for subtilisin dissolved in other protic hydroxyalkylammonium-based ILs and for chymotrypsin in these ILs. Although fluorescence spectroscopy was unable to differentiate protein structural changes, far and near UV spectra pointed out that secondary and tertiary structures of subtilisin were preserved well in diethanolammonium chloride. Wijaya and coworkers [327] reported that lysozyme activity increased in the order of different protic ILs: (2-hydroxy)ethylammonium nitrate < ethylammonium formate < (2-hydroxy)ethylammonium formate < ethylammonium nitrate, and rationalized the trend by the similar water affinity between two kosmotropes or between two chaotropes. Bose et al. [328] observed high thermal stability of *T. reesei* cellulase in tris(2-hydroxyethyl)methylammonium methylsulfate (HEMA) at up to 115°C by using fluorescence spectroscopy and DSC. Similarly, this group [329] noted a higher thermal stability of *endo*-1,4-β-D-glucanase (EG) in HEMA (transition temperature at ∼75°C) than in buffer (∼55°C) by using fluorescence spectroscopy, which was aligned with the high enzyme activity in this IL at the temperature. HEMA is more enzyme-stabilizing than imidazolium-based analogs. The Torkzadeh-Mahani group [330] found an improved activity of urate oxidase in 1% (v/v) aqueous triethanolammonium butyrate, but lower activities at higher IL concentrations (5% and 10%). To explain this, they carried out MD simulations and concluded that intramolecular hydrogen bonds of the enzyme increased in the presence of 1% IL, which reduced random coils and increased α-helix and β-sheet structures for a more compact protein structure. On the contrary, MD simulations by Klähn and coworkers [331] suggested more CALB destabilization was caused by polar methoxyethyl group on IL cations than nonfunctionalized cations, and by decyl side chain than short methyl groups. The destabilization mechanisms are quite different because methoxyethyl group interacts strongly with the CALB surface via Coulomb interactions, but long alkyl chain interacts with the hydrophobic core of CALB via hydrophobic interactions.

In summary, functionalized ILs that are enzyme-compatible share some common structural features: (1) for hydrophilic ILs, they have large molecular structures to minimize hydrogen-bond basicity and nucleophilicity of anions; (2) they are designed to have multiple ether and/or hydroxy groups to enable preferential IL-enzyme interactions. Currently, ILs dual-functionalized with both ether and *tert*-alcohol groups are one of the top-performing nonaqueous solvents that promote enzyme activation and stabilization.

5.4 Summary

Further experimental and computational studies are needed to uncover complex interactions between enzymes and ILs and to identify factors controlling enzyme stabilization and activation. Efforts in both protein engineering and solvent engineering continue to discover innovative methods to improve the enzyme stability and activity in ILs. The effort in solvent engineering includes the use of ILs to modify either the microenvironment or bulk solvent environment of the protein. The ultimate goal is to have IL-compatible/tolerating enzymes and enzyme-compatible ILs to afford high enzyme stability and high catalytic activity (approaching the same order of magnitude as in aqueous solutions).

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CHAPTER

Clean biocatalysis in sponge-like ionic liquids

6

Pedro Lozano[1], Rocio Villa[1], Susana Nieto[1], Antonio Donaire[2] and Eduardo García-Verdugo[3]

[1]Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Chemistry, University of Murcia, Murcia, Spain [2]Department of Inorganic Chemistry, Faculty of Chemistry, University of Murcia, Murcia, Spain [3]Inorganic and Organic Chemistry Department, E.S.T.C.E., Universitat Jaume I, Castelló, Spain

6.1 Solvents, enzymes, and sustainable chemistry

Chemistry is the science that has contributed the most to improving our quality of life. The molecules and materials created by chemists, later implemented in their industrial production, have allowed us to reach levels of comfort unimaginable a century ago. However, the preservation of our environment and sustainability in the use of our resources are the main objectives of 21st century if we want to perpetuate our permanent improvement in the quality of life. In addition to moderation in our consumption habits, a sustainable society will also depend on chemical products and processes, which should be designed according to principles that make them favorable to the preservation of life. Important inherent properties of molecules need to be considered from the design stage to address whether compounds and processes are depleting versus renewable, toxic versus benign, and persistent versus readily degradable [1].

The chemical industry is increasing demand for clean chemical processes to follow a policy of environmental care. In this context, the Twelve Principles of Green Chemistry have been emerged as clear rules to achieve a nonhazardous and sustainable chemical industry, and as minimally harmful as possible for the health of people and ecosystems [2]. In this context, products, feedstock, and chemical manufacturing processes will need to adhere to these principles, which could essentially be focused on two main actions: (i) to minimize, or even to eliminate, the use of hazardous substances that are not fully recoverable, and (ii) to maximize the selectivity in chemical transformations. As result of both actions, the new chemical processes must continue to be economically viable, which involves that new sustainable procedures must not increase the final price of the products. In other words, the production also needs to stay competitive for low-cost processes [3].

Regarding the first action, the use of volatile organic solvents (VOSs) makes an important contribution to the environmental deterioration of chemical processes in terms of health, safety, or impact on air, water and land. It also significantly defines the cost of the process, given the impossibility of its full recovery [4]. Solvents are essential auxiliary materials in the vast majority of chemical synthesis procedures, since they act as a medium for the transport of mass and heat, for the reaction and for the separation of products. These VOSs come to represent between 80% and 90% of the total mass used in any organic reaction, and between 70% and 85% of the waste

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produced [5]. By these facts, the use of some of the more traditional VOSs (e.g., dichloromethane, N, N-dimethylformamide, etc.) is associated with air, land and water contaminations, which represents a clear disagreement with the development of sustainable chemical processes. Furthermore, there are many key criteria, such as product separation and scrubbing, solvent recovery and reuse, energy consumption, environmental impact, toxicity of solvents, unwanted reagents and byproducts, etc., that need to be considered for the designing of clean chemical processes. Due to their volatile nature, VOSs can only be partially recovered for further reuse, although this requires protocols with high energy consumption, that contributes even more to increase their environmental footprint. Supercritical fluids, Deep Eutectic Solvents, organic solvents derived from bio-renewable raw materials (e.g., 2-methyltetrahydrofuran, γ-valerolactone, cyrene, etc.) are reported in other Chapters of this book as emerging green solvents, able to acts as substitutes to classical non-aqueous solvents. They are shown as useful approaches for developing different clean chemical processes (e.g., reactions, separations, extractions, etc.) with low environmental impact, including its easy recovery for further recycling [6].

With respect the second key action, it should be noted that the art of chemical synthesis has reached a considerable degree of maturity, and it is possible to prepare any target organic molecule regardless of its complexity. However, the lack of selectivity in chemical transformations is probably the biggest cause of the economic, environmental and social impact of chemical processes, because of the undesirable by-products. Formation of wastes in organic synthetic processes is crucial, and often linked to the traditional use of a stoichiometric amount of reagents, particularly in custom synthesis and fine chemicals. Furthermore, when considering usual chemical protocols intended for synthetic transformations from a scaling-up perspective, very few are viable, because key criteria such as product separation and purification, recovery and reuse of catalysts and reaction media, energy consumption, environmental impact, toxicity of solvents and reagents, etc., are not taken into account at the outset. Thus, for scaling a production system of interest, a complete redesign and optimization of the synthetic methodology is often required, opening up the transfer from research laboratory to commerce [7,8].

Nature has always been a source of inspiration for chemists. The ability to transfer the exquisite catalytic efficiency shown by enzymes in nature to chemical processes in nonaqueous system is probably the most important challenge for green chemistry [9]. The chemical transformations in living systems are carried out by informational biopolymers with defined monomer sequence (i.e., enzymes and RNAs). They may be considered as the most efficient catalysts for green chemical processes, because they are biocompatible, biodegradable, obtained from renewable resources, and more important, they are fully suitable for the design of multicatalytic processes of industrial interest, as it is the usual way they work in living cells [10]. This later was smartly demonstrated by Edward Buchner (Nobel Prize in Chemistry 1907) when carried out the ten-step biocatalytic path of alcoholic fermentation for transforming glucose to ethanol in a test-tube by using an extract of died yeasts as multicatalysts [11].

In this regard, biocatalysis-based processes have enormous potential in chemical industry, as they are able to increase stereo-, chemo- and regio-selectivities of a vast number of chemical transformations [12]. More than 13,000 enzyme-catalyzed reactions have been successfully demonstrated at laboratory scale, offering clear advantages for the synthesis of enantiopure fine chemicals against any other kind of catalysts. As results, the chemical industry is exploring the great potential of biocatalysis to manufacture both bulk and fine chemicals [13]. This fact is improved by the

continuous developments in genomics, directed evolution and the exploitation of our natural biodiversity, because great improvements in the activity, stability and specificity of enzymes, as well as a huge increase in the number and variety of their industrial applications, are discovered every day [14]. Furthermore, the technological applications of enzymes are greatly enhanced in nonaqueous environments, rather than in their natural aqueous reaction media, because of the catalytic promiscuity that results in the expansion of the repertoire of substrates and chemical transformations. Most compounds are insoluble in water, and water frequently gives rise to unwanted side reactions and degrades common organic reagents. Also, the thermodynamic equilibria of many processes are unfavorable in water, and product recovery is sometimes difficult from this medium [15].

Lipases (triacylglycerides hydrolases; EC 3.1.1.3) are an enzyme family of serin hydrolases, which catalyze the hydrolysis of carboxylic ester bonds in hydrophobic compounds, such as triglycerides, under natural aqueous conditions. Besides, in nonaqueous media, biocatalysts exhibit a wide specificity to recognize very different natural and unnatural substrates [16], carrying out synthetic reactions based on alcoholysis, esterification or transesterification approaches by transferring acyl groups from esters to different nucleophiles (e.g., thiols, amines, etc.) under anhydrous conditions (see Fig. 6.1) [17,18].

In this context, the efficiency, selectivity, and sustainability benefits offered by enzymes are enticing chemists and chemical industries to consider biocatalytic transformations to complement or even supplant traditional synthetic routes. Increasing demands for efficient and versatile synthetic methods, combined with powerful new discovery and engineering tools, has prompted innovations in biocatalysis, especially the development of new enzymes for precise transformations of high interest in chemistry [19].

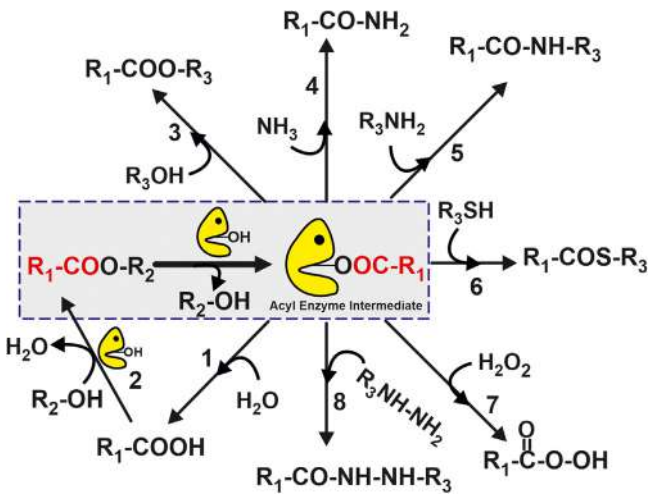

FIGURE 6.1

Schematic of lipase-catalyzed different chemical reactions. 1, Hydrolysis; 2. Esterification; 3, Alcoholysis; 4, Ammoniolysis; 5, Aminolysis; 6, Thiolysis, 7, Perhydrolysis; 8, Hydrazynolysis.

6.2 Essentials of ionic liquids

Since the beginning of this century, ionic liquids (ILs) have been shown as a new class of solvents with the potential to ensure the sustainability of an extremely broad range of chemical processes [20]. ILs are not molecular solvents, they are salts, and so exclusively composed by ions, which are liquid at temperatures below 100°C. Using NaCl as an example, molten NaCl (up 800°C) is an ILs, while an aqueous solution of this salt is an ionic solution [21]. Unlike most organic solvents, ILs have very low vapor pressures and, hence, are nonvolatile. They are also quite inert and nonflammable, and have excellent chemical and thermal stability. In this context, when using ILs, the risk of exposure and the possibility of damage the atmosphere are practically inexistent. This green label of ILs permits their full recovery for further reuse [22]. Due to these exceptional green features and to their versatility as tuneable solvents, they have a vast array of applications [23].

Typical ILs are based on organic cations, for example, dialkylimidazolium, tetraalkylammonium, etc., paired with anions that may have a strongly delocalized charge (e.g., [PF_6], bistriflimide, etc.), some of which are depicted in Fig. 6.2. Due to its ionic nature and the enormous versatility of the ions, ILs are organized into a network of heterogeneous nanostructures, in which coulombic attractions between ions, hydrogen bond formations, solvation effects and dispersion forces between hydrophobic chains are responsible for the interactions that occur in the network, which essentially are responsible for their unique properties [24]. In fact, an extremely wide range of features (such as hydrophilicity/hydrophobicity, viscosity, melting point, electric conductivity, thermal stability, large liquid range, among others) can be obtained just through a combination of specific ions. Hence, a tailored IL can be designed for almost every chemical process (e.g., reaction, extraction, etc) to achieve an optimal operation. By the appropriate selection of cation and anion, the polarity and hydrophilicity/hydrophobicity of ILs can be tuned, modifying its miscibility with molecular solvents (i.e., water, organic solvents, etc.), which has been applied for developing useful approaches of products recovery from the reaction mixture [25]. When ILs are analyzed by using relative Reichardt's scale polarity of molecular organic solvents, ranging from tetramethylsilane as

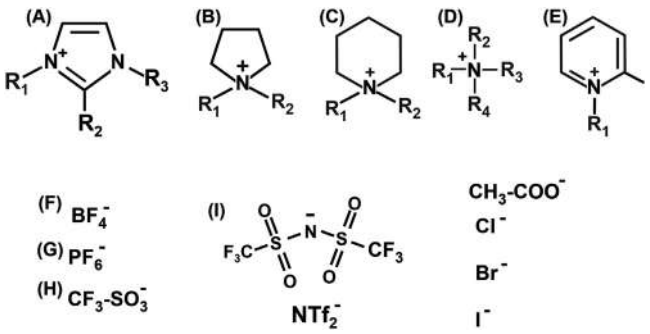

FIGURE 6.2

Some cations (A) alkylimidazolium; (B) dialkylpyrrolidinium; (C) diakylpiperidinium; (D) tetraalkylammonium; (E) N-alkylmethylpyridinium), and anions (F) tetrafluoroborate; (G) hexafluoroborate; (H) trifluorosulfonate or triflate; (I) bistriflymide or [NTf_2]; acetate, chloride, bromide and iodide) found in the most widely used ILs.

the lowest polar, and water as the highest polar solvent, it becomes evident that ILs are polar solvents (see Fig. 6.3) [26].

As can be seen, the polarity of these green solvents is quite similar to those organic solvents miscible with water (e.g., ethanol), although this fact contrasts with the nonmiscibility with water of some of ILs. Another amazing feature is the hygroscopic character of water-immiscible ILs, being able to absorb certain amount of water (e.g., up to 1.4% w/v for both the 1-butyl-3-methylimidazolium bistriflimide [Bmim][NTf_2] [27], and the 1-decyl-3-methylimidazolium bistriflimide [Dmim][NTf_2] [28]), which is an intriguing characteristic when designing enzymatic chemical transformations in water-immiscible ILs.

Fundamental structural studies evidence that ILs can segregate into polar and nonpolar domains, where the polar domains are formed by the charged groups of the cations and anions, while the nonpolar domains are essentially the alkyl side chains of cations. These domains have been characterized by different experimental techniques as well as by theoretical simulations, which provides a good insight into the degree of segregation and the possible morphology of the domains [29]. As a function of these features, ILs can be regarded as nano-structured materials, allowing neutral molecules to reside in less polar regions, while ionic or polar species undergo faster diffusion in the more polar ones. Hence, the nanoscale structural heterogeneity could influence not only the ILs properties but also transport properties and availability of reagents and (bio)catalysts [30].

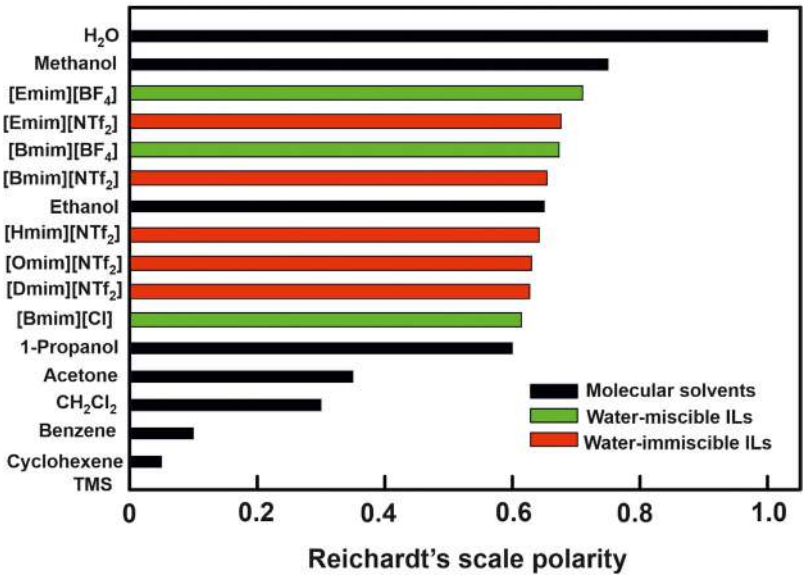

FIGURE 6.3

Comparative representation of the polarity of some water-miscible and water-immiscible ILs with respect to different molecular organic solvents, according to the Reichardt scale of polarity.

Data obtained from ref J. Dupont, On the solid, liquid and solution structural organization of imidazolium ionic liquids, J. Braz. Chem. Soc. 15 (2004) 341–350.

6.3 Understanding biocatalysis in ionic liquids and beyond

The success for any enzyme-catalyzed chemical transformation begins by understanding the singularity of biocatalysis, where the protein nature of enzymes should always be taken into account, because they could easily be irreversibly deactivated by many physical or chemical factors. Enzymes are biological macromolecules based on unique sequences of amino acid monomeric units, which are designed and produced by cells to catalyze reactions in aqueous media, and within a narrow range of environmental conditions (e.g., temperature, pH, etc.), which in fact determine the limits of the life. As result of its informational sequence, each active enzyme show a unique 3-D structural organization, that it is maintained by a high number of weak internal interactions (e.g., van der Waals, salt bridge, hydrogen bonds, etc.), as well as interactions with other molecules (i.e., substrates, effectors, water, etc.) [31]. Enzymes typically fold in such a way that nonpolar residues are buried in a hydrophobic core, while polar residues tend to move to the surface, where they are hydrated. In all nonconventional media, water is a key component for maintaining the active conformation of enzymes.

The exploitation of the tremendous catalytic potential of enzymes for organic chemistry, by switching reaction media from water to nonaqueous environments, is not an easy task, because the disruption in the native enzyme conformation through unfolding leads to biocatalysts inativation [10].

Dry proteins are very rigid and completely inactive in the absence of water, because it acts as a molecular lubricant for enzyme dynamics, being accepted that a few clusters of water molecules are required for the catalytic function [15]. As one example, studies on myoglobin hydration by using dielectric relaxation spectroscopic and high-resolution X-ray crystallography shows that the optimal hydration shell consists of *ca*. two layers of water that surround proteins, which involves up to 1911 water molecules (0.5 nm thick around the protein) [32]. Thus, the internal structures at the active site of proteins are constantly shaped by strong interactions with the hydration shell and bulk water motions. In this context, hydrophilic solvents strip the essential water molecules interacting with enzymes, resulting in fast biocatalyst deactivation [33]. On the contrary, water-immiscible solvents typically afford higher enzymatic activity than hydrophilic ones, being assumed that a hydrated enzyme placed in a dry hydrophobic system is trapped in the native state maintaining its catalytic activity.

Free or immobilized enzymes are commonly used as lyophilized preparation, because the essential water shell around the protein is preserved. As enzymes fold by placing the nonpolar residues into the hydrophobic core, while polar residues tend to move to the hydrated surface, a "memory" phenomenon is observed when a lyophilized enzyme preparation is placed in a dry hydrophobic system. In water-immiscible organic solvents, the lyophilized biocatalyst may be trapped in the native state as a consequence of the low dielectric constant of the medium, and this intensifies intramolecular electrostatic interactions and enables the maintenance of its catalytic activity [15,34]. Sometimes the dehydration process changes the enzyme structure and results in diminished catalytic activity in organic solvents. This detrimental effect can be avoided by lyophilizing enzymes in the presence of lyoprotectants (e.g., polyols, salts etc.) [35], as protective agents against unfolding, or even by the direct addition of small quantities of water to enzyme suspensions in anhydrous water-immiscible solvents [36].

As nonaqueous reaction systems, the behavior of ILs with respect to water, as the essential solvent to preserve enzyme activity, may be considered as an important rule for the success of any biocatalytic process [37]. Usually, water-miscible ILs behave as polar and water-miscible organic solvents, producing enzyme deactivation at low water content by water stripping. However, the biocatalytic activity in water miscible ILs is highly dependent on the nature and concentration of ions. As an example, the alcalase-catalyzed enantioselective resolution of N-acetyl amino acids was improved greatly by using 10% v/v of N-ethylpyridinium trifluoroacetate ([EPy][TFA]), but drastically fall at higher IL concentration [38]. A similar behavior of bell-shaped activity profile with respect the concentration of IL in water was observed for other enzymes (e.g., lacasse, chloroperoxidase, formate dehydrogenase, beta-galactosidase, etc.). Although, these liquid reaction media are really constituted by ionic solutions in water, and not true ILs, the deactivation was also attributed to the water-stripping phenomenon [39].

On the opposite, all the water-immiscible ILs assayed (e.g., [Bmim][NTf_2], [Bmim][PF_6], [Btma][NTf_2], etc.) have been shown as suitable reaction media for biotransformations at low water content, emerging as viable alternatives to molecular organic solvents for organic synthesis [10,17,40]. Under anhydrous conditions, these ILs provide an appropriate microenvironment to enzymes because of the preservation of the essential hydration shell, resulting in an enhancement in activity and stability.

The study of secondary structures for different proteins, like monellin [41], α-chymotrypsin [42], and *Candida antarctica* lipase B—CALB [43]) in water-immiscible ILs (e.g., [Bmim][NTf_2], [Btma][NTf_2], [Bmpy][NTf_2], etc.), by both circular dichroism and fluorescence spectroscopies, demonstrated that the stabilization of enzymes in water-immiscible ILs is correlated with the maintenance of the native protein conformation. Water-immiscible ILs form a strong ionic matrix which retains enzyme molecules in an adequate microenvironment, resulting in a supramolecular net able to keep the protein conformation active because of the preservation of its essential water shell.

Fig. 6.4 shows a model of the structural organization of imidazolium ILs in solid and liquid phases, being described as an extended network of cations and anions connected together by hydrogen bonds. The monomeric unit is always constituted by one imidazolium cation surrounded by at least three anions and, in turn, each anion is surrounded by at least three imidazolium cations, where the strongest hydrogen bond always involves the H placed at C-2 position of the imidazolium ring, as the most acidic proton. The supramolecular net is also maintained by classical π/π stacking interactions between imidazolium rings [45]. Thus, the incorporation of other molecules into the IL network induces changes in the structural organization, and in some cases (e.g., water) may cause the formation of polar and nonpolar regions. Wet ILs are shown as nano-structured materials, which allow neutral molecules to reside in less polar regions, and ionic or polar species to undergo faster diffusion in the more polar or water rich regions [24,30]. Accordingly, enzymes in water-immiscible ILs should also be considered as included into hydrophilic gaps of the network, where the observed stabilization of enzymes may be attributed to the maintenance of this strong net around the protein (see Fig. 6.4B) [44,46]. Furthermore, considering a classical enzymatic process that occurs in water, protein unfolding at increased temperature can also be attributed to the disruption of the structural organization of the medium, as a consequence of the increase in the kinetic energy of water molecules when heated. The extremely ordered supramolecular structure of ILs in liquid phase act as a "mold" that hinders the free movement of water molecules, maintaining an active three-dimensional structure of the enzyme in aqueous nano-environments, and avoiding the

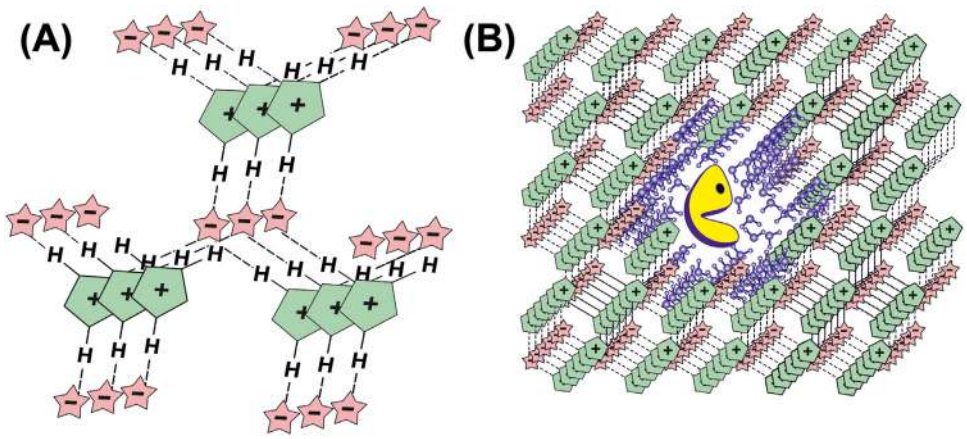

FIGURE 6.4

(A) Model of the supramolecular structure of imidazolium IL net based on hydrogen bonding interactions [24,30]. (B) The active enzyme with the native folded conformation into a wet gap of the water-immiscible IL network [10,17,44].

classical thermal unfolding [47]. As a practical example, the immobilization of *Pseudomonas cepacia* lipase in the solid IL [1-(3'-phenylpropyl) − 3-methylimidazolium)][PF_6] was carried out just by the simple suspension of the enzyme solution in the IL at its melting temperature (53°C), then cooling to room temperature. Afterwards, the resulting solid mixture was sliced in small pieces, obtaining functional immobilized enzyme derivative with markedly enhanced enantioselectivity and without losing any significant activity toward reuse [48]. By this pioneering approach published in 2002, this switchable solid/liquid IL system was used for the entrapment of active enzyme molecules in a nonaqueous environment based on ILs, pointing out the broad possibilities for developing biocatalytic processes in water-immiscible ILs.

Biocatalytic processes in water-immiscible ILs usually are carried out by the direct addition of the free or immobilized enzyme to the reaction medium containing previously dissolved substrates, where the biotransformation occurs. Fig. 6.5 shows the schema of a pioneering example of lipase-catalyzed kinetic resolution of a *sec*-alcohol in [Bmim][PF_6] IL as reaction medium, by using vinyl acetate as acyl donor [49]. The excellent suitability of this enzyme/IL reaction system (i.e., 99% ester yield, enantioselective E-val $>$ 500) was additionally emphasized as results of the full maintenance of enzyme activity during operation cycles of recovery and reuse. After the separation of products by liquid-liquid extraction with diethyl ether, as an organic solvent nonmiscible with the IL, the enzyme remained "anchored" to the IL phase, because enzymes cannot be separated from ILs by liquid-liquid extraction (e.g., with buffer or aqueous solutions). In fact, it is necessary to filter the enzyme-IL solution through ultrafiltration membranes (e.g., 10,000 Da. cutoff) to separate proteins from the IL [17]. This approach permits reuse of the enzyme-IL system by addition of fresh substrates, without any loss in activity. However, this approach has an evident weakness with regards the overall greenness of any chemical process, although liquid-liquid extraction with VOSs is the most usual technique for the recovery of products after biotransformation in ILs systems.

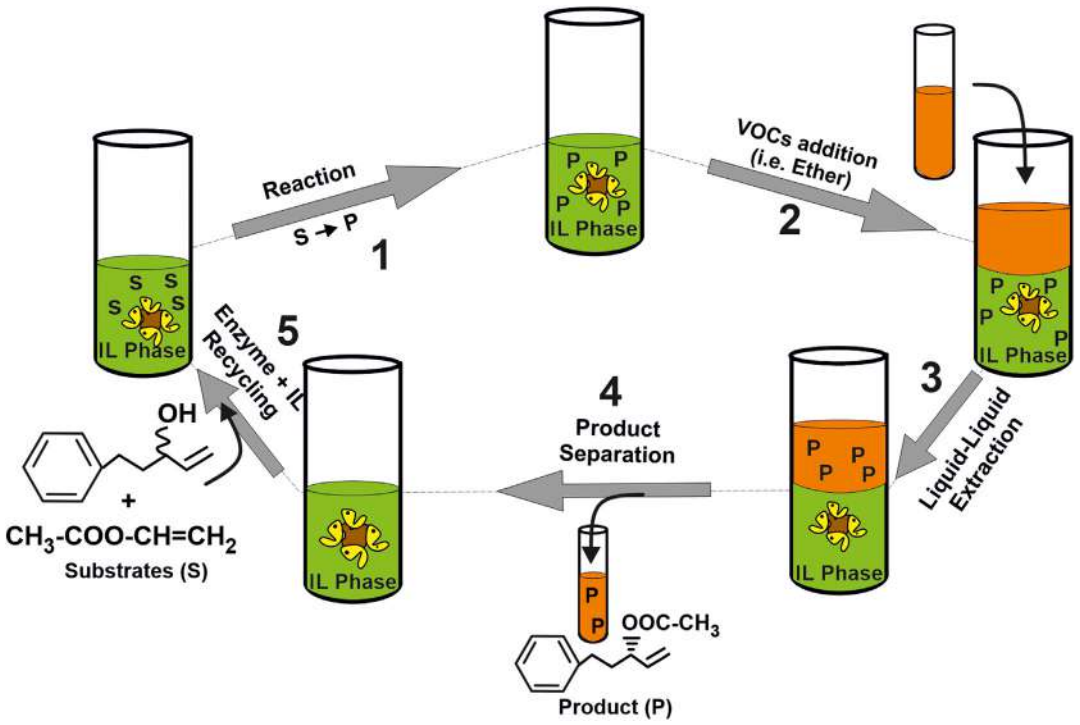

FIGURE 6.5

Step-by-step strategy most usually applied for enzyme-catalyzed chemical transformations in monophasic reaction systems based on water-immiscible ILs, including the product separation, and the recycling of the biocatalyst/IL.

Nowadays, the development of advanced processes suitable to directly provide pure products by integrating all chemical transformations, product separation and recovery steps, as well as the reuse of the solvent and the catalytic phases by straightforward approaches, are key features to build green chemical industries. In this context, additionally to the observed improvements in catalytic efficiency, the combination of enzymes with the IL technologies lead to amazing synergies in the design of smarts approaches for clean product separation [5]. Some of them, such are IL/scCO_2 biphasic reactors, membrane reactors, nano-drop systems, microfluidic devices, supported ionic liquid phases, sponge-like ILs, etc. are described in other Chapters of this book.

6.4 Sponge-like ionic liquids: an enabling green tool to integrate reaction and separation processes

The term *sponge-like ionic liquids* (SLILs) refers to hydrophobic ILs with long alkyl side-chains that behave as temperature switchable ionic liquid/solid phases at a temperature higher than

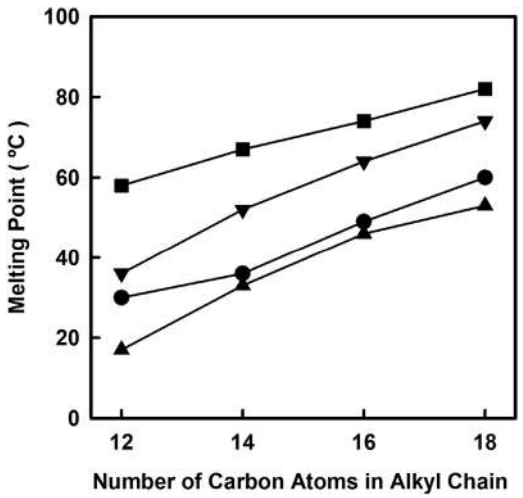

FIGURE 6.6

Changes in melting temperature for ILs based on 1-alkyl-3methylimidazolium cation and different anions (▲, [NTf_2]; ●, [BF_4]; ▼, [PF_6]), as well as for the alkyltrimethylammonium [NTf_2] (■) ILs series.

Data were obtained from refs P. Lozano, J.M. Bernal, E. Garcia-Verdugo, G. Sanchez-Gomez, M. Vaultier, M.I. Burguete, et al., Sponge-like ionic liquids: a new platform for green biocatalytic chemical processes, Green. Chem. 17 (2015) 3706–3717. P. Lozano, J.M. Bernal, M. Vaultier, Toward continuous sustainable processes for enzymatic synthesis of biodiesel in hydrophobic ionic liquids/supercritical carbon dioxide biphasic systems, Fuel 90 (2011) 3461–3467.

room temperature but lower than 100°C [50]. The melting points of ILs have been widely studied, being observed that this physical parameter depends on both the nature of ions, as well as the length of the alkyl chain of cations [22]. Fig. 6.6 shows the changes in melting temperature for a series of 1-alkyl-3-methylimidazolium cations, combined with [BF_4], [PF_6] or [NTF_2] [51], as well as the series of alkyltrimethylammonium cations combined with [NTf_2] [52]. For all cases, the increase in the alkyl length of cation increases the melting temperature of the IL, which could be related with the increase in van der Waals interactions between the aliphatic chains, that may contribute to favor the configuration of polar (charged ionic) and non-polar (hydrophobic) regions of the IL nanostrucuture.

The excellent suitability of ILs, based on hydrophobic anions (e.g., [NTf_2], and cations with long alkyl side-chains, for example, octadecyltrimethylammonium [C_{18}tma], hexadecyltrimethylammonium [C_{16}tma], 1-ocadecyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide, [C_{18}mim]), etc. for dissolving hydrophobic compounds (e.g., vegetable oils [51], biodiesel [52], terpene esters [53], etc.) upon heating near their melting points, has been widely reported. The resulting monophasic and fully clear liquid solutions containing both the compound and the SLIL, became monophasic solid systems after cooling down to room temperature. However, the most awesome feature of these ILs is the fractionation of the components of these solid phase mixtures by simple centrifugation at a temperature lower than room. As a representative example, Fig. 6.7A shows the phase behavior of a 40:60 w/w geranyl acetate/[C_{16}tma][NTf_2] mixture at 50°C (picture 1), at room

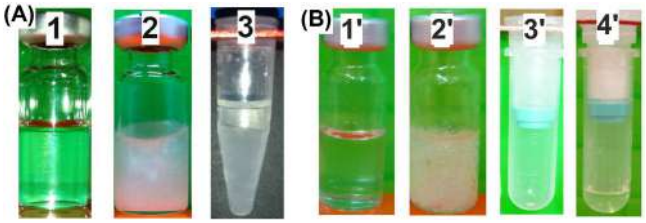

FIGURE 6.7

(A) Phase behavior of a 40:60 w/w geranyl acetate:[C_{16}tma][NTf_2] mixture at 50°C (**1**), at 25°C (**2**), and after centrifugation at 14,000 rpm (30 min) and at 10∘C (**3**). (B) Phase behavior of a 30:70 w/w anisyl acetate acetate:[C_{16}tma][NTf_2] mixture at 50°C (**1'**), at 0°C (**2'**), placed into a centrifugal filter (0.25 μm pore size, **3'**), and after centrifugation at 16,000 rpm (10 min) and at 0°C (**4'**).

Reproduced from ref P. Lozano, J.M. Bernal, C. Gomez, E. Garcia-Verdugo, M.I. Burguete, G. Sanchez, et al., Green bioprocesses in sponge-like ionic liquids, Catal. Today, 255 (2015), 54–59 with permission from Elsevier.

temperature (picture 2) and after centrifugation during 30 min at 14,000 rpm and at 10°C (picture 3). As can be seen in the last picture, the centrifugation step results in two different phases, an upper liquid phase contained the flavor ester, and a bottom of solid IL. At this point, it should be underlined how monophasic mixture solutions of molecular solvents (e.g., 50% v/v ethanol/water, etc.), which become solid though freezing, cannot be then separated by centrifugation. Indeed, the most usual chemical operation for carrying out the separation of liquid mixtures requires heating steps (i.e., distillation). On the opposite, the unique nature of this temperature switchable ionic liquid/solid phase enables the separation of dissolved molecules by cooling/centrifugation.

In another representative example, Fig. 6.7B shows the phase behavior of a 30:70 w/w anisyl acetate/[C_{16}tma][NTf_2] mixture, where the separation process can be improved by introducing the solid mixture cooled to 0°C (picture 2') into a nylon centrifugal filter (0.2 μm pore size, picture 3'). Then, the application of external forces at low temperature (e.g., 10 min centrifugation, 16,000 rpm, 0°C) leads to the retention of the solid IL phase above the nylon membrane, while the liquid flavor filters through the membrane to the bottom of the tube (see picture 4'), as though wringing out a sponge [54].

Because of the simplicity of this separation approach, the residual IL content in the liquid phase (e.g., as determined by using ^{19}F NMR spectroscopy) could be used as appropriate parameter to define the greenness and efficiency of this protocol. By considering the separation of a 50/50 (w/w) [C_{16}tma][NTf_2]/geranyl acetate mixture by a cooling/centrifugation process, it was observed how the decrease in temperature during both the cooling and centrifugation steps resulted in the improvement of the separation efficiency. In this context, although the separation of this mixture after cooling at room temperature provided a top liquid phase essentially based on the flavor molecules, this liquid phase became solid when incubated in an ice bath, as result of the presence of residual IL. However, by following an iterative centrifugation protocol of four steps (10 min, 14,000 rpm) and lowering the temperature from room (25°C approx.) to 4°C, the pure geranyl acetate could be separated as an upper IL-free liquid phase, as confirmed by ^{19}F NMR, while the solid IL remained at the bottom of the tube. Due to the full recovery of the IL, the greenness of the approach was demonstrated. However, it should be noted that the overall yield of the extraction

process after the iterative cooling/centrifugation protocol did not permitted to recover the overall mass-flavor initially dissolved in the IL, that is 51% geranyl acetate, 59% citronellyl acetate, 60% neryl acetate, or 82% isoamyl acetate, respectively [53]. Although the increase in centrifugation time and/or centrifugation speed might improve these result.

To explain all these features, the solid/liquid structural organization of ILs, including the role of dissolved molecules should be take into account. However, as regards the structure of imidazolium ILs, based on an ionic network formed by monomeric units of a cation surrounded by three anions and viceversa (see Fig. 6.4), Dupont describes that the incorporation of molecules in the IL network causes changes in their physical chemical properties, and even the formation of regions with different polarity where these solutes are housed in the net [45]. In this context, in the absence of dissolved solutes, the structural organization of the bare ILs could be considered as an intermediate between that of a classical solvent and that of a liquid crystal. Although, the highly degree of structural organization of ILs is clearly defined at the solid state, it could be considered that the anion-cation interactions partially maintains this structural organization at the liquid phase, even when the liquid IL phase contains dissolved compounds [50]. Other structural studies evidence that neat ILs contains clusters or micro-heterogeneities as result of the chemical structure of ions, resulting in nano-segregation between polar and nonpolar domains. These polar domains are formed by the head groups of the ions, while the nonpolar domains are formed by the alkyl groups. These domains have been characterized by different experimental techniques as well as by theoretical simulations, which provides good insight into the degree of segregation and the possible morphology of the domains. As a function of the length of the alkyl chain in imidazolium cations, it has been found that the segregation between domains was ranged from 8 Å (for the butyl chain) to 35 Å (for the octadecyl chain) [29]. Moreover, the peculiar features of IL clusters are the coexistence of electrostatic between ions, and weak intermolecular forces (e.g., van der Waals, hydrogen bonds, $\pi-\pi$ stacking, polarization forces, etc.), as the mainly responsible of the solute IL interactions for dissolving and/or extraction.

Moreover, by studying the local and long-range bulk liquid structures of different alkylammonium protic ILs (i.e., ethylammonium hydrogen sulfate, ethylammonium formate, etc.), it was demonstrated that these green solvents arrange into a sponge-like bicontinuous nanostructure consisting of polar and apolar domains. The analysis of these ILs by neutron diffraction and computer simulations shows that the lengthening of the cation alkyl chain leads to nanostructures in which the polar and apolar domains are better segregated, while changes in the anion have little effect on their structure [55]. In the same context, the structural organization of alkylpyrrolidinium-based ILs has also been described as an intricately nanostructured net. For the case of ILs containing cations with shorter alkyl chain substituents, they form alternating cation-anion monolayer structures on confinement to a thin film, while cations with a longer alkyl chain substituent leads to bilayer formation [56].

In agreement with the above reported structural features of ILs, a *sponge-like IL hypothesis*, mainly focused on the temperature switchable ionic liquid/solid systems, has been proposed to explain the phase behavior that occurs when dissolving, or extracting, hydrophobic solutes (Fig. 6.8) [50,52–54]. The unique features observed in Fig. 6.7 might be explained as a consequence of this nano-structured organization of $[C_{16}tma][NTf_2]$, where the large alkyl side chains of cations could interact essentially by van der Waals forces, forming hydrophobic holes suitable for the incorporation of alkyl flavor esters (e.g., geranyl acetate) (see Fig. 6.8A). When dissolving nonpolar solutes in these green solvents, they might be incorporated into the nonpolar regions of ILs

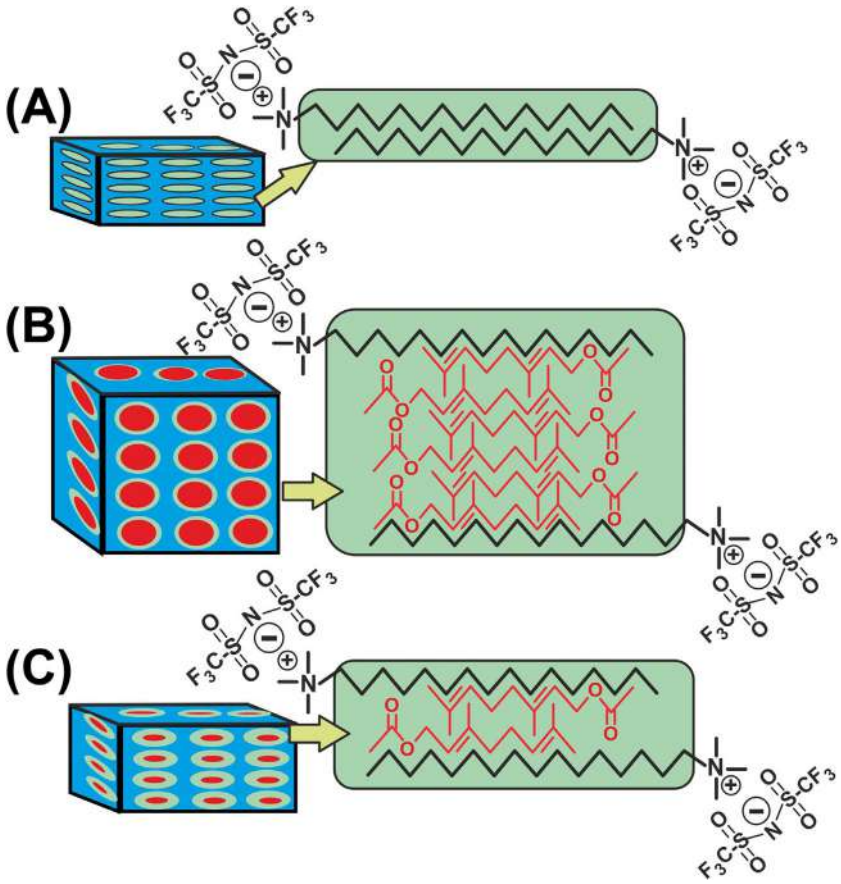

FIGURE 6.8

Schematic representation of the sponge-like IL hypothesis, showing the $[C_{16}tma][NTf_2]$ net as a dry sponge (A), a sponge swollen with geranyl acetate (B), and a "wet" sponge after wringing out by the iterative cooling/centrifugation protocol (C) [50,53].

nano-structure, placed in specific regions that become expanded, which looks like a "sponge that gets soaked" (see Fig. 6.8B). Although the interaction between the alkyl chains should be the main driven force, columbic and H-bond interactions also play a role to define the overall structural self-assembly of the nonpolar solute/IL binary system either as solid or as liquid phase. By using an external mechanical force, such as centrifugation, on this frozen binary system, the solid ionic net would expel the substrate out from these nonpolar regions. This action is enough to induce the segregation of the hydrophobic solute from the solid mixture, allowing "to wring the soaked sponge" with a partial recovery of the compound initially included in the IL net, and leading to a "wet sponge"-like IL, because this behavior resembles to a sponge wringing (see Fig. 6.8C). According to this hypothesis, the SLIL net can be considered as a nano-sponge with holes of variable volume,

which are suitable for housing or releasing hydrophobic molecules as a function of their liquid or solid phase, and the degree of holes compaction [50].

The use of centrifugal filters for the extraction step of hydrophobic compounds from the frozen IL improves the efficiency of the "sponge wringing" phenomenon, because of the resulting higher product yield with respect to the centrifugation process without a coupled filtration [57]. By using a 1:1 (v/v) benzyl propionate/[C_{16}tma][NTf_2] SLIL mixture as representative example (see Fig. 6.9A), when the separation process was carried out following the cooling/centrifugation protocol without the use of membrane, the product recovered was lower than 50%, in agreement with results obtained for the extraction of terpene esters [53]. However, when using membrane filters during the centrifugation step, the recovery of benzyl propionate was clearly improved, being the extraction yield increased with the pore size of the filtration membrane, and obtaining the best results for microfiltration membranes (0.2 μm pore size). It should be noted that, after the separation process, the feature of "wet sponge" was also observed when using centrifugal filters, because of the partial retention of the hydrophobic solute into the IL net.

In another example based on the separation process of methyl oleate from a methyl oleate/[C_{16}tma][NTf_2] mixture, the ability of this sponge-like IL "to soak up" hydrophobic compounds, and then to be "wrung out," was also studied by applying iterative soak/wring cycles (see Fig. 6.9B). By using an initial 55/45 (w/w) methyl oleate/[C_{16}tma][NTf_2] mixture, the extraction

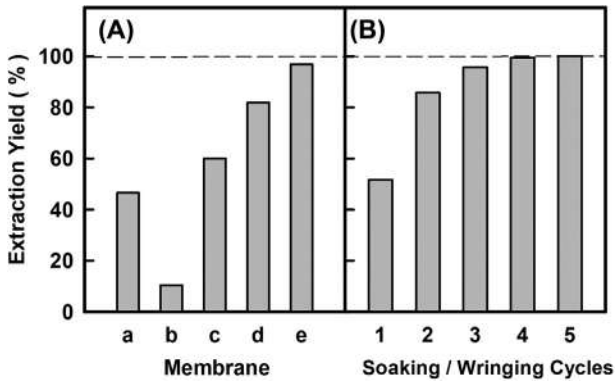

FIGURE 6.9

(A) Extraction yield in the separation process of benzyl propionate from a 1:1 (v/v) benzyl propionate/[C_{16}tma][NTf_2] SLIL mixture by using centrifugation filters with different membrane pore sizes, that is (a) without membrane, (b) 3 kDa cut-off, (c) 10 kDa cut-off, (d) 30 kDa cut-off, (e) 0.2 μm, and following a cooling (−20°C) and centrifugation (15,000 rpm, 15 min, 0°C) protocol (Data obtained from E. Alvarez, J. Rodriguez, R. Villa, C. Gomez, S. Nieto, A. Donaire, et al., Clean enzymatic production of flavor esters in sponge-like ionic liquids, ACS Sustain. Chem. 7 (2019) 13307−13314). (B) Extraction yield in the separation process of methyl oleate from 55/45 (w/w) methyl oleate/[C_{16}tma][NTf_2] SLIL mixture during five consecutive soak/wring cycles using the cooling/centrifugation protocol. After each cycle, the resulting solid SLIL phase was used to dissolve a new methyl oleate sample before to proceed with extraction.

Data obtained from P. Lozano, J.M. Bernal, G. Sanchez-Gomez, G. Lopez-Lopez, M. Vaultier, How to produce biodiesel easily using a green biocatalytic approach in sponge-like ionic liquids, Energy Environ. Sci. 6 (2013) 1328−1338.

yield was determined after a cooling/centrifugation protocol, and the resulting solid SLIL phase was iteratively mixed with a new methyl oleate sample, measuring the extraction yield at the end of each iterative separation cycle. As can be seen in Fig. 6.9B, the amount of the hydrophobic compound released from the solid SLIL phase increased along the initial operation cycles until reach the full extraction, and then remained constant for the following cycles. Once again, the sponge-like behavior of this ILs was observed, whereby three initial cycles of "soaking/wringing," provide a "wet sponge"-like IL able to fully release all the newly added hydrophobic solutes by following the cooling/centrifugation protocol [52].

The unique and genuine nature of this type of ILs, based on long aliphatic chains which are solid at room temperature, allows the straightforward separation of any previously dissolved hydrophobic compound, and through a fast and direct procedure based on cooling, not heating. These new solvents are undoubtedly a clear alternative to VOSs, which involves the application of long and costly distillation processes for the separation of compounds. Moreover, the ionic nature of these SLILs, which determines their negligible vapor pressure when heated, together with their total insolubility in water, and their solid character at room temperature, are unique qualities for a solvent, since their full recovery after any procedure is guaranteed, and their possible dissemination to the environment can be minimized. The reasonable criticism regarding the nongreen character of ILs in term of environmental contamination produced by an accident are clearly mitigated. This set of unique qualities of SLILs permits to consider this green solvent as an interesting bet for a sustainable development of chemistry in a near future [50]. The development of advanced processes able to directly provide pure products by integrating chemical transformations, product separation and recovery and reuse of solvent and catalytic phases by straightforward and smart approaches, is a key feature to build green chemical processes. The SLILs are key enabling technology, leading to amazing synergies when combined with (bio)catalysts, where not only improves the catalytic efficiency (i.e., improved activity and enantioselectivity, enhanced stability, etc.), but also permits the design of smart approaches for product separation. This excellent suitability of the SLIL technology will be illustrated by three different examples of sustainable biocatalytic processes: (i) the synthesis of flavor esters [18,53,57], (ii) the synthesis of first [52], second [58], and third-generation biodiesel [59], and (iii) the synthesis of monoglycerides [60,61].

6.5 Biocatalytic production of flavor esters by using the sponge-like ionic liquid technology

Flavor esters based on short alkyl-chain carboxylic acid (e.g., isoamyl acetate, citronellyl acetate, geranyl propionate, neryl acetate, etc.) are essential compounds for the formulation of most of food, cosmetic and pharmaceutical products, because they provide fruity and floral fragrance qualities highly appreciated by consumers [62]. The continuous increase in global market for flavors and fragrances, being valued at $26.0 billion in 2015, and expected to improve up to $37.0 billion in 2021) [63], is also accompanied by an increased demand for natural products.

US and European legislations determine that "natural" flavor substances can only be prepared either by physical processes (e.g., extraction) from natural sources, or by enzymatic or microbial transformation of precursors isolated from nature.

However, most fragrance esters are industrially produced by classical chemical synthesis, by using nonnatural substrates (e.g., acyl halide derivatives, anhydrides, etc.), and VOSs as reaction media, which set these technological approaches outside of any sustainable criteria. The development of new strategies based on biocatalytic transformations of substrates from natural origin, as well as the implementation of new sustainable extraction and purification processes for the recovery of added-value products at industrial scale, constitute an interesting alternative for obtaining fragrances without increasing the pressure on biodiversity.

Lipases have been reported as the most useful biocatalysts for the synthesis of valuable flavor and fragrance esters by esterification or transesterification approaches in reaction media with a low water content [18,62]. Among all the lipase-catalyzed reactions in nonaqueous environments (see Fig. 6.1), the direct esterification between "natural" substrates, such as carboxylic acids and alcohols from renewable sources, in solvent-free systems could be considered as the best way to obtain "natural" ester products. However, enzyme are fully deactivated in solvent-free system, as results of the high carboxylic acid/alcohol concentration (e.g., a 5.93 M mixture of acetic acid and isoamyl alcohol for the case of isoamyl acetate synthesis), which is a clear limitation for any industrial application.

By following the protocol depicted in Fig. 6.10, the suitability of different SLILs, based on the alkyltrimethylammonium (e.g., $[C_{16}tma][NTf_2]$), or 1-alkyl-3-methylimidazolium (e.g., $[C_{16}mim][NTf_2]$) cations, as a reaction medium for the enzymatic synthesis of flavor esters has widely been reported [53,54,57]. Thus, many different flavor alcohols (e.g., isoamyl alcohol, cinnamyl alcohol, nerol, geraniol, citronellol, etc) and free carboxylic acids have been successfully used as substrates

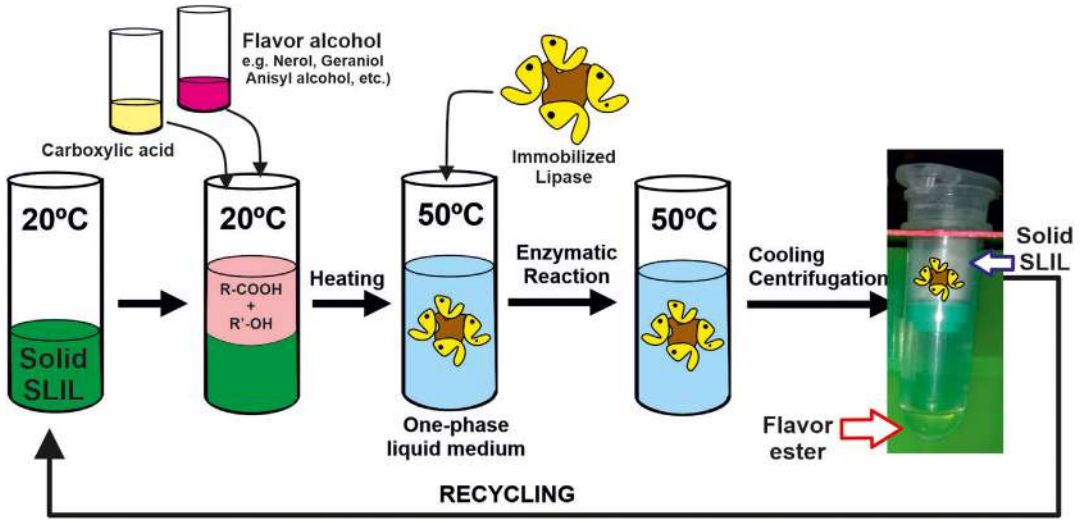

FIGURE 6.10

Green approach for the biocatalytic synthesis of flavor esters in sponge-like ionic liquid (SLILs), and product separation by cooling and centrifugation, including full recovery and reuse of the enzyme—SLIL system [54,57].

for lipase-catalyzed direct esterification of the corresponding ester synthesis. It should be noticed that the immobilized *C. antarctica* lipase B (Novozym 435) was shown as the best biocatalyst for these synthetic processes, providing product ester yield near to 100% in most cases after 2−4 h reaction at 50°C. Moreover, all the assayed SLILs resulted as excellent reaction media for these biocatalytic processes, providing both the maintenance of the catalytic activity of the enzyme toward reuse, as well as the straightforward separation of products. At this point, several empirical rules of these biocatalytic processes for flavor ester synthesis in SLIL can be extracted:

1. The SLIL concentration in the reaction medium is an important controlling parameter for the success of the process. At SLILs concentrations lower than 40% (w/w), the enzyme activity is negatively affected due to the deactivation power of substrates as polar organic solvents. As representative example, for the case of Novozyme-435-catalyzed cinnamyl propionate synthesis by the direct esterification of propionic acid with cinnamyl alcohol for 1 h of reaction at 50°C in $[C_{16}tma][NTf_2]$, an increase from 30% to 90% in the product ester yield was observed by raising the SLIL content from 10% to 40%. The use of SLIL concentrations higher than 70% (w/w) were shown as excellent protective media for the enzyme activity, that provides c. a. 100% product conversion. However, the extraction product yield after the cooling/centrifugation protocol at this high SLIL content was very low (< 20%), because of the strong interaction of the hydrophobic product with the ionic net, as previously indicated in the "wet sponge" phenomenon [57].
2. The length in the alkyl chain in the SLIL cation has a direct effect on both the enzyme activity and the efficiency of the separation process of the flavor product. With respect the enzyme activity, it was found that the enzyme activity was slightly improved by the increase in the alkyl chain of SLIL cation. As example, for the case of Novozyme-435-catalyzed isoamyl acetate synthesis by esterification of acetic acid with isoamyl alcohol (4 h reaction, 50°C) in different alkyltrimethylammonium SLIL (i.e., $[C_{12}\text{-}C_{16}tma][NTf_2]$) at 50% (w/w), it was observed that the ester product yield increased from 92% to 99% when the alkyl chain increased from C_{12} to C_{18} [53]. Nevertheless, as the increase in the size of this chain in SLIL cation results in a higher melting point of the SLIL (see Fig. 6.6), the application of the cooling/centrifugation protocol for product separation provides a lower residual IL content in the final extracted flavor. By using the biocatalytic synthesis of cinnamyl propionate in different 50% (w/w) alkyltrimethylammonium SLIL (i.e., $[C_{12}\text{-}C_{16}tma][NTf_2]$ as example, the separation of the liquid fraction containing cinnamyl propionate was carried out by following three consecutive cooling/centrifugation cycles through filter membranes. It was observed that the residual IL content in the resulting flavor ester fraction was decreased from 11.7% to 0% when the alkyl chain increased from C_{12} to C_{18} [57].
3. The increase in the hydrophobicity in the SLIL anions ($[NTf_2] > [PF_6] > [BF_4]$ improves the enzymatic efficiency for the biocatalytic synthesis of hydrophobic products, as it was observed for cinnamyl propionate [57] and methyl oleate cases [51].

Additionally, to these empirical rules regarding the use of SLILs in biocatalytic synthesis of flavor ester by direct esterification, other factors that could be taken into account for designing efficient reaction media. Thus, the presence of dehydrating conditions (e.g., by adding molecular sieves, etc.), greatly improves the catalytic reaction yield, because of the expected shift of the reaction equilibrium toward the synthetic way (see Fig. 6.1). As an example, for the Novozyme-435-catalyzed the

synthesis of isoamyl acetate by the esterification of acetic acid with isoamyl alcohol (4 h reaction, 50°C) in 50% (w/w) [C_{16}tma][NTf_2]), it was observed that the flavor yield increased from 82% to 99% when molecular sieves were present into the reaction medium [53]. In another example, the synthesis of anisyl acetate, a fragrance ingredient used in many cosmetic and fine fragrance products, was assayed by the direct esterification of acetic acid with anisyl alcohol (at a 1/1 mol/mol acid/alcohol concentration ratio) catalyzed by lipase in 70% (w/w) [C_{16}tma][NTf2]. For this case, the suitability of the SILL reaction system was enhanced under the assistance of 4 W microwave irradiation (which provided a 50°C constant temperature), resulting in up to 82% anisyl acetate yield in 1 h reaction, while the product extraction was improved using centrifugal filters (up to 95% extraction yield) [54].

Furthermore to all these features regarding the excellent suitability of SLILs to carry out the biocatalytic production of nearly pure flavor esters by direct esterification, the operational stability of the enzyme and the recycling of the SLIL are other key criteria to be considered for scalingup. For all reported examples, the remaining solid IL/immobilized lipase system was successfully applied for cyclic protocols of flavor synthesis/product extraction. Noteworthy, the enzyme activity was maintained unchanged upon recycling. The protective effect of hydrophobic ILs, based on [NTf_2] anion and long alkyl chain in ammonium or imidazolium cations, has been related to the ability of these ILs to maintain the native structure of proteins even under harsh conditions [50]. The unique structural features of hydrophobic SLILs not only provide and excellent stabilization medium for enzymatic reaction, but also a simple method for the isolation of the pure products, and the recycling and reuse of the catalytic systems (enzyme/SLILs) by cooling/centrifugation and/or filtration. The ability of these SLILs to melt at temperatures compatible with enzyme catalysis permits the development of a simple and easy green method for flavor ester synthesis that integrated, at the same time, a clean separation protocol.

6.6 Green biocatalytic production of biodiesel by using the sponge-like ionic liquid technology

Unlike fossil fuels, biofuels have the potential to prevent global warming as results of the replace burning of oil-based fuels, such as gasoline and diesel. Due to its functional similarity with petroleum-based fuel and its origin from renewable sources, biodiesel has become a very attractive alternative for use in diesel engines. Biodiesel is obtained by catalytic transesterification of triacylglycerides with methanol (methanolysis), yielding the corresponding fatty acid methyl esters (FAMEs), also named biodiesel, and glycerol as byproduct. The transformation requires three consecutive transesterification reactions for achieving the full conversion of triacylglyceride molecules to biodiesel using chemical or enzymatic catalysts, where an excess of alcohol is commonly used to shift the equilibrium reaction toward the product side. From a technical point of view, biodiesel is successfully produced on an industrial scale using homogeneous chemical catalysts (e.g., KOH, NaOH, H_2SO_4, etc.) at high temperatures (120°C−180°C). However, the undesired side reactions (e.g., soap formation), the glycerol recovery and the removal of inorganic salts from the final product by scrubbing with water, remain as important problems that involve the consumption of large amounts of energy and water that should be cleaned. Although biodiesel is considered as a green

fuel, and it is successfully produced on an industrial scale, the industrial process is not green, sustainable, or clean. Additionally, to the questionable greenness of the industrial production of biodiesel, the sustainability of this biofuel is also weak if agricultural feedstock (e.g., edible oils) are used as raw materials for biodiesel production (first-generation biodiesel), affecting the food market through the increase in food prices. The use of nonedible renewable sources, like waste-cooking oil (second-generation biodiesel), or like microalgae (third generation biodiesel), together with the development of efficient, clean and green procedures is a key challenge for the integral sustainability of the biofuels industry [52,64].

Because of their high catalytic activity and selectivity, the use of enzymes as catalysts for biodiesel synthesis by methanolysis of triglycerides could be regarded as the perfect solution to the problems associated with conventional chemical-catalyzed processes. However, the nonmiscibility of the triglycerides and the methanol substrates is a key limiting feature. The resulting vegetable oil/methanol biphasic systems not only provide low reaction rates, but also cause fast and full enzyme deactivation as a result of the direct interaction of the biocatalyst with the methanol phase. Furthermore, the mutual immiscibility between both final products, the glycerol and the biodiesel, also reduces the biocatalytic efficiency during the process, due to poisoning of immobilized enzyme particles as results of the adsorption of the continuously formed glycerol byproduct onto the support [65].

Against this set of problems, SLILs emerges as the perfect companion of biocatalysts to carry out a true sustainable process for the production of biodiesel. Thus, the immobilized *C. antarctica* lipase B was able to reach the highest level of activity (i.e., up to 100% yield after 4 h reaction) and selectivity for the transformation of triacylglycerols to FAMEs by transesterification in SLILs under mild conditions (60°C). Once again, the SLILs were shown as a key enabling technology in biodiesel production not only because they improve the catalytic efficiency, but also for the synergy resulted from the unique properties of SLILs that lead to the design of a straightforward protocol for the direct separation of pure biodiesel in one step (see Fig. 6.11) [52].

The combination of immobilized lipases with SLILs leads to amazing results for the sustainable production of biodiesel, that can be summarized in following empirical rules:

1. Not all the ILs are able to dissolve a vegetable oil and methanol mixture, nor biodiesel. By using the ILs series based on 1-alkyl-3methylimidazolium cations (i.e., from C_{12} to C_{18}) and [NTf_2],[PF_6] or [BF_4] anion, the phase behavior of different IL/triolein or IL/methyl oleate mixtures, as a function of the nature and concentration the IL, has been reported [51]. According to the statement *like-dissolves-like*, only the SLILs based on cation with large alkyl chains (e.g., [C_{18}mim], [C_{18}tma], [C_{16}mim], etc.), and the [NTf_2], as the most hydrophobic anion tested, were able to provide a fully clear monophasic reaction system when dissolving a 1:3.5 (mol/mol) triolein/methanol mixture. All the remaining combination of ions led to biphasic (liquid-liquid) or multiphasic (solid-liquid-liquid) media, with poor results in biocatalytic synthesis of biodiesel.
2. The SLILs based on the alkyltrimethylammonium cation and the [NTf2] anion shows the best biocatalytic efficiency for biodiesel synthesis. As an example, for the Novozyme-435-catalyzed the methanolysis of triolein, the biodiesel yield reach c. a. 100% yield after 8 h reaction at 60°C for most reaction media (see Fig. 6.12A, [52,54]), while the SLILs based on 1-alkyl-3-methylimidazolium cation only reached up to 80% biodiesel yield for the same reaction time [51].

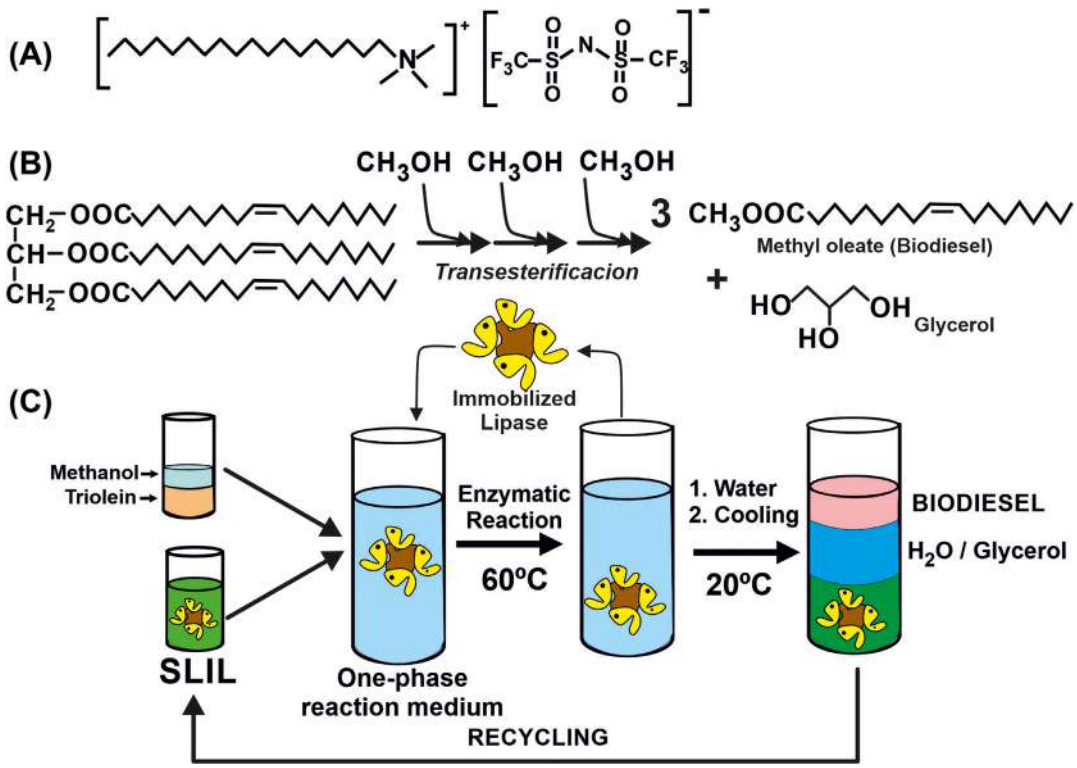

FIGURE 6.11

(A) Structure of the IL $[C_{18}tma][NTf_2]$, as an example of sponge-like ionic liquids (SLILs). (B) Scheme of the immobilized lipase-catalyzed synthesis of both methyl oleate by a transesterification approach. (C) Green approach for the biocatalytic synthesis of methyl oleate in sponge-like ionic liquids (SLILs), and product separation by addition of water followed by cooling and centrifugation.

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3. Although the alkyl chain length in the alkyltrimethylammonium cation seems to have low effect on enzyme activity, the SLIL concentration in the reaction media is a key parameter. As can be seen in Fig. 6.12A, it was necessary to use reaction media containing a [alkyltrimethylammonium][NTf_2] concentration higher than aprox. 45% w/w for achieving the full transesterification of triolein to methyl oleate.
4. The catalytic promiscuity of Novozym 435 permits the synthesis of biodiesel/biofuels in SLILs by using many kinds of vegetable oils (e.g., triolein, waste-cooking oils, cottonseed oil, etc) and free fatty acids as acyl donors. Also, other alcohols (e.g., methanol, ethanol, solketal, etc), are suitable to act as nucleophiles in the transesterification and/or esterification reactions, resulting in a heterogeneous mixture of fatty acids methyl esters and fatty acid solketyl esters with high power energy for engines. As example, Novozym 435 was able catalyze the full transformation

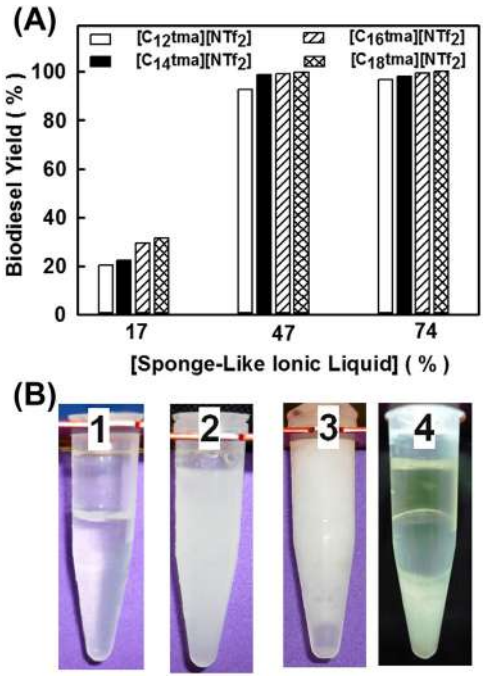

FIGURE 6.12

(A) Effect of the alkyl chain length of the [1-alkyltrimethylammonium] cation of the SLIL on the methyl oleate yield obtained by Novozym 435-catalyzed methanolysis of triolein (1/6 triolein/methanol mol ratio) for 8 h reaction at 60°C and at three different SLIL concentrations. (B) Phase behavior of the reaction mixture containing both methyl oleate and glycerol products at 60°C (**1**), at 60°C after addition of water (**2**), after cooling to 25°C (**3**), and after three consecutive centrifugation steps at 15,000 rpm (1 h) at room temperature, 23°C and 15°C, respectively (**4**).

Reproduced from ref. P. Lozano, J.M. Bernal, C. Gomez, E. Garcia-Verdugo, M.I. Burguete, G. Sanchez, et al. Green bioprocesses in sponge-like ionic liquids, Catal. Today 255 (2015), 54–59 with permission from Elsevier.

of waste cooking oil mixed with free fatty acids to biofuels in [C_{18}tma][NTf_2] after 8 h reaction at 60°C [58].

5. The [alkyltrimethylammonium][NTf_2] SLIL provide an over-stabilization on the immobilized lipase. It was observed that the residual activity shown by Novozym 435 after incubation in 50/50 (w/w) triolein/[C_{18}tma][NTf_2], [C_{16}tma][NTf_2] or [C_{14}tma][NTf_2]) mixtures was higher than 90% after 100 days of incubation at 60°C (i.e., up to 1370 days half life time in [C_{18}tma][NTf_2] [52].

Additionally to this plethora of excellences shown by SLILs for the biocatalytic synthesis of biodiesel, and due to their unique characteristics as solvents, a customized protocol for the pure biodiesel extraction was developed [51,54]. It is necessary to take into account that the biocatalytic transesterification of triglycerides with methanol produces both the biodiesel and the glycerol, although this hydrophilic byproduct should be eliminated. In this context, the glycerol can be

extracted from the reaction medium by washing with water, which is added after the biocatalytic transformation, and before cooling to room temperature (see Fig. 6.12B). The semisolid heterogeneous mixture obtained is then separated by following an iterative cooling/centrifugation protocol (i.e., 15,000 rpm 1 h at room temperature, 23°C and 15°C, respectively), which now results in three phases: an upper IL-free biodiesel phase, a middle IL-free liquid aqueous phase containing the glycerol, and a bottom solid containing the SLIL. The presence of water, a green molecular solvent (nonmiscible with the biodiesel and the SLIL), improves the separation of all phases, providing an easy and sustainable way to separate both glycerol and nonreacted methanol from the biodiesel. Once again, the suitability of the proposed methodology for biodiesel synthesis was demonstrated by the full recovery and reuse of the SLIL/biocatalyst system [50].

In an attempt to validate this proposed straightforward and sustainable technology for the synthesis of third generation biofuels, the transformation of nonedible oils from living microalgae has been successfully carried out. This approach was based on three consecutive steps, consisting on the extraction of oils, their biotransformation to biodiesel, and the final extraction of biodiesel with full recovery of the SLIL for further reuse. For this endeavor, binary mixtures of ILs have been used for developing one-pot systems suitable for the direct extraction and biocatalytic transformation of algal oil to biodiesel without prior isolation. These mixtures were based on the combination of SLILs (e.g., $[C_{16}mim][NTf_2]$) with the IL 1-butyl-3-methylimidazolium chloride ([Bmim][Cl]), taking into account the excellent suitability to carry out the biocatalytic synthesis of biodiesel of the first one, and the ability for dissolving cellulosic biomass of the second [22]. The extraction of oils was carried out by incubating the dry microalgae (*Chlorella vulgaris* or *Chlorella protothecoides*) in the appropriated IL binary mixture at 110°C, which after being cooled at 60°C allowed the transformation to biodiesel by an immobilized lipase. This resulted in a fast and efficient biodiesel synthesis, for example, up to 100% yield in 2 h at 60°C. The subsequent cooling until room temperature and the iterative centrifugation of the resulting semi-solid systems at 20°C and 18°C led to the separation of the liquid algae biodiesel from the solid IL mixture, which was recovered and reused for further operation cycles [59].

6.7 Green biocatalytic production of monoacylglycerides by using the sponge-like ionic liquid technology

Monoacylglycerides (MAGs) are nonionic emulsifiers, widely used in food, pharmaceutical, and cosmetic industries. Besides their bulk applications in food and dairy industries, pure MAGs are also of great interest in medicinal chemistry, due to their biological activity (e.g., antimicrobial, to prevent prostatic hyperplasias, etc.), as well as in the pharmaceutical industry as drug carriers [66]. Moreover, MAGs based on omega-3 fatty acids, like α-linolenic acid, eicosapentaenoic acid and docosahexaenoic acid are much appreciated as nutraceutical, because of the recognized beneficial health effects of omega-3 fatty acids (e.g., as cardioprotective agents). However, the bioavailability of omega-3 fatty acids after their consumption as dietary triacylglycerides is limited for several diseases that involves mal-digestion and malabsorption syndrome (e.g., chronic pancreatitis, Crohn's disease, postsurgery effects, etc.). In this way, the consumption of MAGs based on omega-3 fatty acids are encouraged because their better absorption with respect those delivered as

triacylglycerides. Thus, current strategies to combat lipid malabsorption include dietary supplements containing omega-3 monoacylglycerides [67].

According to all the excellences showed by the combination of SLILs and immobilized lipase for carrying out flavor ester synthesis by integrated reaction/separation green processes, the biocatalytic synthesis of MAGs by direct esterification of fatty acids (i.e., capric, lauric, myristic, palmitic, or oleic acids, respectively) with glycerol was also demonstrated (see Fig. 6.13A). For this green biocatalytic process, different ILs, based on cations with a long alkyl side-chain, and hydrophobic or hydrophilic anions, (e.g., $[C_{16}mim][NTf_2]$, $[C_{12}mim][BF_4]$, etc.) were tested as reaction media. Although all ILs have been shown as suitable reaction media for Novozym 435-catalyzed esterification of glycerol with free fatty acids, a high selectivity of MAGs was only observed in the $[C_{12}mim][BF_4]$ case (e.g., up to 100% selectivity and 100% yield for monolaurin without any loss in activity after reuse for 8 operation cycles). Furthermore, as these ILs are temperature switchable ionic liquid/solid phases that behave as sponge-like systems, a straightforward protocol for IL-free MAGs recovery, based on iterative cooling/centrifugations cycles, was also successfully applied [60].

By using natural sources of omega-3 fatty acids (e.g., fish oil, linseed oil, etc.) as substrates, the synthesis of omega-3 MAGs was carried out by a clean chemo-enzymatic approach based on two

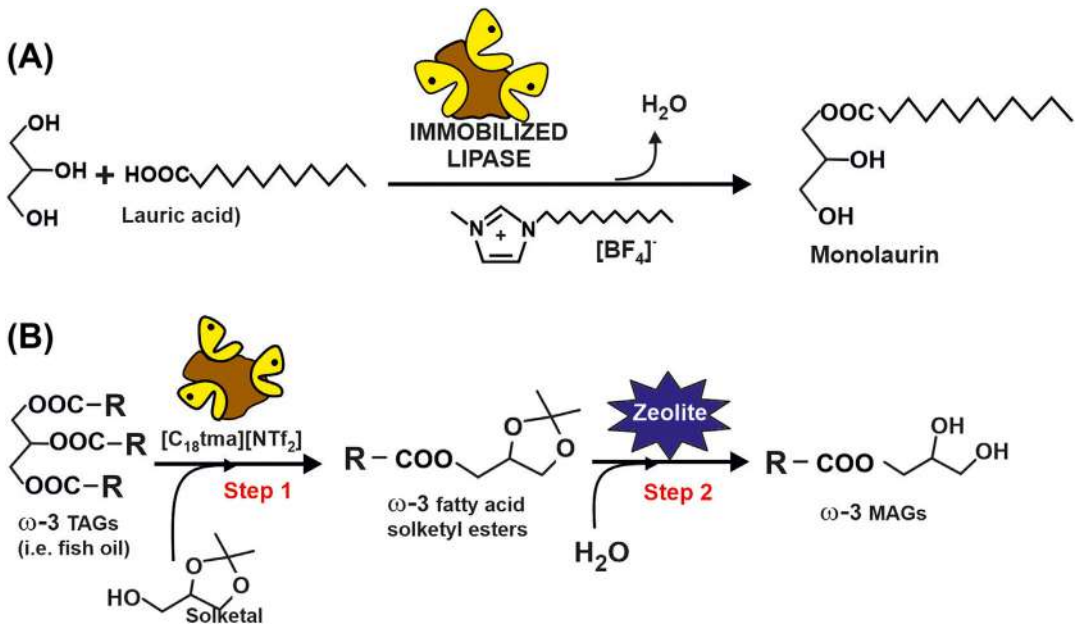

FIGURE 6.13

(A) Scheme of the immobilized lipase-catalyzed synthesis of monolaurin by direct esterification of lauric acid with glycerol in the $[C_{12}mim][BF_4]$ SLIL [60]. (B) B. Schema of the two-step chemo-enzymatic synthesis of omega-3 MAGs. Step 1: Lipase-catalyzed transesterification of omega-3 TAGs with solketal under anhydrous conditions in $[C_{18}tma][NTf_2]$; Step 2: Zeolite-catalyzed selective hydrolysis of the solketal moiety under aqueous condition [61].

consecutive catalytic steps. The first catalytic step consisted in the enzymatic transesterification of raw fish or linseed oil with solketal for producing the corresponding omega-3 fatty acid solketyl esters in 40% w/w $[C_{18}tma][NTf_2]$ under vacuum conditions to avoid any oxidation and at 60°C, leading to a 100% product yield after 6 h reaction for both omega-3 oils. The excellent suitability of the proposed approach was also demonstrated by the recovery of the IL-free omega-3 fatty acid solketyl ester fraction from the SLIL by using a similar approach to that assayed for biodiesel, such as, a washing by water step of the reaction media, and three consecutive cycles of cooling/centrifugation. By this clean and sustainable approach, a nearly pure omega-3 fatty acid solketyl ester was obtained, because the glycerol byproduct and the excess of solketal remained in the aqueous middle phase (see Fig. 6.12B), while the solid SLIL was fully recovered at the bottom of the centrifugation tube. This omega-3 fatty acid solketyl ester was then used as substrate for the synthesis of the omega-3 MAGs as results of the hydrolysis of solketyl ring catalyzed by a zeolite (see Fig. 6.13B), which reached up to 100% omega-3 MAG yield after 3 h reaction at 50°C [61].

6.8 Conclusions

The sustainability goals demanded for the 21st century include the development of a new green chemical industry. To this aim, renewable resources should be efficiently and selectively transformed in the products that shape our quality of life. Nature is the most efficient chemical laboratory, providing substrates and catalysts on which we must build our consumer products with the lowest environmental impact, thus preserving opportunities for future generations.

The new green chemical industry depends on the implementation of selective and efficient chemical transformations, and their integration with clean and sustainable separation processes. Biocatalyst are the most powerful toolbox provided by Nature for selective chemical transformations, which step by step, and reaction by reaction, they are proving their effective usefulness in chemical transformations in nonaqueous media, outside their natural, life-forming environment.

The excellent suitability of hydrophobic ILs to provide appropriate microenvironments for the improvement of activity and stability of enzymes, has been further enhanced by the phase behavior of ILs based on cations with long alkyl chains, so called SLILs. Due to this unique feature, SLILs affords the development of straightforward and sustainable technical protocols for the efficient extraction of hydrophobic organic compound (e.g., flavors esters, biodiesel, monoacylglycerides, etc.) dissolved inside by using a simple cooling and centrifugation approach. These approaches not only lead to nearly pure product, but also the full recovery of the SLIL, as a water-immiscible solid, occurr the same time. All these features have allowed the development of two-step protocols for producing high added value compounds (e.g., flavor esters, biodiesel, or omega-3 monoacylglycerides), with the benefit of: (i) enzyme-catalyzed reactions with a product yield close to 100%, (ii) clean separation of the reaction products by a cooling/centrifugation method, and (iii) full recovery of the SLIL/biocatalyst system without any loss in activity, ready to be reused.

The genuine properties of SLILs, as temperature switchable liquid/solid phase systems, opens up a broad window of opportunities for developing green chemical processes of industrial interest. The implementation of all these features in multienzymatic and/or multichemoenzymatic transformations, mimicking the metabolic pathways found in cells, including straightforward and clean approaches for pure products recovery, are clear steps toward the green chemical industry of the near future.

Acknowledgements

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CHAPTER

Biocatalysis in biphasic systems based on ionic liquids

7

Inês O. Baptista, Ana M. Ferreira, Flávia F. Magalhães, Mara G. Freire, Ana P.M. Tavares and João A.P. Coutinho

CICECO – Aveiro Institute of Materials, Department of Chemistry, University of Aveiro, Aveiro, Portugal

7.1 Introduction

The industrial demand to meet the directives of the green chemistry agenda is promoting intense activity in the field of biocatalysis [1–5]. Biocatalysis allow high selectivity, biodegradability, and mild reaction conditions. Therefore, a number of biocatalytic applications and remarkable developments has been observed in several industrial sectors, from chemical and pharmaceutical industries to food products [6–14]. In parallel, enzymology has become one of the most effective and important areas focused on the research and development sectors of biocatalysis. However, the high structural complexity is the main disadvantage associated with enzymes at industrial levels [15]. The creation of highly active, robust and stable biocatalysts, while avoiding biocatalyst deactivation are the primary difficulties faced in industrial settings. Modern biotechnology, using protein engineering, and directed evolution are creating a revolution, resulting in significant advances in enzymes used in the aforementioned industries [6].

Enzymes have been recognized as highly effective catalysts as they are proven to be very useful in preventing various nasty chemicals forming in conventional processes [4]. Thus, enzymes are also fully compatible with modern standards for safety, health, and the environment, being considered as green catalysts [8]. Biotechnological advances enabled the enzymes to be "made to measure" with novel activity and adaptations to different process conditions, allowing for a wide range of uses.

In this chapter, we present an overview of biphasic systems (BS) as alternative platforms for biocatalysis. Moreover, this chapter intends to highlight the multidisciplinary approach required for the success of this subject, bringing together the scientific and industrial communities to accomplish its application at industrial level.

7.2 Biocatalysis in biphasic systems

Over the past five decades, enzymes as biocatalysts have become one of the pillars of biotechnology with numerous applications due to their mild reaction conditions, high selectivity, and biodegradability [6,16]. Even though they have excellent catalytic properties, enzymes have some

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characteristics that are not suitable for such applications, insofar as: they are unstable and high cost [17]. Although procedures based on the use of enzymes are replacing conventional chemical methods, the commercialization of some enzymes for industrial applications is still at a low pace due to their high costs, low efficiency, and operational catalytic and storage problems [18]. Therefore, rigorous efforts have been made by the scientific community to improve enzymes and to replace unfavorable reactions with green and clean technologies, increasing their performance while developing a more sustainable industrial process [19].

The use of BS constituted by two immiscible solvents, such as water and a water-immiscible organic solvent, offers several advantages in biocatalysis. Their main use has been the separation of the enzyme from the products of the reactions, but it may also be the separation of the enzyme from the reactants if these are deleterious toward the enzyme. Usually, the organic phase is used to recover the product formed, while the aqueous phase contains the enzyme [20,21], as illustrated in Fig. 7.1.

Owing to the peculiar characteristics of BS, they are an attractive alternative offering several advantages, such as high yields, high selectivity, and simultaneous possibility of purification. In fact, the application of BS as a medium for biocatalysis allows for the integration of the enzymatic reaction, separation/purification of products and the reuse of the enzyme, while keeping the technological simplicity and a low cost [22]. The organic solvents commonly used are ethyl acetate and hexane, mainly due to their immiscibility in aqueous media [23]. Table 7.1 summarizes examples of the use of BS as media for biocatalysis.

Lugaro et al. [24] were one of the first authors to use this approach. They showed that laccase from *Polyporus versicolor* catalyzed the 17β-estradiol oxidation in a heterogeneous medium using a BS composed of water and ethyl acetate (EtOAc). The steroidal substrates and metabolites were solubilized in the phase composed of EtOAc while the enzyme remained in the aqueous phase. A similar work was developed by Nicotra et al. [25], where the 17β-estradiol was oxidized into two

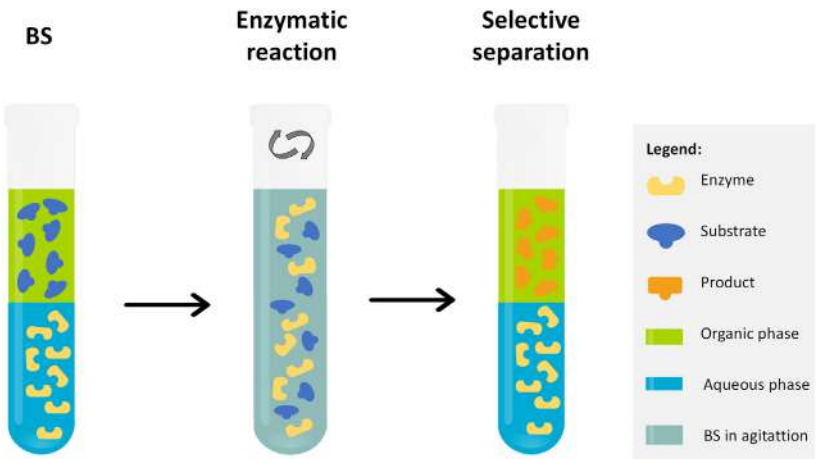

FIGURE 7.1

Schematic of a biocatalytic reaction in a heterogeneous medium using BS.

Table 7.1 Examples of biocatalytic reactions in BS.

Enzyme and source	Substrate	IL BS	Application/product	References
Laccase from *Polyporus versicolor*	17β-estradiol	Phosphate buffer	Oxidation of steroids	[24]
Laccase from *Trametes pubescens*	17β-estradiol	Ethyl acetate and acetate buffer	Oxidation of steroids	[25]
Porcine pancreas lipase type II	Ibuprofen and sorbitol	Hexane and water	Synthesis of bioactive derivatives of ibuprofen	[26]
Peroxidase from *Bjerkandera adusta*	Anthracene	Silicone oil and water	Degradation of anthracene	[27]
Laccase from *T. versicolor*	Anthracene	Silicone oil and water	Degradation of anthracene	[28]
Lipase B from *Candida antarctica*	2-phenethyl acetate	Dioxane and water	Transformatation of 2- phenethyl acetate to 2-phenylethanol and acetate	[29]
Lipase B from *C. antarctica*	1-phenethyl acetate	Organic solvents (acetone, acetonitrile, (1,4)-dioxane and tetrahydrofuran) and water	Transformation of rac-1-phenylethyl acetate to (R)-1- phenylethanol and (S)-1-phenylethyl	[30]

dimers (1c and 1d) by laccase from *Trametes pubenscens*, also using a BS composed of EtOAc. The authors characterized the chemical structure of the products obtained by RP-TLC, NMR and RP-HPLC analysis, showing that the structures of the Lugaro's reaction products were poorly characterized and ambiguously determined [25].

Ibuprofen is a poorly water-soluble drug. To improve its bioavailability, a modification in the chemical structure of ibuprofen was proposed by Zappaterra et al. [26]. In their work, porcine pancreatic lipase B was used for the direct esterification of ibuprofen and sorbitol to produce the ibuprofen sorbitol ester, a water-soluble drug. The enzymatic reaction occurred in a hexane-water BS, where the organic solvent acted as a solubilizer for ibuprofen and the aqueous phase acted as the solvent for sorbitol [26]. The impact of agitation speed on ibuprofen esterification with sorbitol was investigated at various speeds (from 100 to 600 rpm). The results demonstrated that 400 rpm was the best speed since it lead to the highest conversion yield value of 60% [26].

Within the BS perspective is the two-phase partitioning bioreactor (TPPB). This approach consists of applying the two-phase system in a bioreactor, comprising an organic solvent that allows high concentrations of the target water-immiscible substrates to be dissolved and the aqueous phase containing the biocatalyst. Here the substrate migrates from the organic to the aqueous phase at low concentrations, where the enzymatic reaction takes place, as illustrated in Fig. 7.2 [28,31]. This approach is preferred whenever high concentrations of substrate can affect the performance or stability of the enzyme.

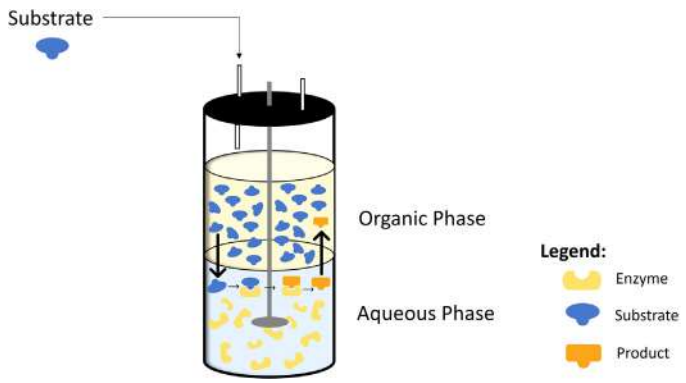

FIGURE 7.2

General scheme for two-phase partitioning bioreactors (TPPB) concept.

Several works using the TPPB have been reported, such as the enzymatic degradation of anthracene, a polycyclic aromatic hydrocarbon [27]. Anthracene was chosen as the poorly soluble substrate, and its degradation was carried out by the enzyme versatile peroxidase from *Bjerkandera adusta* [27]. The effects of solvent (silicone oil) viscosity, agitation rate, and surfactants (Tween 80 and Triton X-100) were evaluated on the removal of anthracene, as well as the mass transfer rate. It was found that in the presence of Triton X-100, the mass transfer coefficients increased from 0.37 to 0.52 min^{-1} when the agitation rate was increased from 200 to 250 rpm. This means that the addition of the surfactant improved the mass transfer of the substrate from the organic to the aqueous phase. At the optimized conditions (silicone oil viscosity of 50 cSt, 250 rpm with the addition of Triton X-100) it was obtained an anthracene oxidation of 88% [27].

Another work evaluated the degradation of anthracene by laccase from *Trametes versicolor* in a TPPB [28]. The organic phase comprised silicone oil and surfactant (Triton X-100) saturated with anthracene. The combined configuration of the TPPB operating with silicone oil as an immiscible solvent allowed to achieve a conversion rate of 16 μmol/L.h of anthracene. Moreover, the results revealed that Triton X-100 benefited the laccase stability [28]. In addition, the reuse of silicone oil to dissolve more anthracene was evaluated in three consecutive cycles of reaction, and a percentage of anthracene removal of 97% was attained. It was also proved that the distribution of anthracene between organic and aqueous phases was facilitated by adding Triton X-100, since it improved the mass transfer of anthracene from the organic phase to the aqueous phase [28].

In addition to BS as a medium for heterogeneous biocatalysis, Broering et al. [29] developed organic–aqueous tunable solvent (OATS) systems. In this approach, the hydrophobic substrate is converted by the enzyme in a single liquid phase (homogeneous biocatalysis). Then, CO_2 is added to induce the BS formation while allowing the selective separation of the product from the enzyme. The hydrophobic product preferentially migrates to the organic phase, while the hydrophilic enzyme partitions to the aqueous rich phase, allowing an easy recovery of the product and the biocatalyst reuse [29], as sketched in Fig. 7.3. The main advantage of this methodology is to solve some issues related to the heterogeneous biocatalysis approach (catalytic reaction in a two-phase system), such as lower reaction rates due to interphase mass transfer limitations [29], and slight

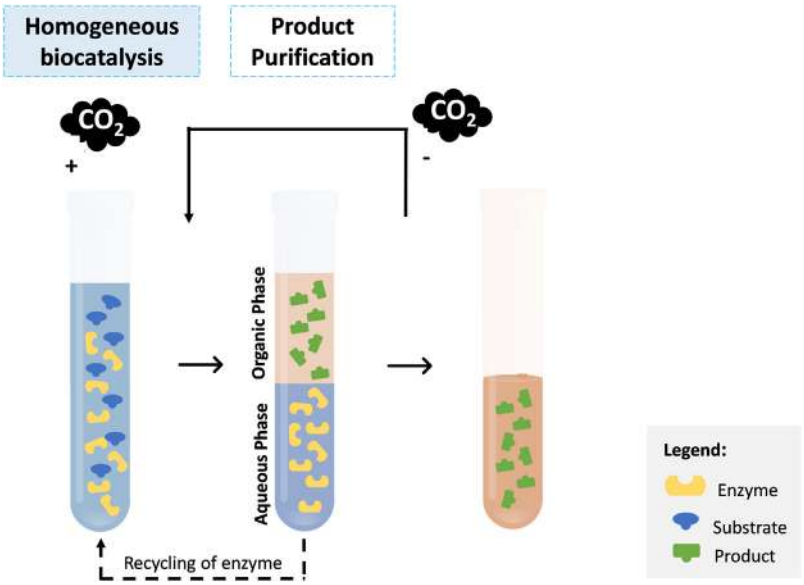

FIGURE 7.3

A proposed organic–aqueous tunable solvent (OATS) process for biocatalyst reuse.

Adapted from J.M. Broering, E.M. Hill, J.P. Hallett, C.L. Liotta, C.A. Eckert, A.S. Bommarius, Biocatalytic reaction and recycling by using CO2-induced organic-aqueous tunable solvents, Angew. Chem.—Int. (Ed.) 45 (2006) 4670–4673. https://doi.org/10.1002/anie.200600862.

loss in enzymatic activity due to the continuous stirring to increase the interfacial contact area between the two phases [29]. Thus, OATS combine the high reaction rates with a simple method for biocatalyst reuse and product separation [29]. However, for an improved performance, some requirements must be designed and optimized, namely the use of an OATS mixture providing an acceptable enzyme reactivity in the CO_2-pressurized separation process, in addition to the enzyme partition to the aqueous phase, while reaction products are retained in the organic phase [29]. As proof of concept, a BS constituted by water and dioxane with responsive behavior through the pressurization with CO_2 was developed. This system was evaluated using the enzyme lipase B from *Candida antarctica* and 2-phenethyl acetate as substrate, allowing 80% chiral product recovery in the organic phase while displaying less than 10% of apparent biocatalyst activity loss after recycling six times [29].

In another work using OATS, the transformation of rac-1-phenylethyl acetate to (R)-1-phenylethanol and (S)-1-phenylethyl was performed using lipase [30]. Different solvents, namely acetone, acetonitrile, (1,4)-dioxane and tetrahydrofuran were studied, from which the OATS based on (1,4)-dioxane was considered to be the most suitable to promote the enzymatic reaction due to its high saturation concentration (17.7 ± 0.8 mM) and increase in the enzymatic reaction rate ($0.014 \pm 0.001\ s^{-1}$) of 1-phenylethyl acetate [30].

In summary, BS increases the solubility of hydrophobic substrates and, therefore, promotes mass transfer, enhancing the overall substrate conversion. The biocatalysis of hydrophobic

substrates can be favored by the presence of a hydrophilic phase, where enzymes are preferentially solubilized, while the hydrophobic phase enhances the substrate solubility. However, it is important to take into account that depending on the organic solvent, high volatility and toxicity may be present, characteristics that are not desired in the development of a sustainable process, without mentioning that they can affect the performance of biocatalysts, as demonstrated by several authors [24,32,33].

7.2.1 Ionic liquids as alternative solvents in biphasic systems

Depending on the type of the biocatalyst, its stability may be limited; therefore, the use of alternative solvents such as ionic liquids (ILs) may improve their performance when properly designed [34–46]. ILs are ionic compounds that belong to the molten salts group, present a melting temperature below 100°C by general definition. ILs are typically composed of a large and unsymmetrical cation and an organic or inorganic anion [47]. The low melting temperatures of ILs result from the weak intermolecular interactions derived from the large size ions and their charge distribution, and lack of an ordered crystalline structure [47]. The ionic nature of ILs is responsible for some of their unique properties, such as their negligible vapor pressure under atmospheric conditions, low flammability, high thermal and chemical stabilities, large liquid temperature range, high ionic conductivity and excellent microwave-absorbing ability. Among the large range of ILs that can be synthesized, the most studied are nitrogen-based and phosphonium-based [48], with some examples of their cation chemical structures given in Fig. 7.4. The cation can be of a different nature and additionally designed by changing the size of the alkyl side chains and the addition of functional

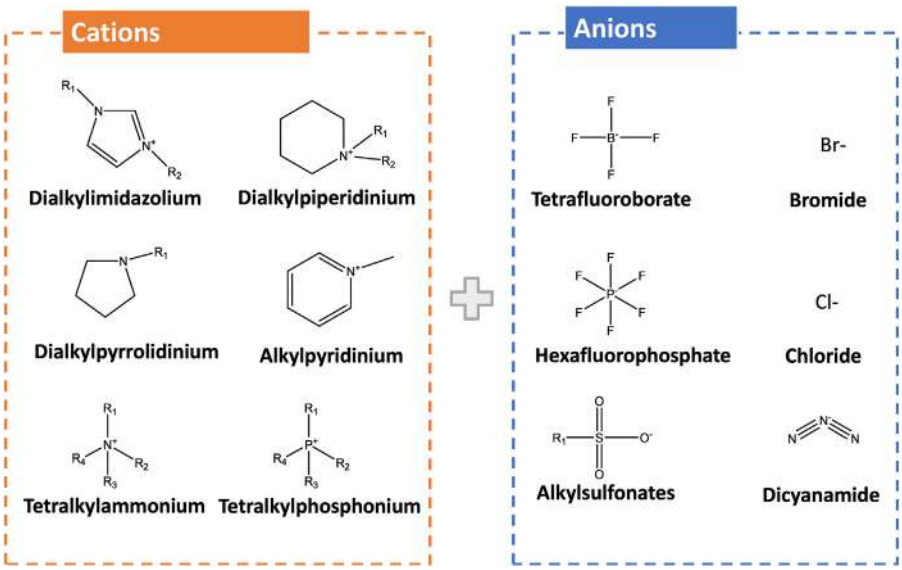

FIGURE 7.4

Chemical structures of common IL cations and anions usually used in biocatalysis.

groups [49]. Furthermore, the anion can have a different chemical nature, such as halogens, sulfates, cyano-based, fluorinated, etc. (Fig. 7.4).

Based on the vast number of cation/anion combinations, it is possible to tune their physicochemical properties for designing a specific IL for a target application, and so, ILs are commonly described as "designer solvents". This feature overcomes the limited selectivity of common volatile organic solvents, allowing the design of more effective solvents for extraction purposes and more efficient separation platforms. Based on these excellent chemical properties, ILs are well-known reaction media. In addition, when properly designed, ILs are a friendly alternative (green solvents) due to their combination of nonvolatility and nonflammability [50], eliminating solvent losses to the atmosphere, and consequently decreasing both the environmental footprint and the cost of the process [50]. However, the negligible vapor pressure is not enough to assure that these compounds are in fact "green," although losses to the atmosphere are completely prevented when compared to traditional volatile organic solvents. Properties such as toxicity, cytotoxicity and biodegradability must also be accessed. For instance, even the most hydrophobic ILs have a non-negligible miscibility with water, which can result in the contamination of aqueous streams [51]. In recent years, several studies were conducted to evaluate the toxicity and biodegradability of ILs [52–58], either by the combination of different anions and cations or by changing the alkyl side chain length and number of alkyl groups at the cation ring. These studies showed that the ILs toxicity is primordially determined by the cation nature and increases with the increase of the length of the alkyl side chain (increase in hydrophobicity) [57,58]. Although ILs cannot enter into the environment by evaporation, they can enter into the biosphere by water streams. Therefore, the synthesis of "greener" ILs and studies on their applications are nowadays one of the major topics of research within the IL community [59]. Starting materials must be nontoxic and ideally should be renewable. Low-cost synthetic routes and easy preparation should also be filled. In this direction, some novel ILs have been reported, such as those composed of cholinium- [60,61] and glycine-betaine-based cations [62,63], combined, for example, with anions derived from amino acids and carboxylic acids [60,61] (Fig. 7.5).

The use of ILs in biocatalysis brings many advantages. Compared to some organic solvents, when suitably designed, ILs do not inactive enzymes [64] and no changes occur in the enzyme structure [65]. The stability and improved activity of enzymes in ILs lead a large number of works, as detailed in recent review articles [34–36,39–46]. These works point out the importance of ILs

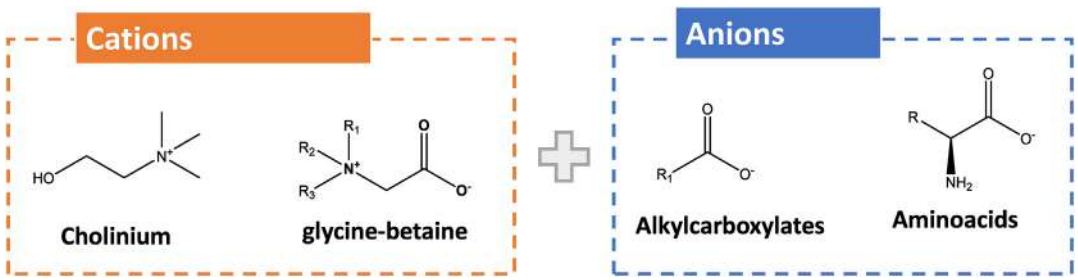

FIGURE 7.5

Chemical structures of "greener" IL cations and anions.

in biocatalytic reactions for enzyme-related applications. Depending on the IL chosen, the bioprocess can be improved due to high conversion rates and high selectivity [66]. For example, some enzymes in ILs composed of $[BF_4]^-$, $[PF_6]^-$, and $[NTf_2]^-$ anions retain activity, while in ILs constituted by Cl^-, NO_3^-, $[CF_3SO_3]^-$, $[CF_3CO_2]^-$ or $[CH_3CO_2]^-$ anions lead to decreases in the enzyme activity [64]. More recently, Bisht et al. [38] demonstrated the use of "greener" ILs, namely cholinium-based ILs, as potential media for enzymes, since these ILs lead to a remarkable enhanced activity and improved stability of enzymes. Besides, ILs can also alter the polarity of the media, which can improve substrates solubility or alter substrate specificity, leading to faster enzymatic reactions, and reduce by-products formation by suppressing side reactions [64,67]. ILs can also enhance enantioselective of enzymatic reactions [64]. Previous studies on enzymatic catalysis in BS composed of ILs and salts/buffers are summarized in Table 7.2.

In the study reported by Zou et al. [68], a novel integrated in situ enzymatic process using *Escherichia coli* whole-cell nitrilase-catalyzed asymmetric hydrolysis of nitriles was developed by the introduction of a biocompatible ionic liquid biphasic system (IL BS), composed of 1-butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF_6]) and potassium phosphate buffer [10% (v/v)]. This work applied a BS with ILs to enhance the nitrilase-catalyzed asymmetric hydrolysis of

Table 7.2 Examples of ionic liquid biphasic systems (IL BS) in biocatalysis.

Enzyme	Substrate	Solvent system	Application/ product	References
E. coli whole-cell nitrilase	o-chloromandelonitrile (CMN)	[BMIM][PF_6] and phosphate buffer	Hydrolysis of CMN to racemic o-chloromandelic acid	[68]
Saccharomyces cerevisiae whole-cell Pyruvate decarboxylase	Benzaldehyde and glucose	[BMIM][PF_6] and fermented broth	Synthesis of (R)-phenylacetylcarbinol	[69]
Recombinant β-glucuronidase from *Aspergillus oryzae*	Glycyrrhizin	[BMIM][PF_6] and acetate buffer	Synthesis of β-D-mono-glucuronide-glycyrrhizin and glycyrrhetinic acid	[70]
Epoxide hydrolases from mung beans	Styrene oxide	[BMIM][PF_6] and phosphate buffer	Biosynthesis of chiral vicinal diols	[71,72]
E. coli whole-cell P450 monooxygenase	1, 4-fluorothioanisole 3, ethyl phenyl sulfide 5, methyl p-tolyl sulfide 7, and 4-methoxythioanisole 9	[$P_{6,6,6,14}$][NTf_2] and potassium phosphate buffer	Asymmetric sulfoxidations of substrates	[73]
E. coli whole cells co-expressing yeast reductase and glucose dehydrogenase from *Bacillus subtilis*	3-chloro-1-phenyl-1-propanone	[BMIM][NTf_2] and Tris-HCl buffer	Biosynthesis of (S)-3-Chloro-1-phenyl-1-propanol ((S)-CPPO)	[74]

o-chloromandelonitrile (CMN) into racemic o-chloromandelic acid (R-CMA). A water-immiscible IL was applied to form a biphasic reaction system. The effects of substrate concentration on the asymmetric hydrolysis of CMN to R-CMA in aqueous and [BMIM][PF_6]-buffer BS were investigated. The results proved that the BS supported higher substrate concentration than the single aqueous phase. Besides, the hydrolysis reaction in [BMIM][PF_6]-buffer BS led to higher enzyme activity. Finally, it was shown that the *E. coli* whole-cell nitrilase retained 85% of activity after 7 cycles reaction [68].

The synthesis of the pharmacological precursor (R)-phenylacetylcarbinol [(R)-PAC] was carried out in the BS constituted by the IL [BMIM][PF_6] and the fermentation broth, using *Saccharomyces cerevisiae* whole-cell as the biocatalyst for the in situ reaction with pyruvate decarboxylase [69]. The *S. cerevisiae* cells present toxicity to the substrate benzaldehyde; thus, the BS was used to maintain the substrate in the hydrophobic IL phase, while the reaction occurs in the fermentation broth phase. When comparing biphasic biotransformation with the traditional monophasic system, the yield and productivity of (R)-PAC increased 1.5 times, whilst the benzyl alcohol (by-product) was reduced [69].

Recombinant β-glucuronidase from *Aspergillus oryzae*, immobilized on zinc-based nanoparticles, was used to catalyze the biotransformation of glycyrrhizin into β-D-mono-glucuronide-glycyrrhizin followed by its transformation into glycyrrhetinic acid [70]. The enzyme activity was independently evaluated in buffer and IL media, and the best activity was achieved in 20% (v/v) of [BMIM][PF_6]. Then, the reaction was studied in a BS composed of [BMIM][PF_6] and acetate buffer, where β-glucuronidase presented high stability and improved performance than in pure IL [70]. The BS also presents the advantage to allow the final product purification since glycyrrhizin and β-D-mono-glucuronide-glycyrrhizin were soluble in the buffer-rich phase, while glycyrrhetinic acid migrated to the IL-rich phase [70]. In this work, the recovery of IL was studied, and 76% of IL was recovered and reused after eight repeated batches without affecting the enzymatic reaction [70].

Cross-linked enzyme aggregates was used to hydrolysis styrene oxide by epoxide hydrolases in an IL BS [71,72]. The enzymatic reaction was evaluated in different ILs families, such as the imidazolium-, pyrrolidinium-, piperidinium-, phosphonium-, and cholinium-based ones. Among them, both works [71,72] verified that [BMIM][PF_6] was the IL presenting the best biocompatibility with the enzyme, and was thus selected to prepare a BS with phosphate buffer [71,72]. The enzymatic reaction presented a yield of 49% and an enantiomer selectivity of 96%−97% [71,72]. Moreover, Yu et al. [71] went one step further, performing a control with an n-hexane BS for comparison, observing that substrate concentration in the IL BS was up to 4.0 times higher than that in the n-hexane-based one. Finally, the enzyme reuse was evaluated during 5 cycles of reaction, showing that the IL BS maintained 70% of the initial activity, while the n-hexane BS only maintained 31% of the initial enzyme activity [71].

As an example of a bio-oxidation, Gao et al. [73] demonstrated an aqueous-IL BS to increase the enantioselectivity and productivity of asymmetric sulfoxidations catalyzed by sulfide monooxygenases P450 (1, 3, 5, 7 and 9). *E. coli* (P450pyrI83H-GDH) coexpresses monooxygenase and glucose dehydrogenase, and was designed for asymmetric sulfoxidations of thioanisole 1, 4-fluorothioanisole 3, ethyl phenyl sulfide 5, and methyl p-tolylsulfide-methoxythioanisole 9, respectively. Because the substrates and their products are hazardous to cells and inhibit sulfoxidation, a biphasic potassium phosphate-IL buffer system was designed to address these issues. The inhibition of reactions, as well as the toxicity of substrates and products to cells, were investigated, and all of

them were avoided by using the potassium phosphate buffer-[$P_{6,6,6,14}$][NTf_2] BS. This represents a biphasic reaction system with excellent biocompatibility with cells and high solubility with substrates and products [73]. The sulfuroxidations of 1, 3, 5, 7, and 9 used *E. coli* cells in potassium phosphate-[$P_{6,6,6,14}$][NTf_2] buffer at a 3:1 ratio (v/v). In such a BS, $>$ 99% of substrates and 35% to 60% of products remained in IL, significantly decreasing substrate toxicity and inhibiting product toxicity. [$P_{6,6,6,14}$][NTf_2] demonstrated good biocompatibility for *E. coli* cell growth and the capacity to shield the cells from much of the substrate toxicity, making it an appropriate cosolvent in the KP buffer BS. In summary, the BS with IL provides an effective reaction platform for biooxidation, while improving cell and enzyme compatibility [73].

(S)-3-Chloro-1-phenyl-1-propanol ((S)-CPPO) is a chiral building block for the synthesis of pharmaceutical drugs. There are enantioselective enzymes capable of transform 3-chloro-1-phenyl-1-propanone (3-CPP) in (S)-CPPO; however, this substrate presents low solubility in aqueous media [74]. Choi et al. [74] proposed the use of an IL BS to overcome this limitation. *E. coli* whole cells coexpressing the enzyme methylglyoxal reductase (YOL151W) and glucose dehydrogenase from *Bacillus subtilis* were used as biocatalysts to perform the transformation of 3-CPP in (S)-CPPO using glucose as a cofactor. After a selection from a variety of ILs, [BMIM][NTf_2]-Tris−HCl buffer BS was chosen for the reaction using Tween 40 as an additive. In the proposed BS, 3-CPP was solubilized in the [BMIM][NTf_2] phase, while glucose was dissolved in the buffer phase, and the biocatalyst located on the interface, where the reaction occurred. After 8 h of reaction, 100 mM (S)-CPPO was synthesized with an enantiomeric excess of $>$99% [74].

7.2.2 Deep eutectic solvents as alternatives solvents in biphasic systems

Deep eutectic solvents (DES) are an emerging type of alternative solvents that resemble ILs, preferabaly biomass-derived, that are formed by eutectic combinations of a hydrogen-bond acceptor (typically a quaternary ammonium halide salt) with a hydrogen-bond donor, such as saccharides, alcohols or carboxylic acids [75]. The formation of a DES is characterized by their melting point, which present reduced melting temperatures than both forming compounds, which may be liquid at room temperature [75,76]. If properly designed, DES present improved solvent ability and peculiar physicochemical properties, which are additionally adjusted by shifting its composition [77]. The fact that biodegradable and inexpensive compounds can be used to form DES and their peculiar physical proprieties have triggered their research in the area of biocatalysis, with an increasing number of applications being reported [76,78−81]. DES have been evaluated as alternative solvents or cosolvents in biocatalysis. DES properties can influence the biocatalyst structure and activity and the biocatalytic process, such as their viscosity and water content [78,81,82]. When well designed, DES can improve enzyme stability and activity, enhancing reaction efficiency [82]. More information can be found in the works from Xu et al. [82], Tan and Dou [81] and Pätzold et al. [78]. Xu et al. [82] identified the downstream separation of the target compound from DES as one of the challenges for this solvent to be applied. Here, BS enter as a promising option to include DES as a solvent in biocatalysis reactions and as an integrated step to separate the target compounds. So far, all examples found in the literature explored lipase-catalyzed reactions in DES, which are summarized in Table 7.3.

Lipase B has been used to synthesized biodiesel [83,84]. However, the typical enzymatic reaction has some limitations, such as mass transfer and accumulation of the by-product glycerol. The use of BS

Table 7.3 Examples of deep eutectic solvents biphasic systems (DES BS) in biocatalysis.

Enzyme	Substrate	Aqueous biphasic system composition	Application/ product	References
Penicillin acylase G from *Escherichia coli*	Waste Oils	[Ch]Cl:Gly, water and n-hexane	Synthesis of biodiesel	[83]
Penicillin acylase G from *E. coli*	Yellow horn seed oil	Several DES and oil	Synthesis of biodiesel	[84]
Penicillin acylase G from *E. coli*	Fatty acids	[Ch]Cl:Sugars and ethyl acetate	Synthesis of glycolipids	[85]

to overcome these problems has been explored as recently reported by Merza et al. [83]. In this work, biodiesel was produced by lipase B from *C. antarctica* using waste oils as substrate [83]. The reaction occurred in DES composed of cholinium chloride and glycerol ([Ch]Cl:Gly), followed by the introduction of *n*-hexane to extract the fatty acids methyl esters produced. The content of water was also studied, with the results showing that [Ch]Cl:Gly (1:2) with 3 wt.% water allowed to achieve the high yield of 44%. When comparing the use of the DES [Ch]Cl:Gly with the typical IL [BMIM][PF_6], a 63% higher yield was observed and a 71% higher yield with the addition of 3 wt.% water [83]. The enzyme reuse was analyzed in five consecutive cycles, that is, the reuse of the DES system was improved by removing the by-product glycerol and using 1-butanol as a washing solvent [83].

In the same line, Zhang et al. [84] produced biodiesel using Novozym 435 (immobilized lipase B from *C. antarctica)* and yellow horn seed oil as substrate in DES media with microwave irradiation method. The authors evaluated 11 DES as alternative solvents in the reaction, and compared the results with *t*-butanol and the [BMIM][BF_4] IL, being the best conversion yield of 75% achieved in ChCl:Gly (1:2). BS was formed with DES, where the substrate acts as the upper phase; after the reaction, the upper phase was the biodiesel produced and the bottom phase was the DES phase, with the enzyme partitioning between the two phases [84]. Under the optimum conditions (8% of *Novozym 435*, methanol/oil ratio 6:1, microwave power 400 W, 50°C, and reaction time 120 min), 95% conversion yield was achieved [84]. Furthermore, the recovered enzyme was used for four successive reaction cycles with a small enzyme activity loss (10% decline of conversion efficiency was observed after two times recycling). However, the conversion yield still kept 80% above and did not dramatically decrease until the 5^{th} recycling [84]. The decrease in the catalytic activity of the enzyme after 4 runs might be due to the structure destruction of the enzyme caused by methanol and long-time stirring [84].

Syldatk and coworkers [85] studied a reaction system based on a DES consisting of [Ch]Cl and several sugars to perform the enzymatic synthesis of glycolipids [85]. The DES-based solvent system acts simultaneously as substrate and solvent and is also suitable for the enzymatic synthesis of novel tailor-made glycolipids [85]. All DES were prepared by mixing equimolar ratios of [Ch]Cl with one of the sugar components. The mixtures were constantly stirred and heated at 100°C until a colorless liquid appeared. The reactions studied were performed by adding lipase Novozyme 435 and fatty acid to the corresponding DES [85]. BS was formed after the reaction by adding ethyl acetate for glycolipid extraction, while the enzyme remains in the DES phase. With this reaction system, it was possible to form a DES-based on [Ch]Cl and levoglucosan and to successfully use it

with lipase for an enzymatic synthesis reaction [85]. This finding should help to create a novel class of glycolipids with more beneficial characteristics [85]. It is also interesting to note that the used lipase was not specific for one glycolipid product. Besides sugar-mono-laurates, many polyacrylate sugars were formed during synthesis [85].

7.3 Biocatalysis in aqueous biphasic systems

Albertsson introduced, in the mid-1950s, aqueous biphasic system (ABS) as a separation technique [86], being a particular type of BS. ABS is an efficient and clean approach for biocatalysis since both phases are mostly composed of water, and thus may be a biocompatible medium for biologically active molecules, like enzymes [50]. ABS are formed when two water-soluble components, (e.g., polymer−polymer, polymer−salt or salt−salt combinations) are mixed in water above given concentrations, leading to the formation of a biphasic aqueous system [50]. In general, the compounds will compete for water molecules with the formation of hydration complexes, with the salting-out effect leading to the formation of ABS.

For the design of an effective ABS in biocatalytic reactions, their phase diagrams and respective tie-lines (TLs) are required. This information is crucial to define ABS mixture compositions able to form two-phase systems and know the coexisting phases compositions. All ABS have a singular phase diagram under a group of conditions, like temperature, pressure and pH. Fig. 7.6 depicts an example of a triangular and an orthogonal representation of the phase diagram for an ABS composed of a polymer, a salt, and water, and the respective binodal curve (points TCB, Fig. 7.6) [50].

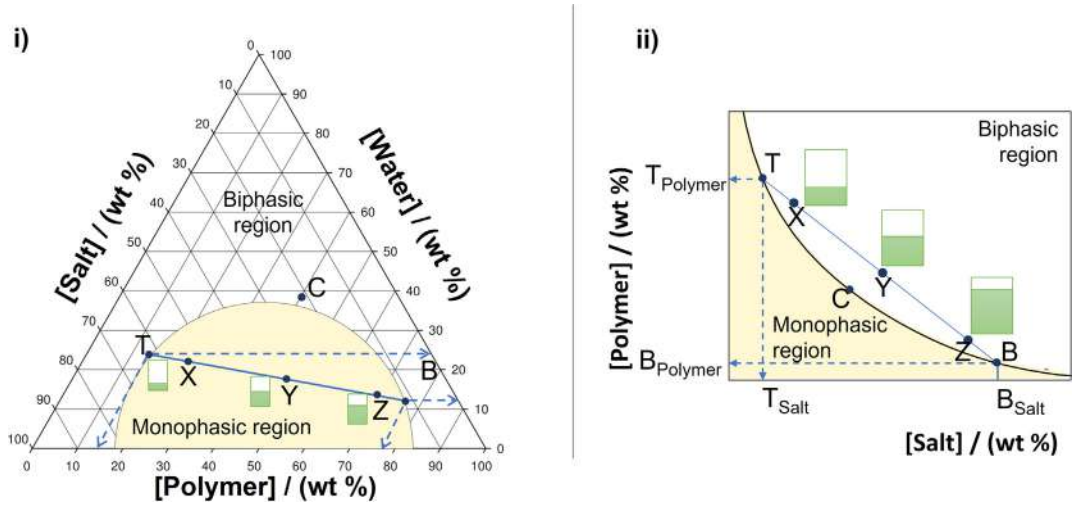

FIGURE 7.6

Schematic of an ABS phase diagram in an (i) triangular and (ii) orthogonal representation. TCB-binodal curve; C-critical point; TB-tie-line; T-composition of the top phase; B-composition of the bottom phase; and X, Y, and Z-initial mixture compositions of biphasic mixtures.

The binodal curve (points TCB in Fig. 7.6) represents the separation between the miscible and immiscible regions, that is, above the binodal curve is located the biphasic or heterogeneous region (the mixture suffers phase separation and forms new two coexisting phases), while below it is the monophasic or homogeneous region [87]. The larger the biphasic region, the higher the ability of the phase-forming components to form BS. Three mixture compositions at the biphasic region are also identified as X, Y and Z in Fig. 7.6. These mixtures are located along the same TL, meaning that all these mixtures present the same top ($T_{Polymer}$, T_{Salt}) and bottom phase compositions ($B_{Polymer}$, B_{Salt}). The determination of the TLs is usually carried out by a gravimetric approach as proposed by Merchuk et al. [88], which has been described in detail in several articles [89,90]. The TL length (TLL) is a numerical indicator of the composition difference between the two phases and it is often used to correlate with the trends observed in solutes partitioning between the phases. The critical point of the ternary system is Point C, where the two binodal nodes meet, that is, the compositions of the two coexisting phases become equal and the BS ceases to exist.

The factors that influence the formation of two phases in ABS and have an impact on the bioreactions are the molecular weight and concentration of polymer, hydrophobicity, pH and temperature [50,91]. If properly designed, the simplicity of ABS, water-rich environment, improved biocompatibility and low cost of phase-forming compounds make them appropriate for biocatalysis. Comparing to classical BS, the aqueous media present in both phases is adequate for aqueous soluble biocatalyst, substrates and products. In addition, ABS can combine production, extraction and recovery techniques into a single step when the biocatalyst is retained in one phase and the reaction product in the opposite phase. Recent developments are found in the literature regarding the use of ABS for biocatalysis, as summarized in Table 7.4.

The work of Guisan et al. [92] studied the reaction between 7-amino-deacethoxy-cephalosporanic acid (7-ADCA) or phenyl glycine methyl ester (PGME) with penicillin acylase G (PGA) from *E. coli* in an ABS composed of polyethylene glycol (PEG) (600−20,000) and ammonium sulfate or dextran to produce cephalexin, a soluble compound. Different compositions of PEG (top-rich phase) and ammonium sulfate or dextran (bottom-rich phase) were evaluated. It was found that the highest partition coefficient ($K = 23.0$) for cephalexin occurred in the ABS constituted by PEG 600/ammonium sulfate (3 M), at pH 6.5, which allowed a synthesis yield of 90% of cephalexin. Using the same enzyme (PGA) and substrates (7-ADCA and PGME) Li et al. [93] evaluated

Table 7.4 Examples of aqueous biphasic systems (ABS) in biocatalysis.

Enzyme and source	Substrate	IL-based ABS	Application/product	References
Penicillin acylase G from *Escherichia coli*	7-ADCA and PGME	PEG and ammonium sulfate or dextran	Synthesis of cephalexin	[92]
Penicillin acylase G from *E. coli*	7-ADCA and PGME	Copolymers: P_{ADBA} and P_{MDB}	Synthesis of cephalexin	[93]
Penicillin acylase G from *E. coli*	7-ADCA and PGME	PEG and phosphate buffer	Synthesis of cephalexin	[94]
Urease from *Canavalia ensiformis*	Urea	PEG and dextran	Production of $(NH_4)_2CO_3$	[95]
Urease from *C. ensiformis*	Urea	PEG and dextran	Study of cell mineralization $CaCO_3$	[96]

the ABS composed of two copolymers (P_{ADBA} and P_{MDB}) in the production of cephalexin. First, the authors verified that the partition coefficient of cephalexin in this ABS was 2.57. Then, the synthesis of cephalexin in the ABS achieved a yield of 98.2%, significantly higher than the enzymatic reaction in phosphate buffer media (73.6% yield). Finally, the recyclability of phase components (copolymers) was successfully achieved (P_{ADBA} recovery = 97.4% and P_{MDB} recovery = 97.2%) by changing the pH of the system [93], showing the use of ABS in biocatalysis to be a promising approach to be applied in the biochemical industry.

Besides all the advantages that ABS offers for biocatalysis, its potential can be improved if the enzymatic reaction occurs in a continuous mode. For this, a microfluidic device can be used [94,95]. In this technology, a parallel individual laminar flow containing phase constituents, enzyme and substrate are introduced in a double Y-branched microfluidic device to continuously promote the enzyme reaction and product separation (Fig. 7.7). The reaction product generated is diffused into the preferential phase, while the biocatalyst is retained in another phase. Then, the phases are separated at the end of the microfluidic device [94,95].

Using microfluidics, a continuous-flow process based on a properly designed ABS (constituted by PEG 4000 and phosphate buffer) was evaluated in the production of cephalexin [94]. In this study, the device offered the advantage of recycling the reaction phase containing the enzyme since the PGA preferentially migrated to the salt-rich phase, while cephalexin migrated to the PEG-rich phase. On the other hand, continuous dialysis of the recycled phase was required to avoid enzyme precipitation. At the optimized conditions, the cephalexin concentration at the outlet was initially 40 mM and then decreased to 35 mM after 300 min. With phase and enzyme recycling, the microfluidic device operated for 5 h in continuous mode. Comparing to the previous work of Guisan et al. [92], where the same reaction and conditions were performed in a classical ABS, the main advantage of using a microfluidic device was to combine the cephalexin production and separation in a single step and continuous mode, with enzyme recycling [94]. Another example of biocatalysis in microfluidic is the production of ammonium carbonate by the conversion of urea with urease from *Canavalia ensiformis* [95]. Compared with the conventional ABS, a reaction rate 500 times higher was obtained in the microfluidic system under agitation. Moreover, the conversion rate of

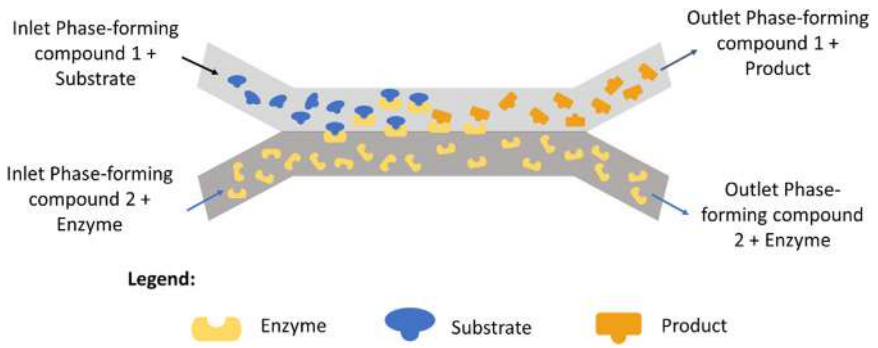

FIGURE 7.7

General scheme of an ABS microfluidic device for enzymatic reaction and product separation.

the substrate to the product was improved with the recirculation of the enzyme, obtaining an increase of about four times in four cycles [95].

ABS are also being used in cellular biology studies due to their capability to simulate organelles in liquid environments within less complex systems by forming artificial microcompartments for biomolecules and bioprocesses [97]. This approach permits understanding natural bioprocesses in cells and their interactions with the environment. To understand the environment interactions and phase compartmentalization on enzymatic mineralization on cells, Cacace and Keating [96] studied the enzymatic hydrolysis of urea to produce calcium carbonate ($CaCO_3$). The reaction was catalyzed by urease from *C. ensiformis* in an ABS composed of PEG 8,000 and dextran 10,000. Urease has preference for the dextran-rich phase ($K = 0.12$), where the enzymatic reaction takes place and $CaCO_3$ precipitates, despite Ca^{2+} concentrations being the same in both phases. In this work, the volume of the dextran-rich phase was reduced while maintaining the same ABS volume, which improves local reaction rates (47% increase in $CaCO_3$ precipitated) [96].

7.3.1 Biocatalysis in ionic-liquid-based aqueous biphasic systems

Rogers et al. [49] first reported the formation of ABS constituted by inorganic salts and ILs. Since then, IL-based ABS have been used as novel alternatives for diverse applications and have been the focus of a significant amount of research, as critically reviewed by Freire et al. [50]. Among these applications, biocatalysis has been highlighted in the last years. While water is the traditional solvent for biocatalysis, some enzymes have been shown to be more active in hydrated ILs [37,67,98]. Thus, IL-based ABS have been proposed as viable alternatives for biocatalysis (as summarized in Table 7.5) with a set of other important advantages such as: lower viscosities than polymer phases, quick phase separation, and high and tailored extraction efficiency. When ILs are used in ABS, the polarity of both phases of the ABS may be adjusted, the separation process can be improved, and the biomolecule partition can be regulated [50,99,100].

Meyer et al. [101] proposed a thermomorphic ABS composed of ILs and salt, forming a homogeneous media for the biocatalysis reaction, followed by an increase of temperature to occur phase separation and thus separate products and the enzyme. After testing several cholinium-, imidazolium- and pyridinium-based ILs, an ABS constituted by [BMIM][BF_4] and sodium phosphate (NaPi) buffer has selected to perform an enzymatic reaction with Lipase B from *C. antarctica* [101]. The hydrolysis

Table 7.5 Examples of IL-based ABS in biocatalysis.

Enzyme and source	Substrate	IL-based ABS	Application/product	References
Lipase B from *C. antarctica*	1-phenylethyl acetate	[BMIM][BF_4] and NaPi Buffer	Hydrolysis of 1-phenylethyl acetate	[101]
Laccase from *T. versicolor*	Dye	PPG 400 and [Ch][DHC]	Degradation of dye	[61]
Laccase from *T. versicolor*	Rutin	PEG 600 and [Ch][DHph]	Synthesis of oligorutin	[60]
Laccase from *T. versicolor*	ABTS	Ammonium-based ZIs and PEG	Oxidation of ABTS	[87]

of 1-phenylethyl acetate was performed with 49.9% conversion and 99.9% enantiomeric excess. The enzyme was reused for 6 consecutive reaction cycles with minimal activity losses [101]. However, it should be remarked that this IL is not the best option for the formation of the ABS as tetrafluoroborate-based ILs are not water-stable compounds, that is, they can hydrolyze in contact with water [102].

To overcome some of the toxicity and biodegradability concerns associated to imidazolium-based ILs, ABS formed by biocompatible phase-forming components, for example, constituted by cholinium-based ILs and polymers, have been developed by Ferreira et al. [61]. The authors studied the use of IL-based ABS for the decolorization of dyes. The enzymatic degradation occurred in the BS using laccase as the biocatalyst. Among the phase constituents evaluated, PEG 400, cholinium dihydrogen phosphate ([Ch][DHP]) and cholinium dihydrogen citrate ([Ch][DHC]) were selected since the biocatalyst presented improved performance when compared to polypropylene glycol (PPG 400) and cholinium acetate ([Ch][Acet]). The partition of the dye and the enzyme was evaluated, and the enzyme and the dye/products were partitioned to opposite phases. After optimizing the best ABS conditions and composition for Remazol Brilliant Blue R (RBBR) decolorization, the system composed of PPG 400 and [Ch][DHC] was selected to evaluate the recovering and reuse of the IL-rich phase containing the biocatalyst, as summarized in Fig. 7.8. The phase containing the

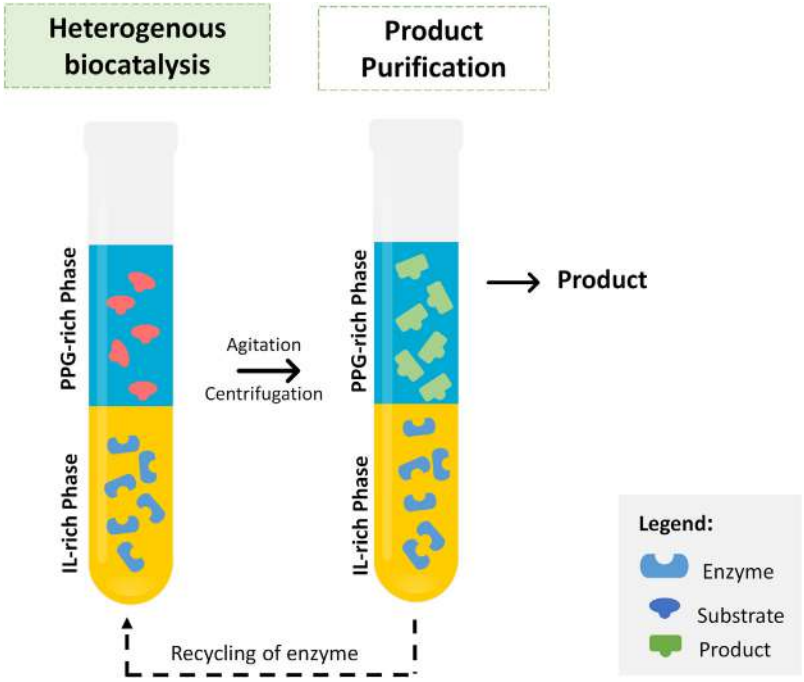

FIGURE 7.8

Integrated process using IL-based ABS for dye decolorization and enzyme reuse.

Adapted from A.M. Ferreira, A.I. Valente, L.S. Castro, J.A.P. Coutinho, M.G. Freire, A.P.M. Tavares, Sustainable liquid supports for laccase immobilization and reuse: degradation of dyes in aqueous biphasic systems, Biotechnol. Bioeng. 400 (2021) 0–1. https://doi.org/10.1002/bit.27764.

biocatalyst was able to perform six consecutive cycles of dye decolorization with a decolorization of RBBR (> 96%).

In the same line, Muñiz-Mouro et al. [60] showed the viability of cholinium-based ABS as an integrated platform for laccase-catalyzed processes. Although the laccase-catalyzed polymerization of rutin is a promising strategy, since it allows the control of the polymeric structure by the nature of the reaction medium, the biocatalyst currently represents one of the highest costs of the process. PEG-IL-based ABS have been investigated with the aim of combining the biocompatibility and low toxicity of PEGs, with increased efficiency and selectivity of the separation step associated with IL-based ABS, while decreasing the viscosity of the media when compared to polymer-based ABS [60]. Notably, using PEG 600 and [Ch][DHP] as ABS constituents, it was successfully achieved an adequate separation of oligorutin for the PEG-rich phase (60% of extraction efficiency of oligorutin ($EE_{oligorutin}$)) and of the enzyme to the IL-rich phase. The synthesis of oligorutin by laccase was performed in a biphasic medium that allowed simultaneously to carry out the reaction, the product recovery, and the enzyme reuse without any further external stimuli [60]. The synthesized oligorutin migrated to the PEG-rich top phase (67% of

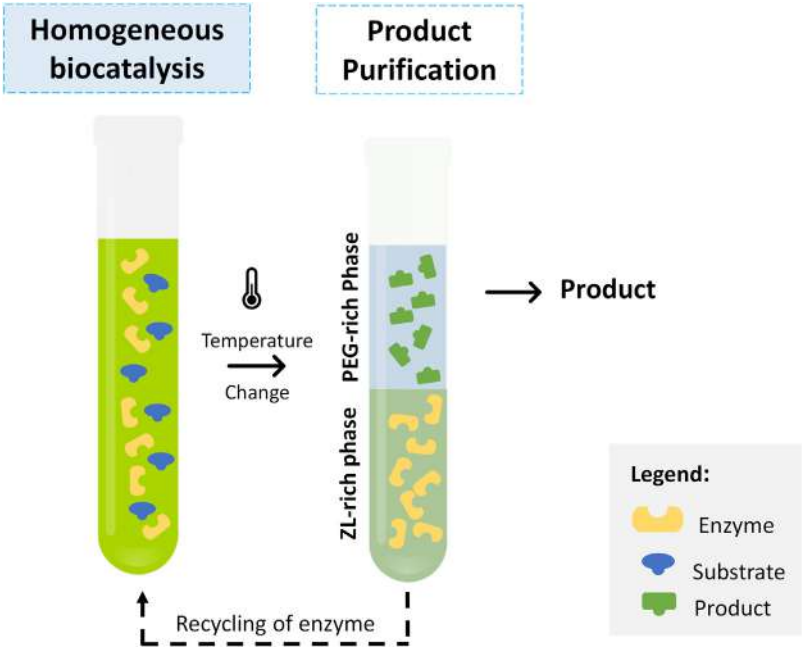

FIGURE 7.9

Flowchart of the integrated reaction-separation process developed, including the enzyme and ZI-rich phase recyclability.

Adapted from A.M. Ferreira, H. Passos, A. Okafuji, A.P.M. Tavares, H. Ohno, M.G. Freire, et al., An integrated process for enzymatic catalysis allowing product recovery and enzyme reuse by applying thermoreversible aqueous biphasic systems, Green. Chem. 20 (2018) 1218–1223. https://doi.org/10.1039/c7gc03880a.

$EE_{oligorutin}$), which was then recovered by separating the two phases after the first cycle of reaction. The IL-rich phase containing laccase (95% of $EE_{laccase}$) was recovered and reused up to three times in a new biocatalytic reaction cycle.

The use of ILs as alternative solvents and constituents of ABS is a promising topic under development. Therefore, there is still much space to research and to carry out the development of such a friendly process, for both the environment and enzymes. Other alternative solvents based on ions, namely zwitterions (ZIs), have also been considered as replacements for classical organic solvents and ILs. ZIs are compounds where a cation is covalently bonded to an anion, preventing the occurrence of ion exchanges between the coexisting phases in an ABS [103]. The authors shown that ABS can be formed by ZIs and polymers, and that these systems display a thermoreversible behavior [87]. These ABS allow the extraction and separation of the enzymatic reaction products for the polymer-rich phase, while the enzyme migrated to the zwitterion-rich phase (Fig. 7.9) [87]. The biocatalytic reaction occurred in a homogeneous media, with subsequent formation of the BS induced by small changes in temperature, which led to the complete separation of the enzyme from the products in a single step. Ferreira et al. [87] also demonstrated that these ABS allow the reuse of the phase containing laccase at least 5 times without decreasing the catalytic activity.

7.4 Concluding remarks

Based on the available literature, BS can be a sustainable platform for biocatalytic reactions; they allow an easy recovery and reuse of the biocatalyst while ensuring the reaction products separation. When properly designed, BS can be biocompatible, emerging as novel alternatives in the field of biocatalysis under "greener" conditions. Suitable ILs and DES have been emerged as excellent solvents for enzymatic reactions due to their tunable ability, especially for insoluble or partially soluble substrates in water and other common organic solvents. Moreover, the combination of ILs or DES with enzymes in BS provides excellent reaction yields, allowing integrated processed to be developed, efficiently separating the products and recycling the phase containing the biocatalyst. More importantly, some works proved that enzymes can be easily recovered from the BS and reused without significantly losing activity. In summary, the published studies show that BS are promising platforms as simultaneous reaction media and separation processes for several processes. However, their use and application are still limited to the laboratory scale. Thus, scale-up studies should be carried out to evaluate the scalability of the process to be implemented at an industrial scale. Moreover, as ILs and DES can be tailored to fit the requirements of other biocatalytic processes, we expect that BS based on ILs or DES may gain recognition, attract further interest, and play a significant role in developing enzymatic sustainable bioprocesses.

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CHAPTER

Biotransformations of carbohydrates in ionic liquids

8

María J. Hernáiz

Biotransformation Group, Department of Chemistry in Pharmaceutical Science, Faculty of Pharmacy, Complutense University, Madrid, Spain

8.1 Introduction

In recent decades, there has been an increasing effort to reduce the use of fossil fuels and oil derivatives, to decrease environmental pollution and to counteract global warming. The use of biomass as a raw material is becoming a major alternative to fossil fuels, as it is widely abundant and relatively inexpensive [1–3]. Carbohydrates, that is, cellulose, starch, chitin, chitosan, uronic acids, sucrose, glucose and fructose (Scheme 8.1), are important raw materials in the chemical industry because they are produced from biomass, which is readily available in large amounts, facilitating their large-scale application [4].

Carbohydrates display important functions in cell physiology and at the nanoscale in cell membranes, as part of glycoconjugates (glycoproteins, glycolipids, and polysasaccharides) comprising the glycocalyx. Consequently, carbohydrates have important roles in many biological processes, including bacterial and viral infection, cancer metastasis, apoptosis, neuronal proliferation, and many other crucial intercellular recognition events [5–9].

Carbohydrate-based compounds are also widely used in the pharmaceutical, cosmetic, detergent, and food industries. While these compounds are mainly produced by chemical methods, the use of enzymatic methods has been investigated over the past 20 years as a greener alternative to organic synthesis. On one hand and due to the low solubility of enzymes and carbohydrates in traditional organic solvents, research has focused on the chemical and enzymatic synthesis of carbohydrates in polar green solvents such as water, supercritical fluids (SCFs) and ionic liquids (ILs) [10]. On the other hand, traditional carbohydrate synthesis and modification frequently involve multiple protection and deprotection steps, the use of hazardous and harmful chemicals and solvents, and other harsh conditions that adversely impact the environment and human health [11–17]. There is a need to find new ways to produce carbohydrate-based products using more environmental friendly conditions.

According to the green chemistry principles [18–21], biocatalysis and solvents play an important role in green processes, since they provide valuable alternatives to classic organic chemistry [22]. First, enzymes offer suitable tools for industrial reactions, which can be carried out under milder conditions, without using heavy metals and with a great control over chemo-, regio- and stereoselectivity. Secondly, there are numerous potential advantages in employing enzymes in

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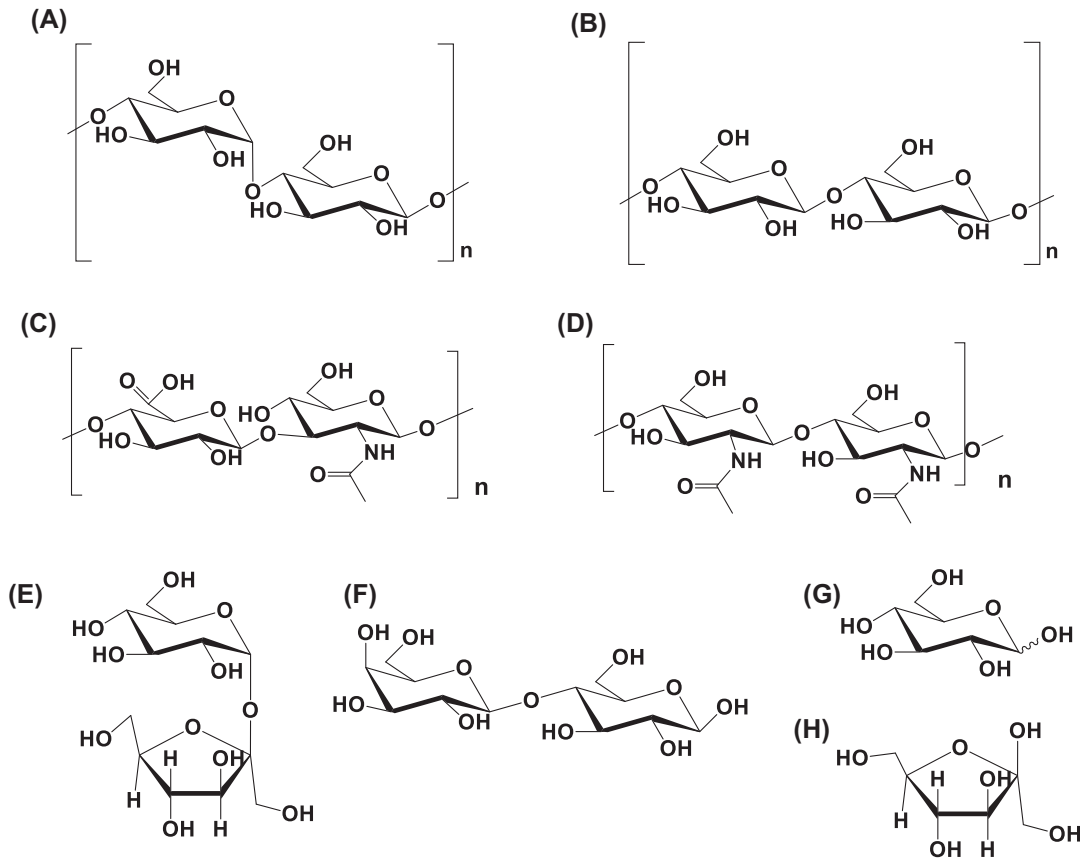


SCHEME 8.1

Chemical structure of some important carbohydrates for industrial applications: (A) starch (α-amylose section), (B) cellulose, (C) chitin and (D) chitosan (E) sucrose, (F) lactose, (G) glucose and (H) fructose.

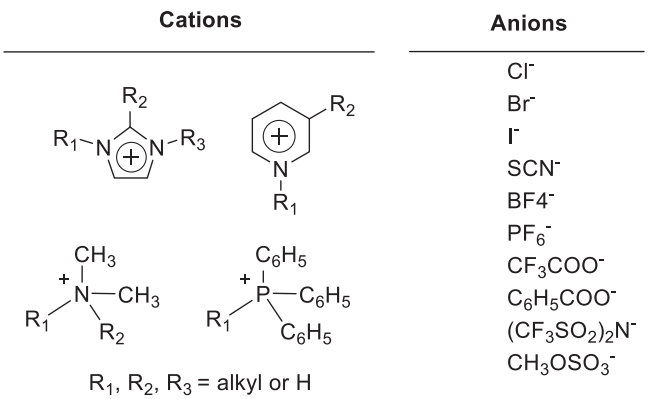
organic solvents, such as increased solubility of nonpolar substrates, shifting of thermodynamic equilibria to favor synthesis over hydrolysis, decrease of water-dependent side reactions, enhanced thermal stability of enzymes, and elimination of microbial contamination, among others [23]. On the other hand, the presence of organic solvents in enzymatic reactions can alter the activity and specificity of the enzyme. There are numerous examples in the literature where solvent physical properties such as, dielectric constant, dipole moment and hydrophobicity are related to various effects on enzyme activity, specificity and enantioselectivity [24–26].

Green chemistry offers the tools to build a sustainable industrial and research effort. Renewable feedstocks, biocatalyzed reactions carried out under mild conditions, at room temperature, in water or other green solvents such as ILs and with high atom efficiency are among the strategies employed to achieve such goals [18,27–30].

8.2 Ionic liquids

ILs have been emerged as a potentially attractive green alternative to environmentally harmful organic solvents [31,32]. ILs are composed entirely of ions (generally consisting of organic cations, namely derivatives of *N*,*N*′-substitutedimidazolium, *N*-substitutedpyridinium, tetraalkylated ammonium, and tetraalkylated phosphonium), and either organic or inorganic anions (halide, organic acid, or isocyanate), are liquids at ambient temperature, have negligible vapor pressure and are nonflammable (Scheme 8.2). The most commonly used ILs, their abbreviated names, and their water solubilities are presented in Table 8.1.

For all these properties ILs are more environmentally attractive than volatile organic compounds and also possess many unique and attractive physicochemical properties, including multiple solvation interactions with organic and inorganic compounds, excellent chemical and thermal stability, high ionic conductivity and a large electrochemical window [33–35]. Importantly, the enormous diversity of ILs makes them suitable for a large number of industrial applications [36]. In fact, the physicochemical properties of ILs, such as viscosity, hydrophobicity, density and solubility can be tuned by simply selecting different combinations of cations and anions, as well as attached substituents, leading to the use of the terms "designer" and "task-specific" ILs [37,38]. For more than a decade, ILs have been increasingly used as solvents and/or (co)solvents and/or reagents in a wide range of applications due to their superb properties: the combination of green properties with their tailor-made chemical, physical, and biological properties. Therefore, ILs have a significant advantage over conventional solvents for use in chemical engineering (e.g., separation, extraction and membranes) [39], chemistry (e.g., organic synthesis, catalytic reactions, nanomaterial synthesis and polymerization reactions) [40,41] and biotechnology (e.g., biocatalysis, biomolecules purification and biofuel production). Their applications continue to expand [42,43].



SCHEME 8.2

Chemical structures of common ionic liquids.

Table 8.1 Nomenclature of commonly used ionic liquids and their miscibility with water.

Cation	Anion	Common notation	Water miscibility
1-Butyl-3-methylimidazolium	Tetrafluoroborate	[BMIM][BF_4]	Yes
	Hexafluorophosphate	[BMIM][PF_6]	No
	Bis[(trifluoromethyl)sulfonyl] imide	[BMIM][Tf_2N]	–
	Trifluoromethanesulfonate	[BMIM][Tfms]	Yes
	Glycolate	[BMIM][$OHCH_2COO$]	–
	Octylsulfate	[BMIM][$OctSO_4$]	Yes
1-Butyl-2,3-dimethylimidazolium	Tetrafluoroborate	[BDMIM][BF_4]	Yes
	Hexafluorophosphate	[BDMIM][PF_6]	No
	Trifluoromethanesulfonate	[BDIM][Tfms]	
1-Butyl-4-methylpyridinium	Tetrafluoroborate	[BMP][BF_4]	Yes
1-Ethyl-3-methylimidazolium	Tetrafluoroborate	[EMIM][BF_4]	Yes
	Bis[(trifluoromethyl)sulfonyl] imide	[EMIM][Tf_2N]	No
	Trifluoromethanesulfonate	[EMIM][Tfms]	Yes
1-Ethylpyridinium	Tetrafluoroborate	[ETPY][BF_4]	–
	Trifluoroacetate	[ETPY][CF_3COO]	–
1-Butylpyridinium	Bis[(trifluoromethyl)sulfonyl] imide	[BUPY][Tf_2N]	–
1-Butyl-1-methylpyrrolidinium	Bis[(trifluoromethyl)sulfonyl] imide	[BMPY][Tf_2N]	Partly miscible
1-Hexyl-2,3-dimethylimidazolium	Tetrafluoroborate	[HDMIM][BF_4]	Partly miscible
1-Hexyl-3-methylimidazolium	Tetrafluoroborate	[HMIM][BF_4]	Partly miscible
	Hexafluorophosphate	[HMIM][PF_6]	No
1-(3-Hydroxypropyl)-3-methylimidazolium	Hexafluorophosphate	[HPMIM][PF_6]	
	Glycolate	[HPMIM][$OHCH_2COO$]	
	Chloride	[HPMIM][Cl]	
1,3-Dimethylimidazolium	Methylsulfate	[MMIM][$MeSO_4$]	Yes
3-Methyl-3-nonylimidazolium	Hexafluorophosphate	[MNIM][PF_6]	No
1-Methoxyethyl-3-methylimidazolium	Tetrafluoroborate	[MOEMIM][BF_4]	–
	Hexafluorophosphate	[MOEMIM][PF_6]	
1-Octyl-3-methylimidazolium	Tetrafluoroborate	[OMIM][BF_4]	Partly miscible
	Hexafluorophosphate	[OMIM][PF_6]	No
1-Octyl-3-nonylimidazolium	Hexafluorophosphate	[ONIM][PF_6]	No
Methyltrioctylammonium	Bis[(trifluoromethyl)sulfonyl] imide	[MTOA][Tf_2N]	No

Table 8.1 Nomenclature of commonly used ionic liquids and their miscibility with water. *Continued*

Cation	Anion	Common notation	Water miscibility
Butyltrimethylammonium	Bis[(trifluoromethyl)sulfonyl] imide	[BTMA][Tf_2N]	No
3-Hydroxypropyl-trimethylammonium	Bis[(trifluoromethyl)sulfonyl] imide	[HTMA][Tf_2N]	–
3-Cyanopropyl-trimethylammonium	Bis[(trifluoromethyl)sulfonyl] imide	[CPRTMATf_2N]	–
Butyltrimethylammonium	Bis[(trifluoromethyl)sulfonyl] imide	[BTMA][Tf_2N]	–
5-Cyanopentyl-trimethylammonium	Bis[(trifluoromethyl)sulfonyl] imide	[CPTMA][Tf_2N]	–
Hexyltrimethylammonium	Bis[(trifluoromethyl) sulfonylimide]	[HTMA][Tf_2N]	–

8.3 Ionic liquids can dissolve carbohydrates: properties and descriptions

Carbohydrates are highly polar molecules that are generally soluble in water, strong acids or organic solvents capable of forming hydrogen bonds, such as dimethylsulfoxide, dimethylformamide, pyridine or 2-methylpropanol. With the exception of water, these solvents have many undesirable properties and there is a need of finding new green solvents environmentally benign to dissolve them. A major advance occurred when ILs employed to dissolve carbohydrates were first used to dissolve a water insoluble carbohydrate, cellulose. Cellulose forms a hydrogen-bonded supramolecular structure that makes it insoluble in water and most organic solvents. Graenacher [44] showed that benzylpyridinium chloride or *N*-ethylpyridinium chloride had the ability to dissolve cellulose with the formation of solutions of various viscosities. However, alkyl pyridinium salts usually have high melting points, thus limiting their applications as solvents.

Since then, many reports on dissolution of cellulose in ILs [45,46] have shown that cellulose is more easily dissolved in ILs containing ammonium [47], imidazolium [33] and pyridinium cations [48]. Also, ILs such as 1,3-dialkylimidazolium formates that are halogen free have been used to dissolve cellulose and various polysaccharides, including amylose [49], have been dissolved at high concentrations under mild conditions. It is important to be able to dissolve cellulose since it is a renewable resource and its current industrial processing is not a green process [50].

Other carbohydrate polymers, such as starch (Scheme 8.1), were found to be soluble in ILs [51], such as [BMIM][Cl] and 1-butyl-3-methylimidazolium dicyandiamide [BMIM][DCA] in concentrations up to 10% (w/w), at 80°C [52]. Higher concentrations resulted in solutions with a viscosity too high for stirring.

There are several review articles that discuss different aspects of the use of ILs in carbohydrate chemistry, in particular, dissolution [46,53], and functionalization of simple sugars [54], cyclodextrins [55], cellulose [56], starch [57], and chitin/chitosan [56]. Sheldon et al. [58] have reported the

solubilities of common monosaccharides, disaccharides and polysaccharides in weakly coordinating IL such as 1-butyl-3-methylimidazolium tetrafluoroborate [BMIM][BF_4] and 1-butyl-3-methylimidazolium hexafluorophosphate [BMIM][PF_6] as well as other synthesized ILs such as 1-methoxyethyl-3-methylimidazolium tetrafluoroborate [MOEMIM][BF_4]. For example, D-glucose dissolves at much higher concentrations in [MOEMIM][BF_4] than in *tert*-butyl alcohol. The solubility of glucose is influenced much more by the nature of the anion than that of the cation. ILs containing the [DCA] anion dissolves glucose more than an order of magnitude better than their tetrafluoroborate counterparts. The behavior of the 1-(2-methoxyethyl)-2,3-dimethylimidazolium chloride and hexafluorophosphate salts were compared [59] with the analogous [BMIM] salts to examine the influence of the ether oxygen on salt thermal properties for a typical constituent cation used in the preparation of ILs. MacFarlane et al. [60] have shown that the high solubility of carbohydrates can be attributed to the H-bond acceptor properties of the [DCA] anion [58], which has been recently recognized as a prerequisite for dissolving complex molecules.

Their density and solubility was assayed in five different ILs: 1-ethyl-3-methylimidazolium dicyanamide [EMIM][DCA], [BMIM][DCA], Aliquat dicyanamide, trihexyltetradecylphosphonium dicyanamide [Aliquat][DCA], and 1-ethyl-3-methylimidazolium trifluoroacetate [EMIM][CF_3COO], between 288 and 339K. ILs based on [Cl], [DCA], formate [HCOO], and acetate [OAc] anions [61] could dissolve up to 10%−20% (wt.) cellulose, and >100 g/L other carbohydrates such as D-glucose, sucrose, lactose, and cyclodextrin.

Also, sugar alcohols, such as xylitol and sorbitol [62], represent a class of compounds that could play an important role in biorefining. More recently, ILs have been used in dissolving and processing biomass [60]. For example, [BMIM][Cl] could dissolve considerable amounts of cellulosic materials and lignin from different wood samples over 24 h at 100°C. The solubilization of carbohydrates in ILs has enabled numerous chemical derivatizations of these natural products in homogeneous systems, as well as the cellulose regeneration for a variety of applications (such as enzymatic hydrolysis, blending with wool keratin, and producing enzyme-encapsulated films). Unfortunately, since carbohydrate-dissolving ILs are typically composed of [Cl], [DCA], [HCOO] and [OAc] anions, these anions form strong hydrogen bonds with carbohydrates for dissolving them. For this reason, these ILs are more likely to denature enzymes [63] preventing a further enzymatic modification of dissolved carbohydrates in ILs. Zhao et al. [64] reported newly designed ILs that are capable of dissolving carbohydrates without considerably inactivating enzymes. Since the high molar concentration of anions in ILs is responsible for the enzyme denaturation, perhaps that a lower anion concentration could reduce the enzyme-inactivating nature of ILs. Therefore, a longer substituent on the cation would increase the molecular weight and thus decrease the anion concentration of an IL. However, a longer alkyl chain on the cation dramatically increases the melting point and viscosity of the resulting IL. In contrast, glycols and their derivatives are known having low melting points and low viscosities. Therefore, poly(ethylene oxide)s could be incorporated into cationic or anionic units to produce the liquid state of ion conductive polymers. In particular, various ILs have been synthesized by grafting alkyloxy substituents (ether or alcohol groups) onto the imidazolium ring or pyridinium ring. The inclusion of alkyloxy or alkyloxyalkyl groups can lower the melting points of the resulting organic salts, yielding room temperature ILs in most cases. In addition, the oxygen atoms embedded in the glycol chain may act as hydrogen-bond acceptors, interacting with carbohydrates to dissolve them. Following this rationale, a series of imidazolium

and tetraalkylammonium ILs carrying glycol-substituents in the side chain, and then the solubilities of sugars and cellulose in these new solvents were determined.

Carbohydrates are among the most abundant, low-cost natural sources of chiral materials. These considerations prompted the design synthesis and characterization of carbohydrate-based ILs as new chiral solvents [65]. Owing to the presence of many hydroxyl groups, these carbohydrate-based ILs have high coordination ability that can be tuned by varying the electronic density of their oxygen atoms through a proper protecting group pattern. Therefore, carbohydrate-based ILs could be used as coordinating solvents in stereoselective and/or metal-catalyzed reactions. Also, most carbohydrate-based ILs can be obtained from renewable sources [66,67].

Water is one of the major impurities of IL and has a strong impact on the solubility of carbohydrates in ILs because it modifies the solvation ability of ILs [68]. Therefore, it is very important to report that the real ability of IL to dissolve carbohydrates as water efficiently masks the solubility behavior and is one of the major hindrances in the dissolution of carbohydrates in ILs. The interactions of 1-butyl-3-methylimidazolium carboxylate ILs ([BMIM][HCOO], [BMIM][AcO] and [BMIM][EtO]) with glucose in water were studied using their volumetric properties, viscosity, conductivity as well as NMR spectroscopy [61]. Volumetric interaction parameters were also obtained from the transfer volumes of the ILs. The contributions of the solvent properties and the IL-solvent interactions were extracted, together with molar activation energies (of the ILs for viscous flow of the aqueous glucose plus IL solution). In addition, the ^{13}C and ^{1}H NMR spectra of methyl β-D-glucopyranoside and α-D-glucopyranoside with ILs and D_2O were studied. The NMR results show that no special interactions were observed between glucopyranosides and ILs.

A water-stable IL [69], 1-butyl-3-methylimidazolium trifluoromethanesulfonate, [BMIM][CF_3SO_3], has been used in aqueous biphasic systems with a large range of monosaccharides and disaccharides, and polyols. Binodal curves, tie-lines, densities and viscosities of the coexisting aqueous phases, were determined for each ternary system. The proposed systems are low-viscosity, offering enhanced features over conventional polymer-based aqueous biphasic systems. In addition, the partitioning of model biomolecules, such as L-tryptophan, caffeine, and β-carotene, was also investigated to examine the applicability of such aqueous biphasic systems. These systems are particularly interesting in the recovery of bioactive products from natural sources, while the availability of carbon-based compounds to cells constitutes a major advantage in separations from fermentative media. Moreover, the use of carbohydrates in ionic-liquid-based aqueous biphasic systems constitutes a step forward along the biorefinery concept envisaging sustainable conversions of biomass into a broad spectrum of bio-based products.

A new generation of IL structures based on the conjugation of the organic superbase 1,1,3,3-tetramethylguanidine with carboxylic acids such as formic, acetic, and propionic acids has been reported [70]. This method produces ILs that both rapidly dissolve cellulose to high concentration and are recyclable by distillation with recoveries and purities over 99%.

More recently, Ru and König [71] have proposed alternatives to ILs (sugar melts, deep eutectic solvents) as their impact on the environment is still under debate. The components of a green solvent should exhibit low acute toxicity and be rapidly degraded in the environment. The current consensus is that ILs cannot be generalized as either green or toxic because their environmental impact is strongly dependent on the species of cations and anions used within the IL.

8.4 Ionic liquids in carbohydrate synthesis

Current carbohydrate synthesis under green conditions reported in literature include no solvent conditions, where one of the reagents can dissolve the other substrates in a nonsolvent system, which is considered greener than the classical reactions performed in organic solvents [72–74], or in the absence of liquid medium by using mechanochemical methods [75–78], or the use of microwave-assisted reactions [79–84]. The use of green solvents and cosolvents as reaction media for carbohydrate synthesis and modification (chemical or enzymatic) can involve ILs [28,46,85–88].

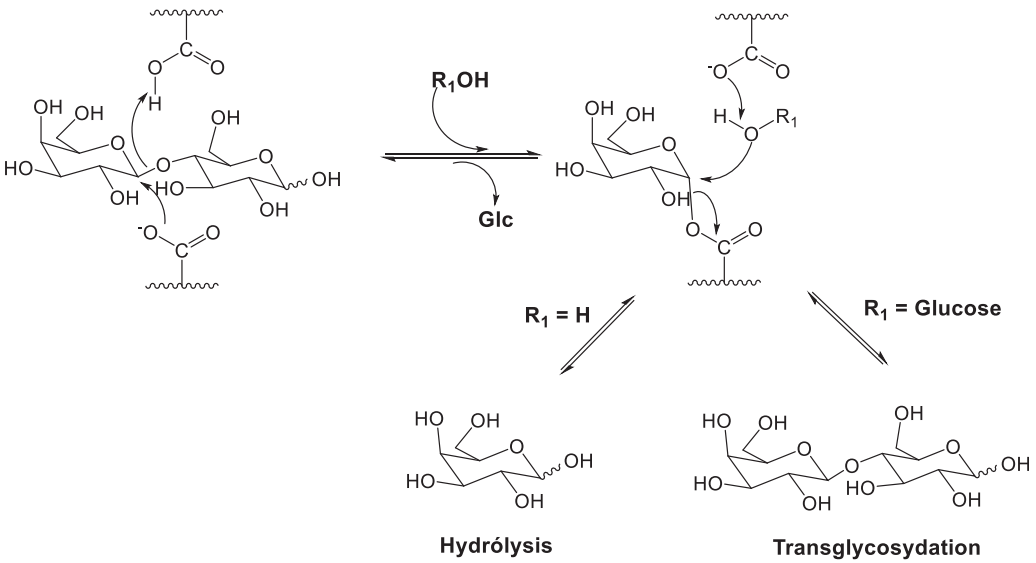
When carbohydrate chemical synthesis and modification requires specific solvents for the desired reaction, particularly ILs are mainly used [46,53,89–92].

Current carbohydrate chemical modifications applied in the chemical industry and described on literature have shown three major areas of use: (1) polysaccharide based materials as substrates; (2) acetylation/de-acetylation of saccharides for building blocks purposes; and (3) IL-catalyzed reactions of carbohydrates.

For the enzymatic synthesis of carbohydrates the main type of green solvents are ILs [10,93–96], biosolvents [28,97], or the absence of solvent. ILs are used, in part, due to their polarity and their multiple possibilities (of cations and anions) for the design of optimal reaction media, allowing the preparation of enzyme stabilizing solutions and solubilizing sugar mixtures [98–100]. Mainly two classes of biocatalysts are for the enzymatic synthesis of glycosides with these solvents; the lipases and the glycosidases.

Lipases (E.C. 3.1.1.3), among the large and diverse group of hydrolases, are probably one of the enzyme families more widely used in the chemical, food and pharmaceutical industries. One of the most important applications of lipases in the carbohydrate field is the synthesis of sugar esters [101,102]. There is an increasing interest in the development of sustainable and nontoxic routes for obtaining these amphipathic molecules, mainly glycolipids, as they are used as nonionic surfactants in food, beverage, cosmetic and drug industries. In this context, lipases are a promising alternative to traditional chemical esterification reactions due to their high regioselectivity, which avoids protection and deprotection steps, and their ability to operate under mild reaction conditions. In a similar way, polysaccharide modification trough lipase-catalyzed acylation/esterification reactions allow the improvement of properties of compounds such as starch for its further industrial use [103,104]. On the other hand, lipase catalyzed regioselective hydrolysis or oligosaccharide peracetylation have also been described as very efficient procedures for the preparation of intermediates in the synthesis of glycoderivatives of biological interest [105].

Other important groups of enzymes useful in carbohydrate synthesis are glycosidases. Glycosidases (E.C. 3.2.1) have been extensively employed in carbohydrates functionalization [106–108]. In general, they are robust and commercially accessible enzymes, tolerant to organic solvents, with a broad substrate range and usually display high stereospecificity. The hydrolysis mechanism starts with the binding of the sugar substrate (donor) to the glycosidase, forming a glycosyl-enzyme complex followed by the hydrolysis of the intermediate to give products. Capturing the glycosyl intermediate with an acceptor alcohol or saccharide can lead to oligosaccharide synthesis, which greatly increases the synthetic applications of these enzymes (Scheme 8.3). Although glycosidases are stereospecific to the hydrolysis (or synthesis) of α or β linkages, they



SCHEME 8.3

Mechanism of glycosidases oriented toward hydrolysis or transglycosydation depending on the nucleophilic group that accesses the active center.

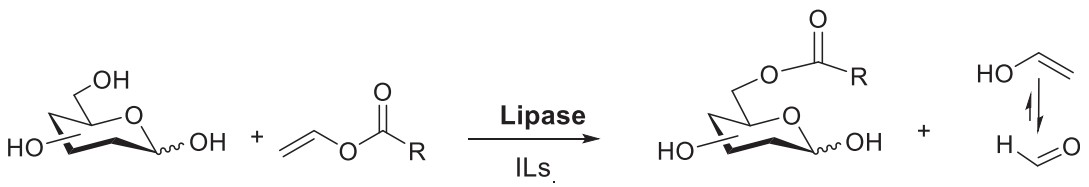
are not always regiospecific. However, engineered glycosidases allow the improvements of selectivity and yields and increase its potential use in the carbohydrate field [106,109].

8.5 Enzymatic processes developed in ionic liquids for carbohydrate synthesis: lipases and glycosidases

8.5.1 Lipases in carbohydrate synthesis

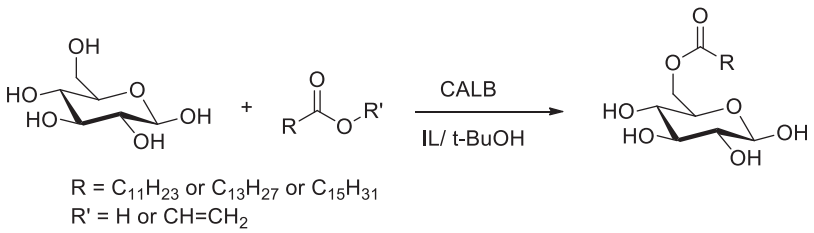
As it was mentioned above, llipase generally catalyzed acylation of sugars by using vinyl esters, which promotes the irreversible reaction toward the acylation of the saccharide (Scheme 8.4) [110–119]. As products of this enzymatic reaction sugar fatty esters are obtained, and these are nonionic surfactants with potential application in several industrial fields [120].

However, the main drawback of sugar esterification processes is the low solubility of carbohydrates in organic solvents. Besides the environmental benefits of ILs, these solvents have allowed a considerable increase of sugar substrate concentration [121]. In addition, these ILs medium have also contributed to enhance lipase activity and regioselectivity [10,122,123]. Park and Kazlauskas carried out the regioselective 6-*O*-acetylation of glucose catalyzed by an immobilized form of *Candida antarctica* lipase (CALB) in different ILs, achieving moderate to high yields [124]. The increase of substrate solubility in the IL also influenced the regioselectivity of the process, avoiding the formation of diacetylated products a by-product in conventional organic media. Another



SCHEME 8.4

Lipase-catalyzed acylation of saccharides.



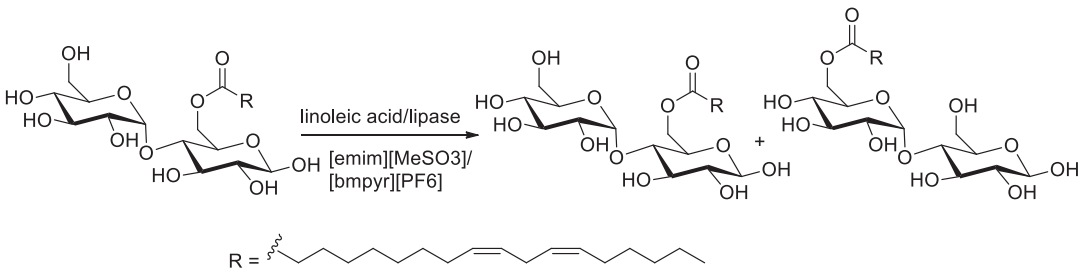
SCHEME 8.5

Regioselective 6-*O*-acylation of glucose catalyzed by CALB in an IL-*t*BuOH media [111,112].

interesting example was reported by Ganske and Bornscheuer, who developed an improved system composed of IL and 40% *t*-BuOH, using fatty acid vinyl esters as acyl donors and commercial CALB, reaching conversions up to 90% and excellent regioselectivity (Scheme 8.5) [111,112].

A few years later, Koo et al. investigated the synthesis of 6-*O*-lauryl-D-glucose through the regioselective acylation of glucose catalyzed by immobilized CALB (Novzyme 435) in ILs mixtures [113]. Subsequently this group developed a new strategy based on the preparation of supersaturated glucose solution in ILs [114]. The sugar was first dissolved in an aqueous solution and transferred into an IL. Then, water was removed, leading to supersaturated ILs which was employed as an excellent media for the CALB catalyzed esterification of glucose using either vinyl laurate or lauric acid as acyl donor. This innovative approach was further optimized, using the supersaturated sugar system in a mixture of ILs. Best conditions permitted the reuse of CALB and ILs, maintaining 78% of initial activity after five cycles [125,126]. As an alternative, ultrasound have also been successfully employed to overcome the mass transfer limit in viscous ILs, accelerating the lipase catalyzed sugar acylation in supersaturated ILs [127].

Not only glucose but also other monosaccharides have been employed as substrates of immobilized CALB catalyzed esterification in the presence of ILs. Other compounds of biological interest such as mannosyl esters can be obtained through this methodology. Galonde et al. developed a procedure to obtain mannosyl myristate by CALB catalyzed transesterification from vinyl myristate in [Bmpyrr][TFO], leading to high yields and regioselectivity [128,129]. Ha et al. reported a very good example of ultrasound irradiation on lipase-catalyzed esterification of fructose with palmitic acid in ILs. They prepared a supersaturated fructose solution of [BMIN][TfO]/[OMIN][Tf_2N] (1:1,



SCHEME 8.6

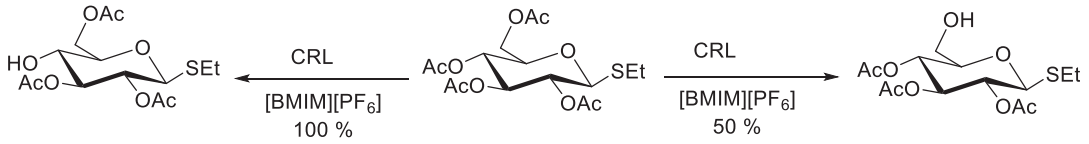
Lipase-catalyzed esterification of 6- and 6′-hydroxyl groups of maltose in a binary ionic liquid system [131].

v/v) and subjected this IL solution of fructose to the lipase-catalyzed transesterification under ultrasound irradiation. This strategy enables a highly enzyme activity and stability in the synthesis of fructose palmitate. After five times reuse of Novozym 435 and ILs mixture retained 84.4% of initial enzyme activity under ultrasound irradiation. These results show that enzymatic reaction in viscous ILs mixture under ultrasound irradiation is an effective method for enzyme activity, as well as, enzyme stability resulting in economic competitiveness of green process [130].

Esterification of longer carbohydrates is a more complicated process than the corresponding acylation of monosaccharides. In this context, binary IL reaction media were used in the esterification of 6- and 6′ hydroxyl groups of maltose with linoleic acid catalyzed by *Pseudomonas cepacia* and CALB (Scheme 8.6) [131]. Lu et al. reported a binary system also for the synthesis of starch palmitate, adding a lipase to the mixture [BMIM][BF_4]/[BMIM]Ac as a green alternative to harmful organic solvents commonly employed in the preparation of starch esters [132].

Adak and Banerjee esterified corn starch with oleic acid but in an environment of little-known ILs [133]. Lipase from *Rhizopus oryzae* was the catalyst, and microwave radiation was used as the heating source. Newly synthesized imidazolium ILs such as [C_{16}MIM][Br], [C_{16}−3-C_{16} IM][Br_2], and [C_{16}−$_{12}$-C_{16}IM][Br_2], which exhibited surfactant properties, were used in this reaction. The significant increase in the efficiency of biocatalyzed starch esterification and achievement of a high degree of substitution was explained not only by the use of nontoxic ILs but also by more efficient microwave heating. Thus, the authors presented a highly efficient and environmentally friendly method of obtaining hydrophobic and thermoplastic starch derivatives in the presence of ILs.

In 2019, Zarski et al. again used immobilized lipase from *Thermomyces lanuginosus* and [BMIM][Cl] to esterify potato starch [134]. This time, hydrolysates of high-oleic vegetable oils (pure and waste rapeseed oil) were used as esterifying agents. However, the reactions were not carried out directly on potato starch gelatinized in IL but on dried gel. Hydrophobic starch derivatives with a much higher degree of substitution were obtained under the most optimal conditions, regardless of the oil hydrolysate. The significant increase in the degree of esterification compared to previously developed methods [135] was explained by conducting the reaction in a three-component system, that is, which consisted of hydrophilic IL, nonionic surfactant, and hydrophobic fatty acids. The surfactant used polyoxyethylene sorbitan monooleate, which was of strategic importance in increasing the biocatalysis efficiency in the IL. The addition of the small amounts to the reaction



SCHEME 8.7

Influence of [BMIM][PF_6] on the selectivity of the CRL catalyzed hydrolysis of a penracetylated thioglucopyranoside [136].

system prevented chloride recrystallization below 60°C and thus enabled the syntheses to be performed at more optimal temperatures for lipase, that is, 40°C−50°C. P80, due to its amphiphilic nature, increased the contact between the hydrophobic and hydrophilic phases.

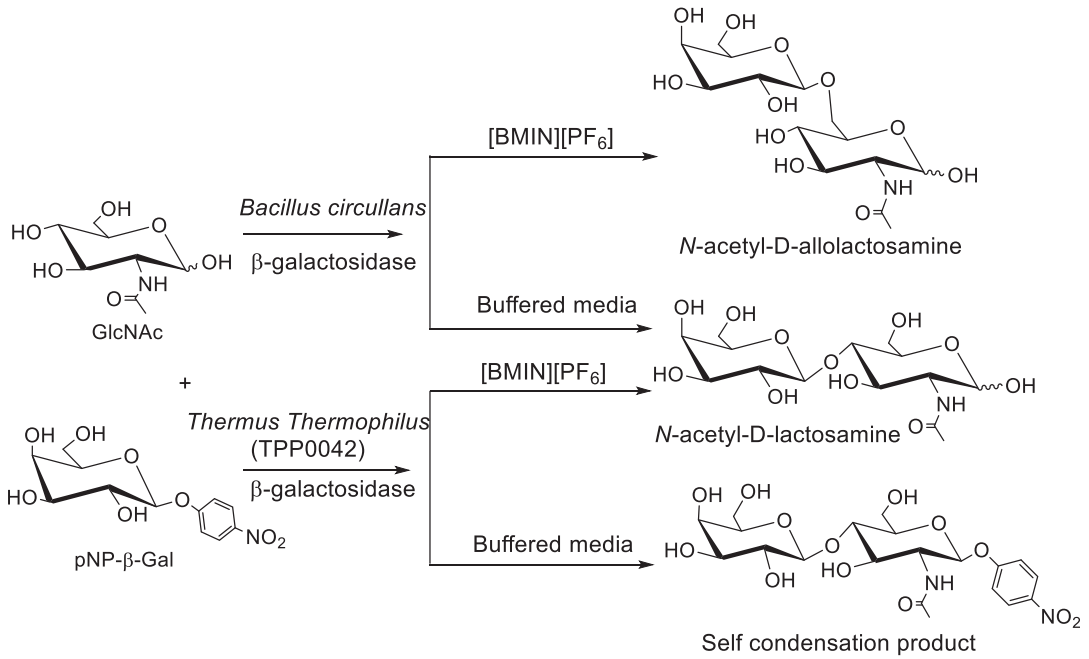
It also limited the access of [BMIM][Cl] to the active center of the enzyme and, consequently, led to its inactivation. Moreover, a new and efficient method of potato starch gelatinization in imidazolium IL was presented in the mentioned studies. Until now, the maximum concentration of starch in gel with [BMIM][Cl] ranged from 5 to 10%. It was proven by using SEM and X-ray methods that a reduced pressure and temperature of 60°C for 30 mins allows for successfully obtaining up to 30% starch gel [134]. Potato starch esters obtained in ILs by Zarski et al. have been structurally, physico-chemically, and functionally characterized. Films extruded from them exhibited increased hydrophobicity and improved mechanical properties while retaining biodegradability and nonphytotoxicity.

Deacylating reactions have also been investigated in ILs. Gervaise et al. studied the influence on the regioselectivity of the hydrolysis of a commercial percetylated thioglucopyranoside catalyzed by *Candida cylindracea* lipase [136]. [BMIM][PF_6] considerably improved substrate solubility and the ratio IL/buffer was determinant for the selectivity of the process, leading to either ethyl 2,3,4-tri-*O*-acetyl-1-thio-β-D-glucopyranoside or ethyl 2,3,6-tri-*O*-acetyl-1-thio-β-D-glucopyranoside by the addition of 50% or 100% of the IL respectively (Scheme 8.7). The same IL turned out to be a useful reaction medium too for the *P. cepacia* lipase catalyzed hydrolysis and alcoholysis of 3,4,6-tri-*O*-acetyl-D-glucal [137].

8.5.2 Glycosidases in carbohydrate synthesis

Chemical synthesis of oligosaccharides is still an open challenge. In this context, glycosidases appear as a very useful tool, as they can be used not only in hydrolysis, but also in glycosidic bond forming reactions under conditions in which a carbohydrate hydroxyl group acts as a more efficient nucleophile than water. The introduction of high concentrations of donor or the addition of certain solvents can shift the reaction equilibrium toward the synthesis of a specific saccharide (Scheme 8.3). In addition, using genetic engineering to create modified enzymes from microorganisms, higher yields can be attained [109,138].

These reactions have been conducted in green solvents, especially ILs, which present different advantages, not only from an environmental point of view, but also because of their effect over the enzyme performance, varying enzyme activity and selectivity [96,118,139].



SCHEME 8.8

ILs Effects in transglycosylation reaction catalyzed by *B. circulans* and *Thermus thermophiles* β-galactosidases.

ILs are a wide range of compounds that have been proven to be suitable as cosolvents in glycosidases reactions [118]. The use of ILs in the synthesis of carbohydrates was reported by Kaftzik et al., in the early 2000s [140]. This group carried out the synthesis *N*-acetyllactosamine [Gal-β(1→ 4)-GlcNAc] by a transglycosylation reaction catalyzed by a β-galactosidase from *Bacillus circulans*. The desired product was also a substrate for the enzyme and therefore the yield was limited to 30% if the reaction was performed in water. Researchers demonstrated that the addition of 25% v/v of [MMIM][$MeSO_4$] as a water miscible IL could suppress the secondary hydrolysis of the product and enhance the yield to 60%.

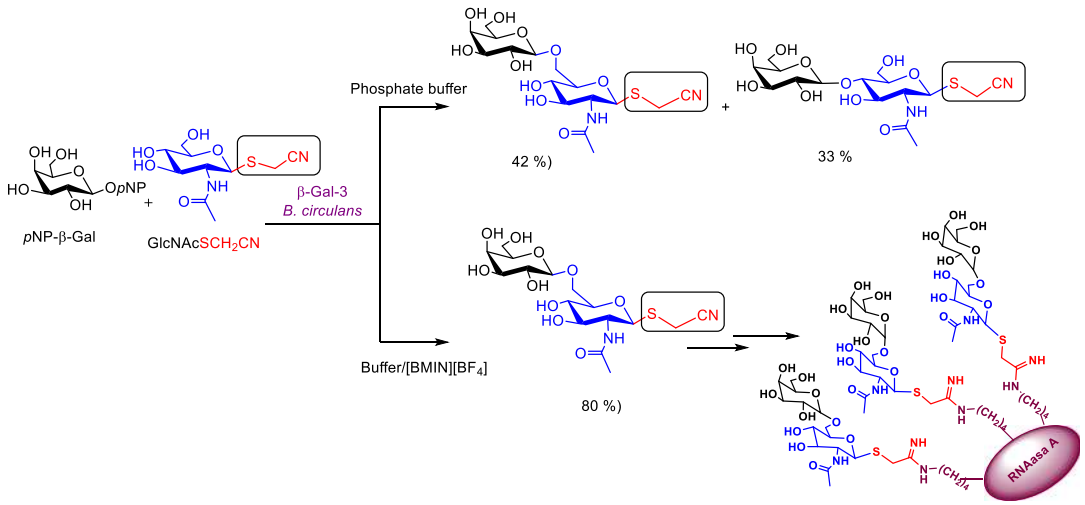
Some other examples can be found in the literature, describing excellent yields obtained with IL as cosolvents in reactions involving *N*-acetyl-D-glucosamine (GlcNAc) as substrate. The thermophilic biocatalyst β-Galactosidase from *Thermus thermophilus* TPP0042 promotes the synthesis of *N*-acetyl-D-lactosamine when the fluorinated IL [BMIM][PF_6] was employed. However, these aqueous conditions lead to high amounts of self-condensation product derived from the donor [Gal-β(1→3)-Gal-β-pNP].

The presence of IL as cosolvents affected the reaction by improving the regioselectivity of the enzyme, reducing self-condensation reactions, and increasing the synthesis of disaccharide (Scheme 8.8). ILs have an effect in the synthesis of disaccharides, as the solvent appears to modify the tertiary and secondary structures of the enzyme. Through deep molecular interactions and molecular modeling studies, authors concluded that the enzyme became more flexible in the presence of the mixture water-IL, this allowed the stabilization of the GlcNAc molecule in the active center [141].

Analogous results have been found with *B. circulans* ATCC 31382 β-galactosidase. This enzyme recognizes GlcNAc and GalNAc as acceptors and is used to synthesize Gal-β-(1→3)-GalNAc and Gal-β-(1→3)-GlcNAc with high regioselectivity but low yield [142]. The synthesis of Gal-β-(1→3)-GalNAc and Gal-β-(1→3)-GclNAc catalyzed by β-galactosidase of *B. circulans* ATCC 31382 was investigated using different ILs as cosolvents, leading to yields up to 97% with an exceptional regioselectivity and nearly-zero hydrolytic activity (Scheme 8.8). In addition, the Il was recycled and the reaction was scaled up to 80 mL and under these conditions yields go up to 85% [143].

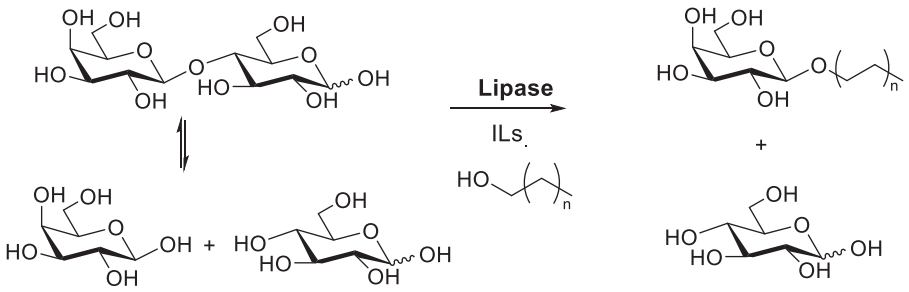
Furthermore immobilization of β-Gal-3 from *B. circulans* ATCC 31382 on glycosyl-agarose support [144] provided an effective and sustainable approach to carry out the enzymatic synthesis of these disaccharides, allowing biocatalyst and IL recycling. The derivative that showed more activity and stability were the ones bound to a Lys rich region on a monofunctional glyoxyl-agarose support. This immobilized enzyme was tested on the synthesis of β-(1→3) galactosyldisaccharides using the [BMIN][PF_6] as cosolvent and leading to yields up to 90% and maintained regioselectivity. Gomez et al. also analyzed the reusability of the immobilized enzyme under identical reaction conditions and finds that immobilized enzyme retains about 90% of its activity after six batches with conversion yields above 75% when [BMIN][PF_6] was used as reaction media. Furthermore, green solvent recovery and recycling were achieved retaining catalytic activity and increased productivity.

Based on these results, these authors have investigated the use of galactosidases in ILs for the synthesis of disaccharides containing the thiocyanomethyl group on the anomeric position, which were further employed in the preparation of *neo*glycoproteins [145]. β-Gal-3 from *B. circulans* and β-galactosidase from *Escherichia coli* recognized *N*-acetylglucosamine (GlcNAc) functionalized with the thiocyanomethyl group at C1 as acceptor and activated-galactose derivative as donor (Scheme 8.9). In the first case, the presence of this activation group induced a change in the



SCHEME 8.9

Regioselective synthesis of activated disaccharide mediated by β-Gal-3 in the presence of [BMIN][BF_4] and further preparation of well-defined *neo*glycoproteins.



SCHEME 8.10

β-Galactosidase (TN1577) catalyzed synthesis of alkyl galactopyranoside.

substrate recognition pattern of β-Gal-3, affording a mixture β(1→6) and β(1→4) glycosidic linkages instead of expected β(1→3). Particularly in this work, the presence of the Ils, specially [BMIN][BF_4], completely shifted the equilibrium toward the synthesis of β(1→6) with high regioselectivity. Then the activated disaccharide was successfully employed in the preparation of well-defined *neo*glycoprotein. As an additional advantage of this sustainable process, the biphasic medium created under these conditions allowed the recycling and reuse of IL.

ILs also have been used for the synthesis of alkyl galactopyranosides using thermophilic β-galactosidase from *Thermotoga naphthophila* RKU-10 (TN1577) as biocatalyst, milk processing waste lactose as galactosyl donor and an amphiphilic tetraammonium-based IL containing tetraammonium cation with C18 acyl and oligoethylene glycol as solvent. This IL proved to be a useful reaction medium for the synthesis of octyl galactopyranoside compared to the buffer system (Scheme 8.10). The general applicability of this system was verified with the synthesis of a series of alkyl galactopyranosides with high yield and regioselectivity [146]. This work developed an efficient approach for enzymatic production of alkyl galactopyranosides by applying an amphiphilic IL mediated reaction system, which offers technical advantages in improving substrate solubility, suppressing hydrolysis, enhancing enzyme working temperature ranges (up to 95°C), reducing reaction time and increasing the yield of the reaction.

8.6 Conclusions

There is currently an increasing demand to provide green solutions that may replace, in a short future, petroleum-based processes. The development of eco-friendly solvents represents an important field of research, as solvents account for a significant part of the pollution that chemical and pharmaceutical processes bring about. Carbohydrate-based compounds are also widely used in the pharmaceutical, cosmetic, detergent, and food industries. While these compounds are mainly produced by chemical methods, the use of enzymatic methods has been investigated over the past 20 years as a greener alternative to organic synthesis. Owing to the low solubility of enzymes and carbohydrates in traditional organic solvents, research has been focused on the chemical and enzymatic synthesis of carbohydrates in polar green solvents such as ILs. This chapter is mainly focused on the use of these ILs in the enzymatic

synthesis of carbohydrates and glycoconjugates. Two classes of biocatalysts (lipases and glycosidases) are discussed. Overall, some interesting proof of concepts have been reported; however, there is still promising room for research and innovation in the area. By combining powerful biocatalytic reactions, applied to carbohydrate chemistry, with the introduction of ILs, many synergies can be found. Remarkably, many ILs provide interactions with enzymes as well, thus leading to other effects that exceed their role as mere solvents. On the basis of the future needs for sustainable chemistry, together with the potential applications and strengths that these strategies may bring, it is expected that more examples of novel applications in carbohydrate chemistry will appear in the coming years with a positive impact on circular bioeconomy.

Acknowledgments

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CHAPTER

Recent progress in ionic liquid-assisted processing and extraction of biopolymers

9

Hamayoun Mahmood[1], Ahmad Shakeel[1,2], Sikander Rafique[1] and Muhammad Moniruzzaman[3,4]

[1]Department of Chemical, Polymer & Composite Materials Engineering, University of Engineering and Technology (UET), Lahore, Pakistan [2]Faculty of Civil Engineering and Geosciences, Department of Hydraulic Engineering, Delft University of Technology, Delft, The Netherlands [3]Department of Chemical Engineering, Universiti Teknologi PETRONAS, Perak, Malaysia [4]Centre of Research in Ionic Liquids (CORIL), Universiti Teknologi PETRONAS, Perak, Malaysia

9.1 Introduction

The polymer science and engineering have recently applauded its 100th anniversary. Plastics have been an indispensable part to the many advanced societal progressions of the 20th century, resulting in great improvement regarding the quality of human life. The exciting material characteristics of a broad range of plastics (a synonym for polymers) and its phenomenal durability have opened many new opportunities in numerous fields including drug delivery, agricultural fields for enhanced crop production, cosmetics, lightweight packaging, detergents, and more other advanced applications [1–3].

Yet, the current scenario of thermoset and thermoplastic polymers is predominantly unsustainable, with environmental impacts continuously expanding with an increase in industrial production capacity and societal needs. As the severe impacts of plastic pollution on air, soils, water, and biota have become obvious, public demand has provoked policymakers to conceive a range of mitigation plans. Nevertheless, the scale of petro-based plastic pollution is likely to boost over the coming years, actuated by the forecasted increase in human population and global plastic use, particularly the recent influx in single-use plastics during the prevalent COVID-19 pandemic [4,5]. Around 359 million metric tons of plastic was produced in 2018 whereas 385 million metric tons was utilized in the same year. Nevertheless, it has reported that of all post-consumer plastic waste that has been generated only about 9% has been recycled while 81% was dumped in landfills or exists in the environment. Undoubtedly, polymer waste disposal is an immense ecotechnological issue, and one of the strategies to cope with this problem is the evolvement of biodegradable polymers [6,7].

The technology of biodegradable polymers has turned up as a subject of extensive scientific and industrial attraction owing to their versatility, environmentally benign compostability, and superior biological properties. Biodegradable polymers are derived partly or entirely from naturally derived matrices, which are abundantly available and are majorly comprised of polysaccharides, fibers, and proteins. Biopolymers are believed to diminish environmental impacts in particular applications and immense exertion is being undertaken to synthesize drug/gene delivery systems from biodegradable polymers.

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Furthermore, biopolymers either inherently or by modification, can confer antibacterial activity and excellent mechanical and resin properties, making them an ideal candidate for biomedical practices [8,9].

Although biopolymers offer a cheap, diverse, abundant, and renewable resources, their extremely low solubility in ordinary solvents render a critical challenge to fabricate materials from biopolymers or furnish their chemical derivatization. This is particularly confronting for polysaccharides, which possess a complex 3D structure fusing amorphous and crystalline parts with strong hydrogen bonding network among the molecular chains. Only a few polysaccharides can be dissolved in exclusive polar solvents such as pyridine, dimethylformamide, formamide, and dimethylsulfoxide (DMSO) [10,11]. For example, starch owing to its supramolecular structure possess very low solubility in virtually any solvent except DMSO. However, these classic solvents are either toxic, flammable or corrosive, or exhibit cumbersome solvent recovery process provoking safety issues to the environment and humans as well. Conversely, the complete dissolution of biomolecules often requires a multistep pretreatment process under extensive mechanical stirring consuming huge amount of energy. In addition, the traditional acid or alkaline methods pose environmental threats, while enzyme-based techniques are infeasible due to high cost and prolonged processing time. These confinements have triggered a quest to find promising and novel green solvents for biopolymer extraction, processing and derivatization [12,13].

Recently, ionic liquids (ILs) have shown immense potential to cope with the limitations mentioned above. ILs are classified as a group of salts comprised of bulky, unsymmetrical ions containing delocalized charge which renders them liquid state at comparatively low temperatures, that is, $<100^{\circ}C$. Specifically, ILs with their melting point below room temperature are termed as room-temperature ILs (RTILs) [14,15]. In contrast to the ordinary organic solvents, most ILs are recyclable, nonvolatile, nonexplosive, and thermally stable and thus can be highly promising and environmentally benign solvents. The ILs mostly employed for biopolymer isolation and processing encompass salts of organic cations including pyridinium, imidazolium, phosphonium or ammonium and their derivatives comprising manifold lengths of side alkyl groups. Conversely, lactate and formate are typical examples of organic anions while inorganic anions include tetrafluoroborate, hexafluorophosphate, chloride and bromide [16].

The use of ILs confronts the broad utilization of biopolymer matrices by coupling at least two important green chemistry principles viz: valorization of renewable feedstock, that is, biopolymers via environmentally benign processing route (ILs). The main objective of this chapter is to sketch a concise prospect of the present state-of-the-art on the use of ILs as processing media and as an enabling tool to afford the potential of more sustainable substitute for petroleum-based plastics for various end-of-use products.

9.2 Ionic liquid-assisted dissolution and processing of biopolymers

9.2.1 Chitin and chitosan

Chitin is the second most abundant biopolymer on Earth after cellulose and is recoverable from numerous resources, principally from crustacean shell waste. Other renewable raw materials rich in chitin encompass fungi, squid pens and fly larvae. About 1.5×10^5 tons per annum has been estimated as the total accessible chitin from different sources. Structurally, chitin biopolymer is a linear

amino polysaccharide composed of β-1,4-linked 2-(acetylamino)-2-deoxy-D-glucose units. Conversely, chitosan as the most important chitin derivative has been recognized as a valuable biomaterial and a substantial alternative to existent commercial materials. The biomedical potential of chitin and chitosan is immense due to their biocompatibility, nontoxicity, suitability for burn and wound healing and are utilized in the preparation of tissue engineering scaffolds and stem cell techniques. Owing to the excellent applications of chitin and chitosan in the biomedical field Global Chitin Market surpassed the valued of 803 million dollars in 2016, with a forecast to attain 2941 million dollar by 2027. The use of ILs not only afford an efficient access to these exciting biopolymers, but ultimately furnish the product platform on the basis of this renewable resource, a significant advancement toward sustainability [17,18].

Seafood represents a major source of animal protein in the world. About 3.4 million tons of shrimp have been caught every year as reported by the Food and Agriculture Organization with Asia being the most notable region for shrimp fishing. Ammonium-based ILs namely isopropylethylammonium propanoate, diisopropylethylammonium acetate and dimethylbutylammonium acetate were reported for the isolation and characterization of chitin from shrimp shells [19]. The extracted chitin exhibited moderate molecular weight with yield (weight of extracted chitin/weight of raw material) as high as 13.4% under 110°C for 24 h operating conditions. The extracted chitin was further transformed into chitosan with 93% degree of deacetylation. The ILs showed remarkable performance in the extraction of chitin from shrimp shells and could be promising green solvent with significant selectivity.

In an interesting study, the possibility of adding thiourea to imidazolium ILs to boost the dissolution ability of solvent to chitin was investigated [20]. The chitin was dissolved in the mixture solvent under room temperature for 24 h followed by heating at 100°C for 24 h with vigorous stirring. The results indicated that both the counter anions of the ILs and substituents on the imidazolium rings steadily affected the dissolution power of the solvent mixture. It was anticipated that the developed solvent could employed as a benign media for functionalization and derivatization of chitin in the future.

The IL [Amim][Br] (1-allyl-3-methylimidazolium bromide) was successfully used for the facile acylation of chitin to synthesize chitin acylates exhibiting high DS values. First, lauroylation of α-chitin was conducted and operating conditions were optimized. Chitin laurate with appreciably high DS value was synthesized using lauroyl chloride in the presence of pyridine in [Amim][Br] with heating at 100°C for 24 h. In addition, chitin acylates with various substituents were also prepared by acylation of α-chitin utilizing different acyl chlorides under the similar reaction conditions [21].

In the chitin biorefinery context, enzymatic access is restrained due to complex 3D structure of chitin, as in the case of cellulose-a highly recalcitrant polysaccharide. In this regard, proficient conversion of chitin into N-acetylglucosamine and N,N′-diacetylchitobiose was successfully achieved employing room temperature ILs [22]. The RTIL was used as a pretreatment solvent under mild conditions prior to enzymatic hydrolysis producing N,N′-diacetylchitobiose (667.60 ± 20.71 mg/g of chitin) or N-acetylglucosamine (185.0 ± 4.0 mg/g of chitin). RTIL were proved to induce structural changes in chitin polymer furnishing an efficient one pot process while significantly minimizing quantity of IL used.

Corresponding to lignocellulose-based refinery, shell biorefinery has been proposed suggesting the remarkable potential of chitin for the production of chemicals [23]. Levulinic acid (LA) has been extensively recognized as one of the top twelve biomass-based platform chemicals serving as a versatile

building block for the sustainable preparation of fuel additives, solvents, herbicides, flavor substances, pharmaceutical ingredients, resins and polymeric matrices. Recently, ILs were demonstrated as affective catalysts to convert chitin into LA up to 67%. The impact of IL structure on chitin conversion process was also elaborated. It was found that both hydrogen bonding capability and acidity of ILs exhibited significant effects on the yield of LA. Furthermore, two pathways viz deacetylation−depolymerization to glucosamine and direct depolymerization to N-acetylglucosamine were suggested based on results of NMR, IR, SEM and degree of acetylation analysis, as possible chitin hydrolysis mechanisms [24].

The use of IL to extract chitin from shrimp shell and subsequent coagulation in water endures the study of unique chitin particles which were dissolved and regenerated, but not dried inducing an open hydrated gel-like structure. A comparison of enzymatic hydrolysis of this chitin hydrogel with dried shrimp shell, chitin extracted from shrimp shells by IL and then dried was conducted using chitinase of *Streptomyces griseus* [25]. The enzymatic hydrolysis of shrimp shells produced only the monomer (N-acetylglucosamine), whereas significantly higher amounts of the dimer N,N′-diacetylchitobiose were noted compared to the monomer when all forms of pure chitin were used. Surprisingly, traces of the trimer N, N′,N′′-triacetylchitotriose were also observed when the IL-chitin hydrogel was employed as substrate.

A factor that impedes the processing of biopolymers like chitin is the highly viscous solution when solid loading of chitin is increased in solvent IL. Kasprzak et al. developed a novel procedure to fabricate homogeneous chitin films in ILs assisted by DMSO as a cosolvent [26]. DMSO was added to the chitin-IL solution prior to biopolymer regeneration to reduce viscosity. The use of cosolvent not only allows the facile preparation of chitin compared to the procedure using neat IL but also reduces the quantity of an IL required during biopolymer dissolution. No considerable difference was noted in the properties of chitin regenerated from neat IL solution and those of prepared with the assistance of DMSO. All the chitin particles showed similar crystallinity degree, morphology, thermal degradation profiles, and wetting behavior.

Recently, Song et al. have described a simple yet efficient method of cellulose coating onto chitosan hydrogel beads and subsequent application as drug carriers was presented [27]. The chitosan beads were coated with cellulose using IL 1-ethyl-3-methylimidazolium acetate as a dissolution media in a one-pot one-step process. Upon contact with the cellulose−IL solution, the moisture contents of chitosan beads diffused outward and enacted the role of antisolvent. This led to the chitosan surface to be well coated with the regenerated cellulose as revealed by FTIR and FESEM analysis. Furthermore, the cellulose-coated chitosan particles induced sustained release patterns of verapamil hydrochloride in SGF and SIF environments when used as drug carriers.

Traditionally, commercial chitosan is principally produced by conventional chemical methods from chitin via strong acidic or alkali treatment [28]. Despite the low cost and high yield, these methods pose serious drawbacks of environmental toxicity, numerous products with heterogeneous characteristics, poor control and especially deacetylation degree. Recently, the work of Qinyuan Ma and coworkers described for the first time the use of IL tetrabutylammonium hydroxide for the efficient conversion of chitin into chitosan at room temperature [29]. Results confirmed the good solubility of chitin upto 18 wt.% in aqueous tetrabutylammonium hydroxide solution at 80°C. Furthermore, efficient chitin deactylation was reported with high concentrations of IL (12 wt.%) and exhibited promising application in chitin conversion. It was also observed that *Rhodococcus equi* CGMCC14861 (ReCDA) activity on chitin was activated by IL pretreatment. A comparison of simultaneous and sequential techniques was also presented, and the simultaneous one-pot deacetylation afforded the highest acetic acid yield of 3.78 mg/g chitin in 24 h.

9.2.2 Agar/agarose, guar gum, and starch

9.2.2.1 Agar/agarose

Agarose is a linear hydrophilic galactan consisted of repeating disaccharide units [30]. It is known to form a network structure, either weak or strong depending upon its concentration, in the presence of a solvent (typically water). This structure makes it an ideal candidate for applications involving electro-kinetic movement and diffusion of biopolymers like DNA. The main sources of agar and agarose are red seaweeds including *Gracilaria* spp., *Gelidium* spp., *Ceramium* spp., *Acanthopeltis* spp., *Campylaephora* spp., and *Pterocladia* spp. [31]. There are different procedures to extract the agar/agarose such as surfactant/solvent induced precipitation, repeated freeze-thaw, alkali treatment, and pressure syneresis [32]. However, owing to high boiling point, recyclability, negligible vapor pressure, wide electrochemical window, etc., ILs are considered as an alternative for different applications such as materials design [33], extraction, and dissolution of polysaccharides [34,35].

Sharma et al. [36] reported, for the first time, an economical and eco-friendly selective extraction method of agarose from hot seaweed extract of *Gracilaria dura* using bio-ILs (bio-ILs) under ambient conditions. Five bio-ILs (choline formate, choline caproate, choline acetate, choline laurate and choline caprylate) were observed to have strong affinity toward agarose and, therefore, selected for precipitation of agarose. The optimum concentration of ILs needed for the extraction of agarose was found to be varied for different bio-ILs. For instance, in case of choline formate, the critical concentration was 8 wt.% while for choline laurate the value of optimum concentration was 4 wt.%. Moreover, the results showed the maximum yield (14.0 ± 0.5 wt.%) of agarose within 4 h by using 4 wt.% of choline laurate, which was observed to be the best candidate for the isolation of agarose. The bio-ILs were reused and recycled for three agarose batches without losing the agarose quality and yield. The obtained agarose exhibited the properties comparable to the commercially available biopolymer, which made them suitable for gel electrophoresis and molecular biological applications.

Likewise, the isolation of agarose from *G. dura* using three different ILs, [Emim][OAc], [Ch][OAc] and [Emim][Dep] under varying conditions of microwave irradiation or heating was studied by [37]. [Emim][OAc] was observed to be highly efficient for the isolation of agarose. Moreover, the extraction efficiency of agarose was increased by increasing the microwave irradiation or temperature. The outcome of the study further showed a higher yield (39 wt.%) of good quality agarose depending upon the nature of IL and experimental conditions. Therefore, the developed methodology for agarose extraction was simple, easy, economic, highly efficient and environmental friendly.

In addition to the extraction of agarose, IL has been utilized to process the agarose for producing different materials including fibers, and ionogels. For instance, Forget et al. [38] investigated the antimicrobial properties of carboxylated agarose fibers produced via electrospinning of high viscosity agarose solution in IL, [Bmim][Cl]. The fiber diameter, fiber swelling, elastic modulus of mesh, and loading/releasing of IL were observed to be influenced by the extent of carboxylation. The prepared fiber meshes displayed excellent antibacterial properties, which made them suitable for applications in wound dressing.

The utilization of ILs for producing ionogels based on agar/agarose has been discussed in literature for different applications including sensing of volatile organic compounds [39], drug delivery [40], bacterial inhibition [41], and super capacitors [42]. For example, Sastry and coworkers [40] examined the drug release properties of hybrid gels composed of IL 1-dodecyl-1-methylpiperidinium acetylsalicylate

([C_{12}mpip][AcSa]) in aqueous sodium salicylate (SS) mixed with the aqueous solution of agar or chitosan. The incorporation of biopolymer in the IL/SS mixture resulted in higher elastic modulus as compared to the viscous modulus without any crossover (i.e., stiff viscoelastic gels), which was attributed to the disruption/dissolution of micellar structures formed within IL/SS system due to the presence of biopolymer chains. The drug release was observed to be higher in case of thin films prepared from chitosan as compared to the agar due to the longer chains of chitosan. This drug release profile was strongly influenced by the biopolymer concentration as well. Likewise, the antimicrobial properties of ionogels synthesized by dissolving either agar or gelatin in [Emim][Cl] or [Omim][Cl] were reported by [41]. The results revealed that the ionogels based on longer IL chain ([Omim][Cl]) exhibited enhanced antimicrobial and antifungal properties due to the improved cell membrane permeability. Instead of a single IL system, agarose ionogels based on the mixture of protic ([HEA][HCOO]) and aprotic ([Bmim][Cl]) ILs were prepared and characterized by Bharmoria et al. [43]. The obtained ionogels displayed a solid-like behavior in rheological analysis, which was observed to vary as a function of ratios between each IL. This study presented a unique way of tailoring physical properties and dissolving capability of ILs toward biopolymers by efficiently mixing the ILs having different nature.

9.2.2.2 Guar gum

Guar gum is a neutral polysaccharide typically isolated from the seeds of the leguminous shrub *Cyamopsis tetragonolobus*. For this biopolymer, the ratio between mannose and galactose lies within the range of 1.3−1.8 [44]. This polysaccharide is widely used in many industries including food, textile, personal care, petroleum, and paper [45]. However, the dissolution of guar gum in common solvents (i.e., water) is limited by the hydrogen bonding between its hydroxyl groups. Therefore, IL has been effectively utilized to dissolve high molecular weight guar gum without affecting the chain integrity [46].

del Agua with coworkers [47] reported, for the first time, the synthesis of a conductive polymeric gel using a nonvolatile continuous phase (i.e., IL). The ionogels were prepared from guar gum, PEDOT (a conducting polymer) and an IL [Bmim][Cl]. The optimum concentration of individual components (i.e., guar gum, PEDOT and IL) resulted in excellent ionic conductivity and better rheological properties. The fabricated ionogels displayed flexibility due to the presence of guar gum, redox properties imparted by PEDOT and a unique combination of negligible vapor pressure and ionic conductivity given by the IL. This simple mixing approach can be applied to other polymers (i.e., polyaniline and polypyrrole) with diverse nature to produce new type of materials with exciting properties. Similarly, Verger et al. [48] fabricated the ionogels based on guar gum by a simple approach, which did not involve any chemical modification of biopolymer. The properties of ionogels were observed to vary as a function of chemical structure of IL, concentration and molecular weight of guar gum and temperature. The rheological properties of prepared ionogels revealed the formation of highly elastic physical ionogels, which was attributed to the synergistic interactions between IL molecules and guar gum. This study presents an interesting way to design guar gum based ionogels for a wide range of applications.

However, the utilization of guar gum-based ionogels was limited by (1) the continuous leakage of IL over time and (2) the requirement of higher molecular weights of guar gum to obtain better mechanical properties. The second constraint may further affect the dissolution efficiency of guar gum in IL and hamper the processability of the materials. Hence, to solve these problems, an additional polymeric component {i.e., polymeric IL (PIL), poly[1-(2-acryloylethyl)-3-methylimidazolium bromide]} was incorporated in the guar gum/[Bmim][Cl] system (see Fig. 9.1) [49].

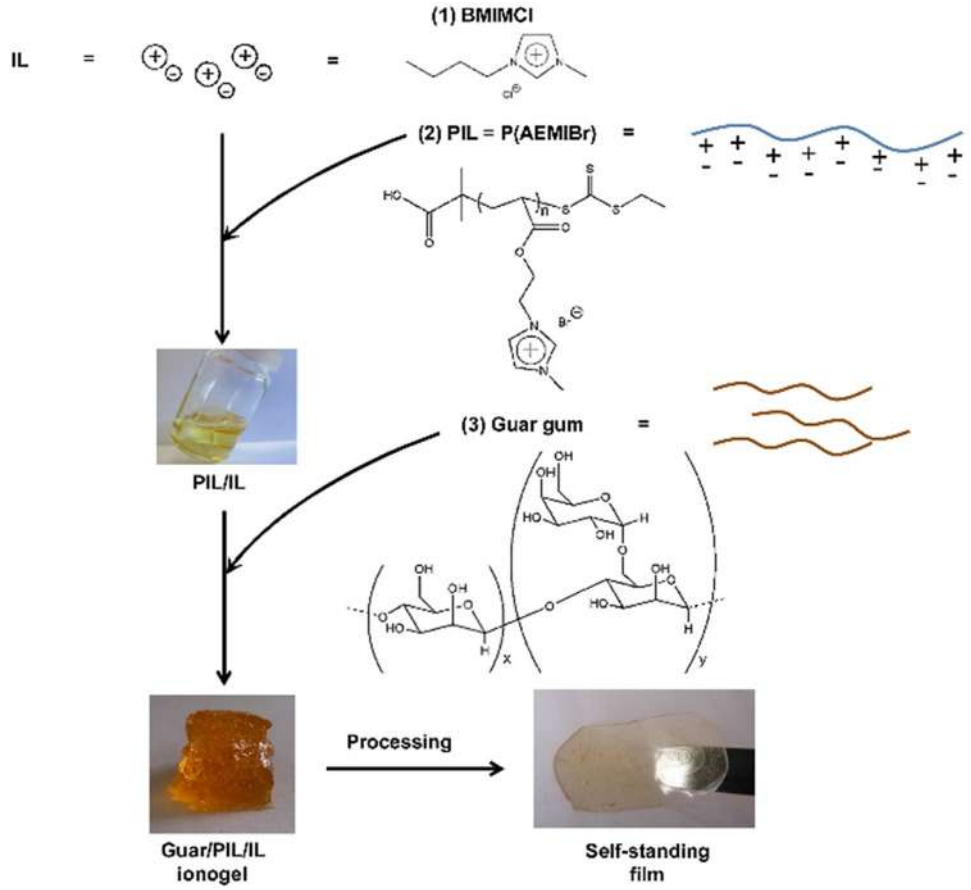

FIGURE 9.1

Fabrication of hybrid ionogels (in the form of bulk material and self-standing film) based on guar gum/PIL/IL [49].

The polymeric chain entanglements and polar interactions between guar gum and PIL resulted in excellent dimensional stability, higher elastic modulus and activation energy and improved thermal stability. The prepared ionogels also displayed enhanced conductivity which was attributed to the homogenous dispersion of IL molecules within the system. These hybrid ionogels offer interesting characteristics for wide range of applications including super capacitors and bio-based battery systems.

9.2.2.3 Starch

Starch is one of the most abundantly available semicrystalline biopolymers typically obtained from the plants (i.e., rice, corn, maize, and potato). Native starch is typically obtained in the form of white granules, which primarily consist of two homo-polymers: amylose and amylopectin [50]. It is

considered as a suitable candidate for many industrial applications such as food, paper, chemistry, fermentation, and film because of its biodegradability, low cost and renewability [51,52]. The formation of strong hydrogen bonds between the hydroxyl groups of starch limits its processing [53] and, therefore, its plasticization is important for many applications.

IL has been extensively used as a plasticizer for processing starch from various sources such as rice, potato, wheat, and corn [54,55]. For instance, Devi et al. [56] studied the sol-gel transition, physical, and rheological properties of rice starch plasticized by using [Bmim][Cl]. The concentration of IL was observed to significantly affect the thermal stability, storage modulus and electrical conductivity of the prepared gel. [57] compared the plasticization efficiency of ILs ([Chol][OAc], [Emim][Cl] and [Emim][OAc]), deep eutectic solvent, DES (choline chloride) and glycerol for maize starch. The results revealed that the lower shear viscosity, higher Trouton number and limited degradation of starch melt were observed in case of [Emim][OAc] as compared to the other studied plasticizers. A similar comparison between ILs, DES and glycerol for plasticization of potato starch was reported by [58]. The lowest viscosity of starch was obtained in case of IL while DES was found to be cheaper with similar properties as IL.

In addition to pure IL, mixture of water and IL is also a promising alternative for starch plasticization. Zan [59] examined the structural disorganization of different starches (i.e., wheat, pea, maize, potato and purple yam) using mixture of water and [Emim][OAc]. The granules of wheat starch were completely destroyed within 1−1.5 h, pea starch granules were disorganized within 6 h and other starches (maize, potato and purple yam) were even more resilient toward aqueous IL system. This plasticization efficiency of aqueous IL system was observed to be varied as a function of IL/water ratio [60], alkyl chain length of cation [61], anionic structure [62], etc. For instance, the dissolution and gelatinization of starch (maize and potato) occurred synergistically and competitively by increasing the [Emim][OAc]/water ratio [63]. Likewise, the longer alkyl chain length of cation facilitated the gelatinization of starch in aqueous IL system with higher ratios of water (IL/water: 1/35, 1/15, 1/10, 1/5) while the IL having cation with shorter alkyl chain length facilitated the starch gelatinization in aqueous IL system having higher ratio of IL (IL/water: 1/2) [64].

To avoid the leakage of IL from the plasticized system, Wang et al. [65] studied the plasticization of corn starch using PIL. The corn starch granules were destroyed by dissolving them in aqueous PIL solution at room temperature, which indicated the strong interaction between PIL and starch chains. This destruction in crystalline structure of starch was further verified by the outcome of XRD and FTIR analysis. Furthermore, the prepared plasticized starch was hot pressed to form a transparent film, which showed significant thermoplasticity of the material (see Fig. 9.2). Hence, instead of IL, PIL can also be efficiently used to plasticize the starch for different sustainable applications.

Kanaan et al. [66] investigated the multiresponsive release of biomolecules from the hydrogels based on semiinterpenetrating network of starch and cationic copolymer of HEMA and IL ([Bmim][Cl]). The experimental outcome demonstrated that the characteristics of hydrogels including ionic conductivity, electronic conductivity and shear modulus were found to vary as a function of relative chemical composition. The sorption/release capacity of biomolecules was significantly influenced by the intensity and duration of applied potential in addition to the chemical composition of hydrogel. The prepared hydrogels exhibited long term functionality and stability due to the presence of lower amounts of IL, which eventually inhibited the leakage/loss of IL. Hence, the designed hydrogels are suitable for producing stable, soft, efficient, degradable, cheaper and multiresponsive materials having possible applications in drug delivery, bioseparation process, electrochemical devices, etc.

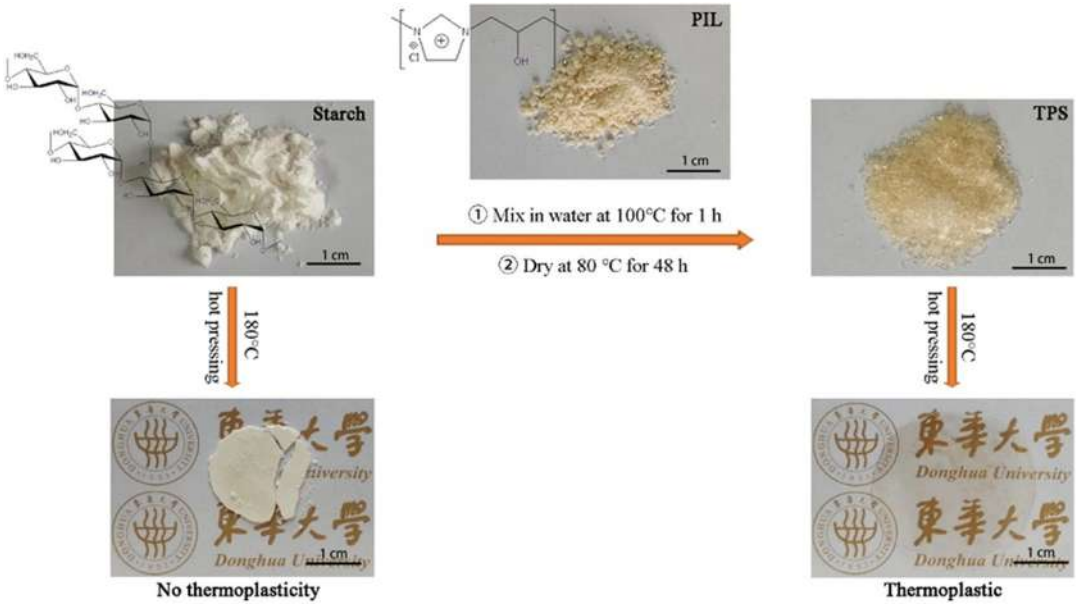

FIGURE 9.2

Fabrication of plasticized starch film using PIL [65].

9.2.3 Silk and hydroxyapatite

9.2.3.1 Silk fibroin

Silk fibroin is a natural biopolymer mainly produced by *Bombyx mori* (silkworm) cocoons. Silk fibroins have attracted significant attention of researchers due to their interesting set of properties such as biodegradability, outstanding mechanical properties, biocompatibility, and amphiphilic chemistry [67,68]. Several physicochemical treatments such as heating, stretching, and electric field can be applied to prepare silk-based films, porous matrices, hydrogels, particles, fibers and three-dimensional scaffolds for different applications including drug delivery and tissue engineering [69]. However, silk fibroin possesses lower solubility in alkali, dilute acid, water, and other common solvents because of strong hydrogen bonding and a higher degree of crystallinity (approx. 1/3 amorphous and 2/3 crystalline) [70]. This opens a new opportunity for ILs to utilize them as an efficient solvent for silk processing.

Samie et al. [71] investigated the extraction and dissolution of silk using choline-based aqueous IL. The maximum dissolution (25%) of silk was observed at optimized processing conditions (i.e., magnetic stirring at 200 rpm and 50°C for 2 h). The crystallinity and thermal stability of the regenerated silk was lower than the degummed silk fibroin, which was attributed to the disruption in polypeptide chains caused by the IL. This study describes the preparation of silk fibroin in the form of nano-sized powders which can be used for different applications.

The dissolution of silk fibroin in IL can be finely tuned by playing with the type of IL, dissolution time, and temperature. For instance, the optimum dissolution time and temperature for silk fibroin in [Bmim][Cl], without the degradation of fibroin heavy chains (which are critical for its

mechanical properties), were found to be 90°C for <30 min or 60°C for <120 min [72]. Likewise, the influence of different precipitants (mono-functional alcohols, ethylene glycol, glycerin, acetone, methyl ethyl ketone, acetonitrile and distilled water) on the dissolution and structural conformation of silk fibroin in ILs ([Bmim][Cl], [Bmim][OAc] and [Bmpy][Cl]) was investigated [73]. The polar precipitants observed to facilitate the ordering of fibroin structure upon dissolution in ILs for producing fibers and films. In case of mono-functional alcohols, the severity of dissolution conditions decreased by increasing the length of the hydrocarbon radical (i.e., from C1 to C4). Moreover, methanol proved to be the best precipitant for transforming α-helix structure to β-sheet segments of fibroin, which is critically important for the mechanical properties of fibroin based materials.

Zhang et al. [74] reported a unique way of preparing the composite silk fibers by dissolving the silk multifilament fibers in IL ([Emim][OAc]) in the presence of methanol as an antisolvent/precipitant. The dissolution process was performed for several temperatures and times, to control the extent of dissolution of silk fibers. The optical micrographs revealed the formation of composite fibers consist of undissolved multifilament fibers bounded by coagulated (dissolved) silk matrix. Furthermore, the dissolution activation energies were estimated, for the first time by using time-temperature superposition, for different experimental data and the average value was around 131 ± 8 kJ/mol. The volume fraction of dissolved silk was also calculated from the XRD data by using the simple rule of mixtures.

Xie et al. [75] fabricated the silk fibroin films by dissolving silk in IL under ultraviolet light. The results showed the formation of stronger films (up to 23.7 MPa strength), which was associated to the structural transformation in silk from α-helix to β-sheet due to the UV-light irradiation. This enhancement in mechanical properties was found to be significantly dependent on irradiation time, wavelength of UV-light and the distance from the light source. Moreover, the prepared films exhibited enhanced wettability, biocompatibility, swellability and cell adhesion due to the increased number of oxygen atoms present on the film surface caused by irradiation. Therefore, the fabricated high strength silk fibroin film is a potential candidate for the biomedical applications.

In addition to the films, the preparation of silk fibroin nanoparticles using IL assisted silk dissolution has also been reported in literature. For example, Carissimi and coworkers [76] studied the secondary structure of regenerated silk fibroin nanoparticles by using infrared spectroscopic technique. The results showed the presence of silk I (i.e., irregular) structure, a 7% reduction in silk II (crystalline β-sheet) structure and a corresponding increase in the content of turns for regenerated silk fibroin nanoparticles. These changes in the structure of silk during dissolution in IL ([Emim][OAc]) suggested an incomplete transition between turn and sheet. The as-fabricated silk nanoparticles were further used to deliver drugs such as Naringenin [77] or Curcumin [78] for cancer therapy. The hydrodynamic diameter and zeta potential of silk nanoparticles were observed to vary as a function of drug loading content, which ultimately affect its suitability for therapeutic applications. The drug loaded nanoparticles retained their antitumor and antioxidant activities toward HeLa cells, without any decrease in the viability of healthy cells [77]. Hence, silk fibroin nanoparticles provide a biocompatible and biodegradable system with the potential to treat cancer cells by local and long-term sustained delivery of drugs.

9.2.3.2 Hydroxyapatite

Hydroxyapatite (HAp) is one of the most important bioactive materials and has gained significant attention in the field of dental materials and orthopedics because of its similar properties with natural bones [79]. HAp has been chemically synthesized from various methods such as hydrothermal reaction, sol-gel synthesis, solid state reaction, coprecipitation reaction and mechano-chemical

methods [80]. However, HAp has also been isolated from different biological sources including bones and fish scales using alkali, acid, or heating [81]. In addition to the environmental concerns, these extraction methods distort the physical structure of the obtained HAp along with the loss of other major components such as collagen [82]. Therefore, the use of a green solvent such as IL which requires mild conditions for extraction of HAp is needed.

Muhammad et al. [83] reported the extraction of HAp by dissolving the waste of fish scales in [Bmim][OAc]. The processing of fish scales in IL resulted in complete dissolution of organic portion while the insoluble inorganic part (HAp) was obtained with the yield of 32 ± 2. The outcome of XRD and FTIR techniques for the prepared sample displayed the characteristic peaks of HAp. The degradation temperature of isolated HAp was higher along with the lower weight loss during heating, which verified the removal of organic part from fish scales during IL treatment. The average particle size, pore volume, BET surface area and pore size of the extracted HAp were observed to be 1870 nm, 0.09 cm^3/g, 37.82 m^2/g and 9.97 nm, respectively. Moreover, the isolated HAp exhibited excellent biocompatibility, tested for both A431 and HEK cell lines.

On the other hand, Sundrarajan et al. [84] described a unique green synthesis process of HAp nano-plates supported by flower extract (*Moringa oleifera*) and IL ([Bmim][BF_4]) (see Fig. 9.3).

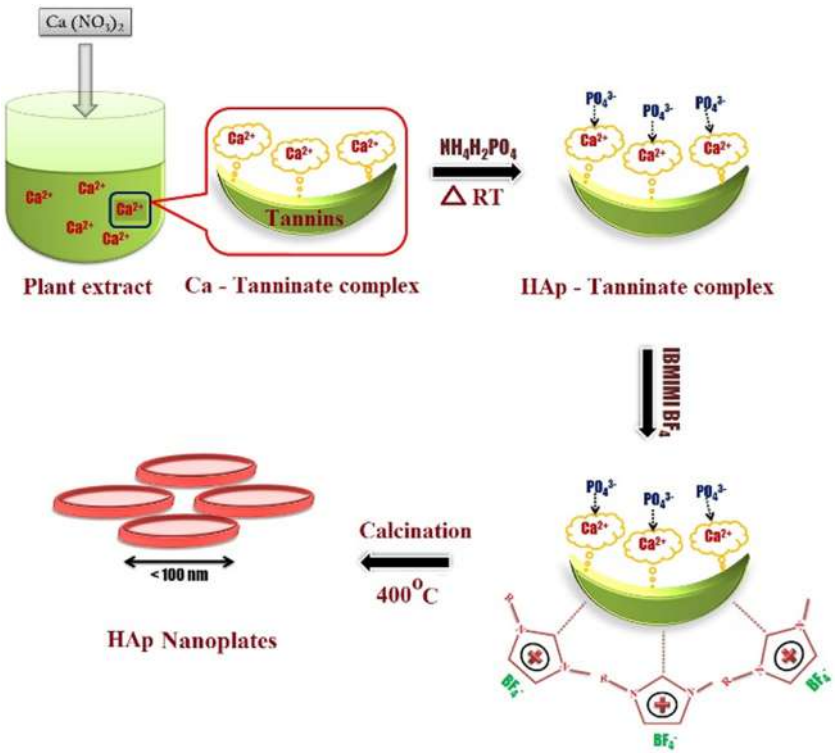

FIGURE 9.3

Schematic of the synthesis of HAp nano-plates [84].

The IL efficiently modified the nucleation and growth of HAp nano-plates while the flower extract acted as a biodegradable chelating agent. The results displayed that the morphology and crystallinity of HAp nano-plates were significantly dependent on the calcination temperature and IL. At higher calcination temperature (i.e., 400°C), the presence of IL reduced the formation of intermediate, which eventually resulted in higher purity (80%) of HAp. Hence, this study presents the use of green solvent (IL) and eco-friendly plant extract to prepare HAp nano-plates for potential applications in biomedical field (i.e., tissue engineering).

Apart from pure HAp, the modification of HAp has also been performed in literature to enhance its properties such as solubility, thermal stability, and surface bioactivity. For example, Moradi et al. [85] investigated the characteristics of strontium-doped HAp nanoparticles prepared through IL assisted hydrothermal process. The utilization of IL ([Bmim][Br]) provided a control over the nucleation and growth process, which eventually resulted in nanoparticles with smaller size (100−200 nm) and higher surface area (around 50 m^2/g). Furthermore, the prepared nanoparticles showed good biocompatibility ($\geq$ 91%), which suggested their use in light curable dental composites. Likewise, the fluorination of HAp using IL has been recently reported by [86], to improve its chemical stability and bonding with bone under extreme conditions. The use of IL for fluorination, instead of a common solvent, offers highly concentrated ionic environment which means efficient fluorination. Two different ILs were tested for the fluorination of HAp including [Emim][BF_4] and [Emim][PF_6] under various conditions. The experimental results revealed that [Emim][BF_4] was efficient for fluorination of HAp as compared to the other one (see Fig. 9.4), which was attributed to its higher hydrophilicity. By increasing the temperature up to 80°C or by decreasing the initial

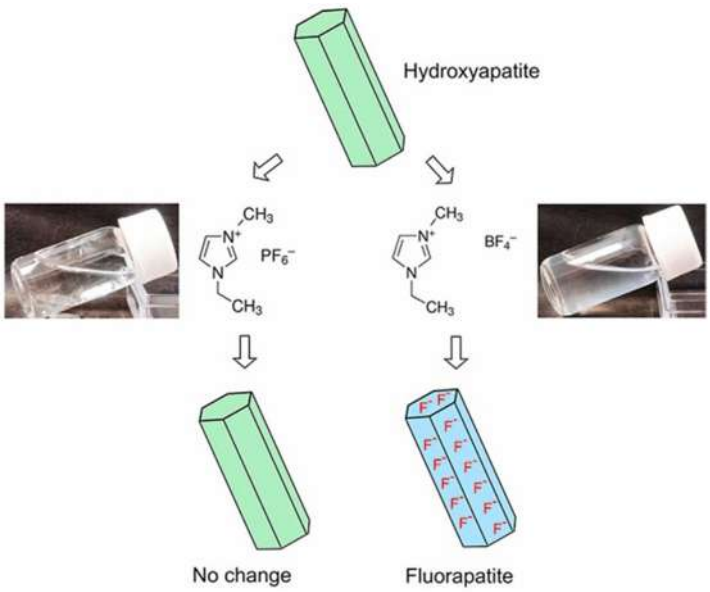

FIGURE 9.4

Schematic of fluorination of HAp using two different ILs [86].

pH up to 7, the formation of calcium fluoride (CaF_2), in addition to the HAp or fluorapatite, was observed. Furthermore, in acidic environment, the solubility of fluorinated HAp was observed to decrease by increasing the amount of substituted fluorine in the crystal lattice.

In addition to the dental applications, HAp has also been utilized as a catalyst for different reactions. For instance, Tang et al. [87] fabricated an IL-supported HAp system through a facile impregnation method and employed as a catalyst for heterogeneous oxidative desulfurization reaction in fuel. The experimental outcome confirmed the efficient immobilization of IL on HAp which was attributed to the porous structure of HAp, which also facilitated the desulfurization reaction. Moreover, the as-fabricated IL supported-HAp catalyst displayed the removal of sulfur up to 97.2% under mild conditions along with the excellent recyclability (i.e., for six cycles). Likewise, the synthesis of pyrimidine derivatives were reported by using imidazolium based IL supported on HAp encapsulated γ-Fe2O3 as a nano-catalyst [88]. The results displayed the excellent yields (80%–95%) in shorter reaction times (7–13 min) by using the prepared catalyst. Hence, this study describes an attractive method for large scale synthesis of pyrimidine derivatives due to the mild reaction conditions, reusability of the catalyst, use of the green solvent (IL), cleaner reaction profile and rapid assembly of heterocyclic molecules.

9.2.4 Collagen and keratin

9.2.4.1 Collagen

Collagen is a structural protein found in connective tissue including rigid connective tissue that makes up the skeleton of vertebrates bone, ligaments, joint, and skin tissues [89]. Collagen is composed of amino acids, mostly glycine (33%) and proline (hydroxyproline) (22%). Collagen is classified into three primary types: collagen type I (present in skins, muscles, and hard tissue), collagen type II (found in cartilage), and collagen type III (found in skin and vasculature). Collagen is used in a wide variety of ways. Due to its excellent biocompatibility, nontoxicity, and biodegradability, it is widely utilized in the dermatological, pharmaceutical, biomedical healthcare, and food businesses [89–92].

Collagen interaction with ILs including imidazolium, phosphonium, and choline dihydrogen phosphate (DHP) has been investigated. Interacting with collagen at several hierarchical levels has been demonstrated [93]. However, choline DHP, a biocompatible IL, stabilizes collagen by exerting an electrostatic force on collagen, making it a possible biocompatible crosslinker. Collagen's interaction with ILs will provide avenues for studying collagen hierarchical orderings in tissue and biomedical engineering. The effect of [Bmim][Cl] has caused substantial alterations in collagen hierarchy [93]. Choline amino acid ILs have shown disruptive effects at the molecular and fibrillar levels [94]. Destabilizing effects related to ammonium or phosphonium base ILs were seen on collagen owing to chaotropic ions [95,96], whereas stabilizing effects of choline DHP were observed due to electrostatic forces [97]. Imidazolium and choline-based ILs have also been studied with collagen [98] Additionally, imidazolium ILs have been used to unhair skin matrix fibers, allowing for cleaner and greener processing [99,100]. Aqueous protein-IL interactions are still poorly understood, with most interpretations being speculative. This underlines the necessity to understand collagen-additives interactions, thereby offering a novel probe for protein-IL interaction. Tarannum et al. [95] investigated the interactions of proteins with IL in which anions played a significant

role. Imidazolium-based ILs containing a variety of anions, including dicyanamide, hydrogen sulfate, dimethyl phosphate, acetate (A), sulfate (S), and DHP, were selected due to the elucidate function of anions in causing collagen destabilization based on the kosmotropic and chaotropic properties. Characterizations showed that imidazolium-based ILs were significant denaturants of collagen's triple helical structure. Imidazolium cations destabilized effects of collagen with varied anions: IDP < IDHP < IA < IDCA < IS < HIS. Electrostatic exchange among collagen's anions and amine groupings enabled these noteworthy effects and alterations. Similar works were carried out by Meng et al. [101] who found partial loss of triple helical structure in [Bmim][Cl] induced collagen during dissolution and regeneration. Zhang et al. performed experimental and computer analyses on cellulose/collagen films through using [Emim] [Ac] and utilized to regenerate triple helical structure, suggestive of potential uses in tissue engineering [102,103].

9.2.4.2 Keratin

Keratin is a fibrous structural protein and most of the components in the human body are composed of keratin, including hair [104–107]. It is notorious for its poor insoluble nature in water and other chemical solvents. Making keratin solution will open new possibilities for manufacturing textile fibers, animal feed additives, leather and wool neatening agents. Every year, thousands of tons of nonspin wool fibers are discarded during wool weaving [108]. Economically and environmentally, it is critical to create techniques for reusing materials.

ILs have made some success in disintegrating keratin [109,110]. Idris et al. [104] examined the capacity to extract keratin from turkey feather by [Bmim][Cl] and novel IL [choline][thioglycolate]. The thiolic group was predicted to reduce disulfide bridges and increased keratin solubility. The method was carried out in 10 h at 130°C with nitrogen resulting in considerable percentage of solubility (up to 45%). A comparison among the utilized ILs and [Amim][Cl] for solubilizing keratin revealed no significant difference. It was found that disulfide cleavage did not show any major role in keratin breakdown. All three IL solutions became too viscous to dissolve at 130°C with 50% feather content. These ILs could dissolve keratin hydrogen bonds thus increasing its solubility in accordance with previous findings [109,110]. In another study, DES based choline chloride (ChCl) and H + bond donors including urea, ethylene glycol (ETG), citric acid (CA), and oxalic acid (OA) were evaluated as solvents for keratin, the main constituents of rabbit hair. The solubility of rabbit hair increased in the raw ChCl-ETG, ChCl-urea, ChCl-CA, and ChCl-OA after 2 hours. As a consequence, DES-based ChCl and OA were chosen for a more comprehensive investigation, which revealed that rabbit hair may be dissolved to an extent of 89% after two hours of stirring at 120°C [105]. Sakaho et al. used a novel technique for dissolving animal hair in two deep eutectic solvents [111]. Sulfur reducing agents and powerful oxidizers were used in aqueous media to dissolve. This process might be utilized for industrial removal of animal hair and epidermis with additional study and scaling up. Based on ChCl- urea and ChCl-urea-hydrogen peroxide adduct yielded eutectic. They were liquid at room temperature and could dissolve animal hair in modest sodium sulfide or ammonium thioglycolate concentrations. The solubility of rabbit hair was improved from 51% to 79%.

In another study, Deng et al. [112] used IL [Bmim][Cl] and ultrasonication to hydrolyze feather keratin, using minimum hydrochloric acid and time than traditional methods. SEM demonstrated changes in surface structure of feathers, increasing keratin polymer conversion via ultrasonication. The ideal parameters were predicted using response surface optimization, and experimental verification resulted in 83.1% hydrolysis of feather keratin.

Millions of tons of chicken feathers are produced annually from poultry waste. Chicken feather dumping pollutes the environment. Chicken feathers contain 90% keratin. In an investigation [113], the dissolved keratin and its capacity to form biofilm were examined by employing two imidazolium-based ionic liquids ([Bmim][Cl] and ([Emim][Ac]). Enzymatic biodegradation by keratinolytic protease from microorganisms was employed to efficiently and environmentally solubilize keratin. Solid-State Fermentation (SSF) with chicken feathers was used. This study was aimed to evaluate soluble keratin from SSF and ability for biofilms formations. It was observed that Keratin biofilms made from soluble keratin had varying hues and surface textures. The skin morphology of soluble keratin matrix was smoother than the dissolved one shown. The surface texture may differ depending on solidification speed. Soluble proteins took longer for evaporation resulting in slower biofilm formation and a smooth surface.

Another study found that human hair, which is a common keratin-rich natural fiber, and an abundant source from human waste and products can be produced from its usage [114]. In general, over 300,000 tons of hair are discarded each year gloabally while keratin can be generated cheaply from leftover hair and could be utilized for the production in biomaterials [115]. The study included the solubility of human hair in various hydrated ILs was because of their outstanding solvation characteristics. Among hydrated ILs, tetrabutylammonium hydroxide (40% aqueous) was used to fully dissolves 25 wt.% w/w waste human hair. The solution yielded 20%−22% melanin and 36%−38% keratin, respectively, which could be utilized to strengthen nitrogen deficient liquid seaweed fertilizers for agricultural usage. Ion Jelly uses gelatin, a commonly accessible, cheap, and extensively researched biopolymer. Gelatin is made by heating collagen following an acid or alkaline pretreatment. Gelatin dissolves in water at 30°C−35°C, causing a coil-helix transition by Bigi et al. [116]. Renaturation influenced mechanical and swelling characteristics of films, which indicated increased Young's modulus and decreased swelling with increasing triple-helix concentration. Crosslinking with glutaraldehyde improved mechanical characteristics of the films, yielding stable films with elastic modules up to 27 MPa. The hydroxyproline residue promotes quaternary structure compactness by forming hydrogen bonds between the hydroxyl and carbonyl groups of the main chain helices. This interaction affects the physical characteristics of gelatin and collagen. Although cheap, biocompatible, and biodegradable, gelatin only interacts strongly with compounds soluble in water [117]. In another work, the influence of glutaraldehyde on the structure, mechanical characteristics, and thermal stability of produced gelatin films were studied [118]. The intermolecular forces (hydrogen and ionic bonds) and triple helix structure of gelatin film were severely disturbed by GTA. Crosslinked gelatin films had the best mechanical and thermal characteristics of all films at pH 4.5, giving 16 MPa tensile strength and 15% TGA residue. The use of ILs with gelatin can greatly expand its application area, since IL can modify the polymer's physical-chemical characteristics and microenvironment. This can solve one of gelatin's flaws, namely the trapping of weakly water-soluble compounds [119,120].

9.3 Relevant properties of ionic liquids for biopolymer dissolution and processing

ILs are defined as ionic compounds with immeasurable alliance of cations and anions and showing extremely important and widely applicable properties. More importantly, the impact of the potency of charges between the cation and the anion, and hydrophilicity/hydrophobicity and the molecular

polarity, altogether contribute to the demonstration of the structure and the essence of substituent groups. Physicochemical attributes of ILs play an indispensable role toward the optimization of application of ILs in carrier materials. The principal parameters of ILs that influence solvation capabilities of ILs for biomolecules, which is essential for the fabrication of carrier materials are described here.

The viscosity and polarity of ILs are the major criterion for the solvation power of ILs. In general, ILs are considered as highly polar solvents due to their ionic nature. It has been established that ILs exhibit polarity in the range those of short- to-medium-chain formamide or alcohols. The anionic part is chiefly responsible for the polarity of ILs and their resulting hydrogen-bond basicity which promotes the dissolution phenomena. Conversely, a high viscosity endeavors a hostile effect, and low viscous ILs are more efficient to dissolve biopolymers molecules. The viscosity of ILs is usually similar to that of oils and reduces with increasing temperature. As in the case of polarity, the viscosity also depends mainly on the anion and can be sharply decreased by use of cosolvents with ILs [121,122].

Furthermore, size of the anion in the IL is also important for dissolving biopolymer matrices. In contrast to the large and noncoordinating anions, chloride (despite being a small hydrogen bond acceptor) is most effective anion to dissolve cellulose. Nevertheless, incorporation of the halide anion embarks a high viscosity problem due to processing difficulties during biopolymer dissolution. ILs containing anions such as formate, acetate, and phosphate exhibit lower viscosities and are extensively applied for numerous applications [123,124].

The high polarity of ILs enable them to be miscible with polar solvents including ketones, dichloromethane, and lower alcohols, but ethers and alkanes are immiscible with ILs owing to their nonpolar nature. ILs can be classified as hydrophilic or hydrophobic on the basis of their water miscibility. As most of the biopolymers are hydrophilic in nature and possess hydrogen bonding ability, the hydrophilicity of ILs favors the dissolution process. By the manipulation of structural functionality on the cation or anion of an IL, its hydrophilicity/hydrophobicity can be amended. For an example, ILs show amphipathicity when incorporated with long chain alkyl group on their cation and show surfactant-like behavior in aqueous solutions. In addition, due to their remarkable water solubility and directional polarizability, surfactant ILs exhibited an interesting behavior to self-assemble into highly structured forms, which could be beneficial for the fabrication numerous nanomaterials. In particular, this self-assembly phenomena in ILs can be rectified just by changing the ion type and the chain length of alkyl group [125−127].

9.4 Conclusions and prospects

Owing to enforced legislative measures worldwide for environmental protection, and public environmental sensibility, biopolymers have eventually started acquiring appreciation in numerous industrial sectors of biomedical, energy, and materials. In this chapter, an overview of the research advancements on the isolation, processing, and functionalization of biopolymer matrices via dissolution and gelation in ILs is presented. Although ILs have facilitated the numerous progressions in the preparation of biopolymer materials, there is a lack of conformity on both peculiar biopolymer's solubility in the ILs and the optimization of ILs structure most appropriate for this purpose. While

various ILs have been tried out for lignocellulose and biopolymer dissolution synthesized from imidazolium, pyridinium, ammonium etc. cations and enormous variety of anions, it is noted that imidazolium ILs with anions, such as [Emim][OAc], [Emim][Cl], [Bmim][OAc], [Bmim][Cl], and [Amim][Cl], are the best suited. Nevertheless, ILs-mediated processing of ILs is a relatively new field, and most of the findings on IL-based biopolymers are only at the laboratory scale, and techno-economic evaluations for scale-up opportunities should be performed. In particular, for biomedical applications, only a few studies could be found regarding in vitro and in vivo biocompatibility of the biopolymers regenerated from ILs. The consistent provision of high-quality biopolymer raw materials and bulk supply of cheap ILs from renewable feedstock coupled with novel recycling techniques would be essential for the commercial realization of sustainable IL technologies. Nevertheless, we believe that fabrication of biopolymeric materials employing ILs have significantly been progressed in recent years as illustratively conferred in this chapter. Thus, such materials would exhibit tremendous applications in the biomedical engineering and environmental benign industries in the future by boosting a transition from nonrenewable fossil-based economy to a sustainable bioeconomy.

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CHAPTER

Ionic liquids for biomass biotransformation

10

María Catalina Quesada-Salas, Marie E. Vuillemin, Catherine Sarazin and Eric Husson

Unité de Génie Enzymatique et Cellulaire, UMR 7025 CNRS Université de Picardie Jules Verne, Amiens, France

10.1 Introduction

Ionic liquids (ILs) are melting salt imperfectly assembled so that they do not form a crystalline lattice making them liquid at temperature below 100°C and sometimes at room temperature (RTILs). With regard to their stability in water and air, they constitute a subclass of melting salts. In general, they are viscous and have low vapor pressure, making them non-volatile and offering thus interesting non-inflammability property as compared to more classical solvents. This low volatility is the basis for the claim of green solvents. Most often, ILs are composed of an organic cation and an anion which can be either organic or inorganic, providing a large number of combinations. Usually, the cations are large and asymmetric while the anions are smaller and with a high electronic density. Depending mainly on the alkyl grafted on the cation, hydrophilic and hydrophobic ILs can be distinguished, and even both classes are hygroscopic. The increase in chain length grafted on the imidazolium cation can be the origin of toxicity, ecotoxicity, and lack of biodegradability. However, not all are harmful and because of the tunability to design targeting application properties, the green requirements can be tunable as well.

Several studies have demonstrated the capability of hydrophilic ILs to solubilize biopolymers from various lignocellulosic biomass (LCB) leading to an increase in their enzymatic degradation [1–3]. This offers a new alternative in biorefinery by combining pretreatment and enzymatic catalysis in nonconventional media such as ILs.

10.2 Ionic liquid pretreatment prior to enzymatic transformation of biomass

One suitable alternative to face the increasing demand of energy, fuels, and platform molecules is the use of renewable feedstocks such as biomass derived from plants and animals that could produce the same type of products through processing in biorefineries [4–6]. Among these resources, LCB and chitin have received attention lately. Indeed, LCB is one the most important plant material on the planet [7] and is the most valuable renewable resource for the sustainable production of biofuels [8–10], biomaterials and platform chemicals [11,12]. 5 to 8% per year of LCB production would cover the annual consumption of fossil oil [13,14]. On the other hand, chitin is the second

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most abundant biopolymer on earth and can be transformed into bioproducts or biomaterials from different species of insects [15–19].

Nevertheless, their transformation process is yet challenging. The valorization of LCB is complex due to the variability and complexity of the raw material. LCB is composed of cellulose (40%–50%), hemicellulose (15%–30%), and the aromatic lignin polymer nested matrix (Fig. 10.1).

Chitin biomass consist mainly in repeated patterns of *N*-acetyl-D-glucosamine (Fig. 10.2) associated with proteins, lipids and minerals [20]. This arrangement results in a low accessibility for fractionation of each polymer not only by enzymes but also by microorganisms.

Owing to the complex structure of LCB and chitin, they are classically treated by using strong acids such as hydrochloric acid (HCl), hydrofluoric acid (HF), or sulfuric acid (H_2SO_4) [21]. Generally, this is called as the pretreatment and is a previous step to the biochemical pathway for second generation bioethanol production. From the last decades, ILs have emerged as promising nonconventional and eco-friendly solvents that can be employed during processes for the extraction and/or conversion of LCB and chitin. The number of researches on ILs pretreatment of LCB and chitin has been growing rapidly in the last ten years (Fig. 10.3).

The main reason behind this recent interest in ILs pretreatment is that contrary to organic solvents, in which lignin and chitin exhibit low solubility, ILs can solubilize both, mostly through hydrogen bonding interactions [20]. Indeed, the pretreatment with ILs has often proven to increase sugar yields after enzymatic hydrolysis [16,22–30] and to increase enzymatic delignification [31,32].

10.2.1 Ionic liquid pretreatment: the key to unlocking biomass recalcitrant structures

Pretreatment involves the alteration of biomass to overcome its recalcitrance so that enzymatic reactions on polymers can be achieved more rapidly and with higher yields. During IL pretreatment, the biomass is dissociated, making it possible for enzymes to hydrolyze cellulose and hemicellulose more rapidly and with higher yields. This includes the removal of lignin which subsequently increases the surface area and reduces the crystallinity of cellulose [14,33]. Research studies related to pretreatment of LCB using ILs have already been the subject of numerous reviews. They documented the impact of the biomass source, the type of IL, the biomass loading, the water content, the particle size and the operating conditions as process time and temperature on the lignin and sugars recovery performance [7,34–40]. Moreover, the ability to recycle and reuse ILs without significant loss of its ability to dissolve biomass and maintain a similar performance in enzymatic saccharification makes them interesting from an ecologic and an economic point of view. On this subject, some studies have reported that the dilution of ILs with water in specific percentages not only reduced the viscosity of the IL but also maintained the pretreatment efficiency [28,41–44].

The most commonly used ILs for biomass pretreatments are hydrophilic and the cations are mostly organic, whereas the anions can be both organic and inorganic (Fig. 10.4) [35].

They are mostly based on imidazolium, pyridinium, pyrrolidinium, cholinium, tetrabutylammonium, tetrabutylphosphonium and alkylalkyloxyammonium for cations, and on halogens, carboxylates, amides, imides, thiocyanates, phosphates, sulfate, sulfonate and dichloroaluminates for anions [14,45,46] (Fig. 10.4A). Although a large variety of ILs are used in literature for pretreatment purposes, numerous studies focus on imidazolium-based ILs for their effective solvation properties [26,47].

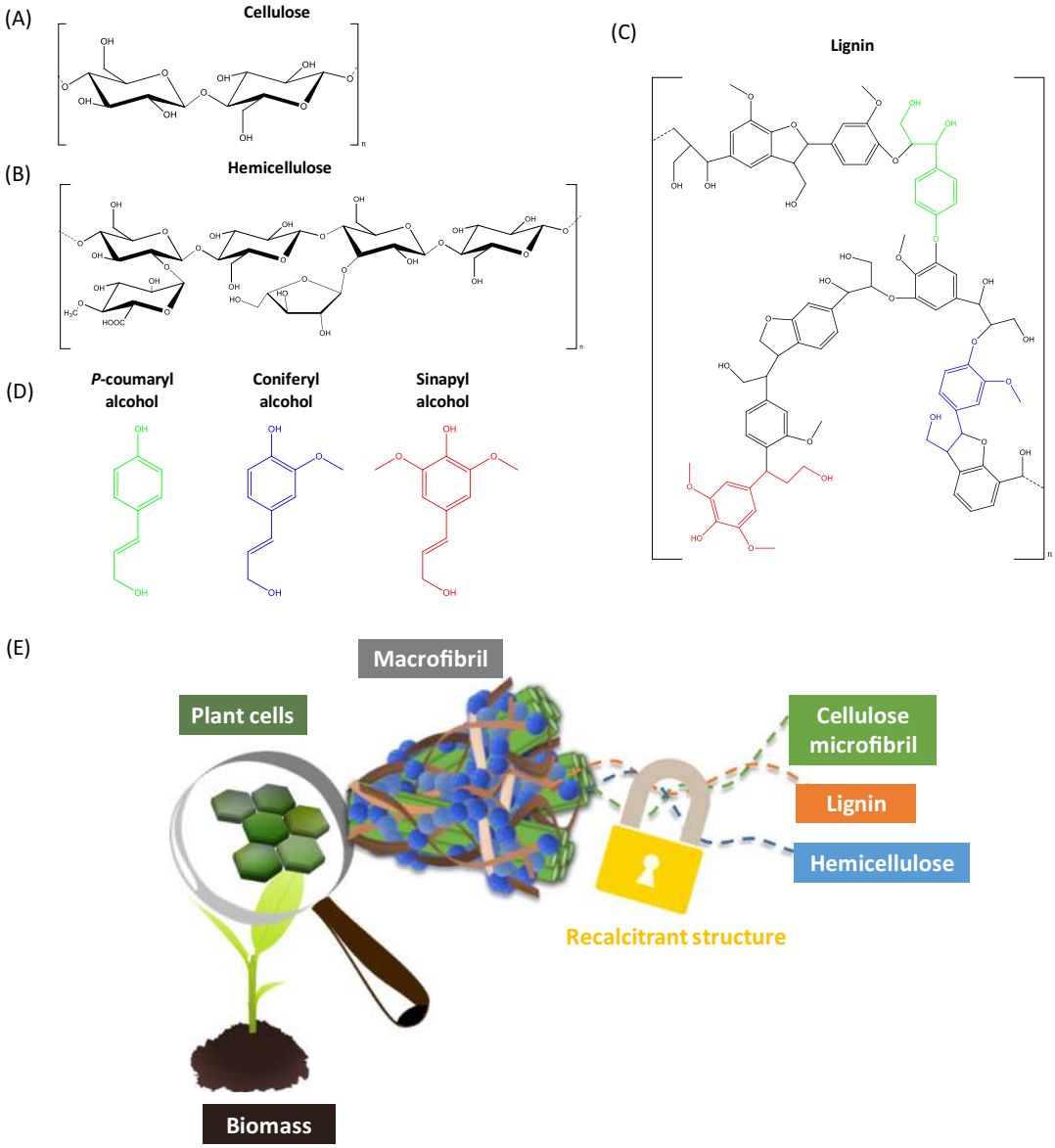

FIGURE 10.1

Representation of (A) cellulose, (B) hemicellulose and (C) lignin structures, (D) phenylpropenoid units of lignin and (E) lignocellulosic biomass structure recalcitrance due to the arrangement of polymers.

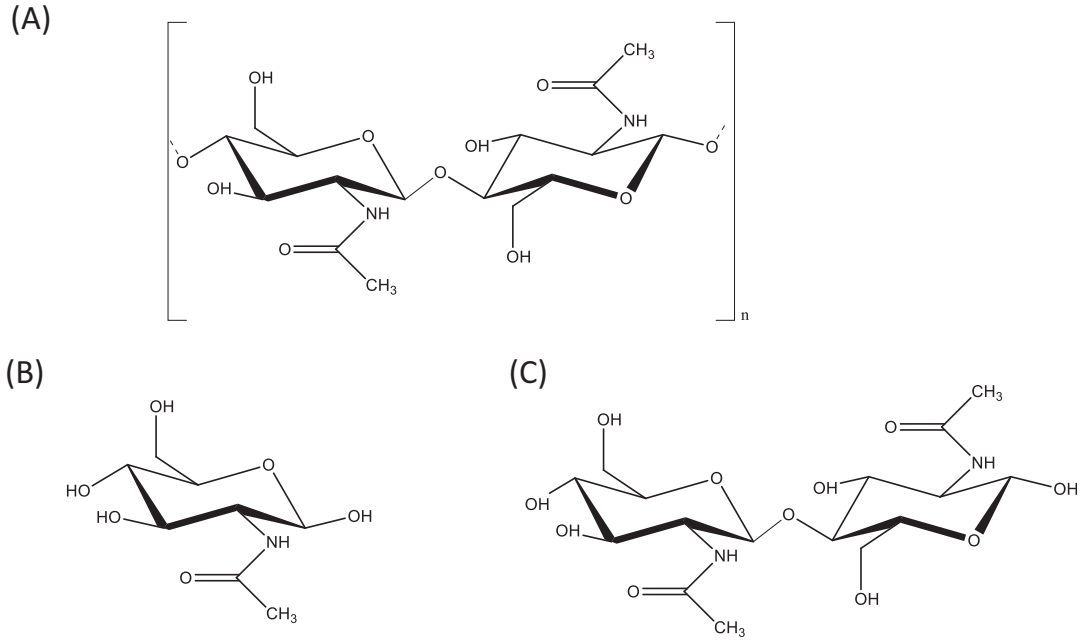

FIGURE 10.2

(A) Chitin chemical structure and its (B) monosaccharide *N*-acetylglucosamine (DP1) and (C) chitooligosaccharide *N,N′*-diacetylchitobiose (DP2).

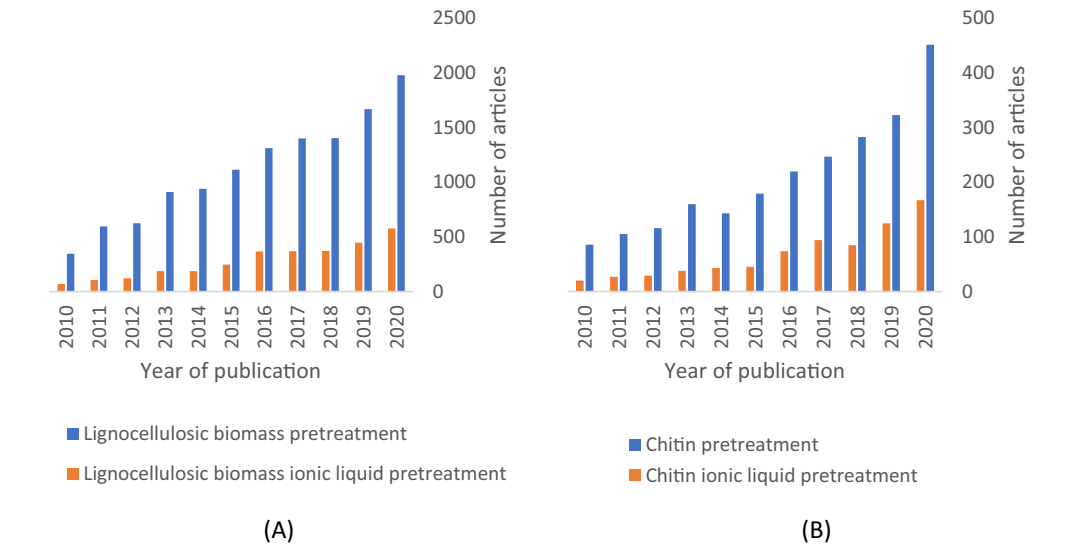

FIGURE 10.3

Number of articles found from "SienceDirect" by using keywords (A) "Lignocellulosic biomass pretreatment" and "Lignocellulosic biomass ionic liquid pretreatment" and (B) "Chitin pretreatment" and "Chitin ionic liquid pretreatment" between 2010 and 2020.

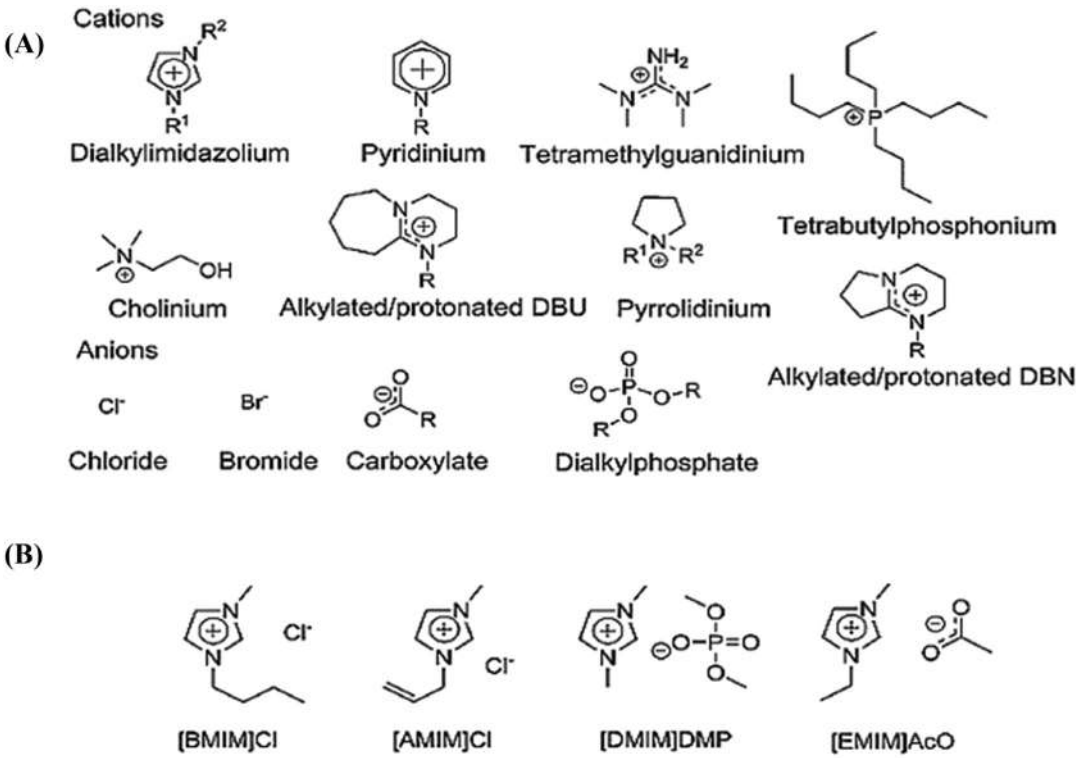

FIGURE 10.4

(A) Common cations and anions used in ionic liquids. (B) Most common studied ILs.

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10.2.2 Understanding ionic liquids power to dissolve lignocellulosic biomass and chitin

Several studies have looked into LCB dissolution in ILs. Dissolution is a physical solvent–solute interaction which can be governed by the nature of both components of the IL and its H-bond basicity. The exact mechanism behind dissolution of the three LCB polymers is still debated in the scientific community. Indeed, there have been difficulties to compare studies, because it is clear that there is an impact of the ILs solvation parameters (nature of the cation and anion, hydrogen bond acidity-basicity, melting point, stability and viscosity), but also of the processing conditions (time, temperature, particle size, biomass loading) and the biomass type [48]. However, there is a consensus on the fact that the dissolution phenomenon is related to the formation of bounds between ILs and biomass polymers, especially through H-bonding [47,49,50]. A way to understand the solubility of biomass in ILs is by using the COSMO-RS and Kamlet-Taft (KT) parameters.

The COSMO-RS parameter (COnductor-like Screening MOdel for Realistic Solvents) calculates thermodynamic properties of ILs based on quantum mechanical data. This parameter has been used by Liu and collaborators to determine cellulose solubility in different ILs (21 different anions and 17 cations) and they demonstrated, via enthalpy calculation, that H-bonding between the anion of ILs and cellulose was the driving force behind cellulose dissolution [50]. On the other hand, the KT parameters can separate the polar nature of components based on their acidity (α), basicity (β), and dipolarity/polarisability (π^*). The acidity or parameter α is a measure of the ability to give a proton in hydrogen solvent-solute bonds or to accept electrons. The basicity or parameter β is a measure of the ability to accept a proton in hydrogen solvent-solute bonds or to give electrons [51]. The KT basicity (β) value of the ILs is often regarded as a measure of cellulose and lignin solubility in them, although other operating parameters must be also considered, such as temperature, biomass loading, process time, biomass source and composition, and water content [47]. However the higher the β value (> 0.8) (Fig. 10.5) of the IL, the better the solubilization of cellulose and lignin (Fig. 10.5) [47,52,53].

The same solubility behaviour has been reported for chitin. To dissolve chitin, its hydrogen bonding network has to be disrupted; therefore, ILs require to have high β-values ($\beta > 0.5$) [54,55]. There is hardly a consensus on chitin solubility in ILs in literature data, because it is strongly related to chitin quality, source and extraction methods [56]. However, the mechanism of chitin

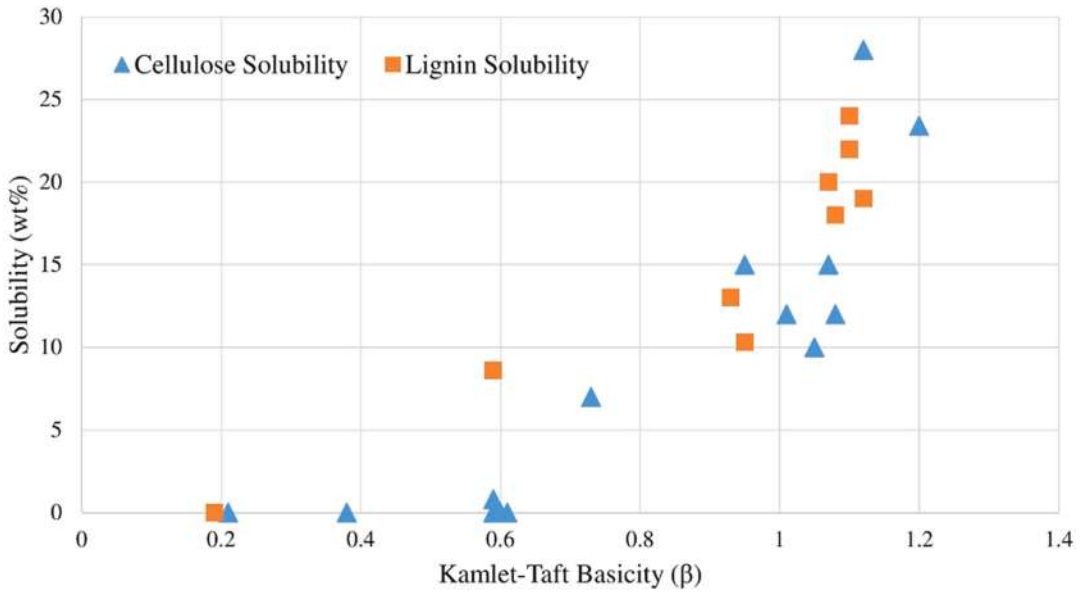

FIGURE 10.5

Correlation of lignin and cellulose solubilization with Kamlet-Taft Basicity.

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interaction with ILs is related to its ability to produce a strong hydrogen bonding network between hydroxyl and *N*-acetyl groups of the polymer and the anion of ILs. NMR spectroscopy studies has been performed on *N*-acetylglucosamine solvation in 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]) [57]. The results clearly suggest that hydrogen bonding is formed between the hydroxyls of *N*-acetylglucosamine and both the anion and the cation of the IL, resulting in chitin solubility.

10.2.3 Enhanced enzymatic hydrolysis of lignocellulosic biomass and chitin in ionic liquids

The efficacy of ILs pretreatment can be regarded as its ability to disorganize the cellulose/hemicellulose/lignin matrix. It is often evaluated by the accessibility of the polymers after pretreatment, which can be approached by measuring the increase in sugars yields after hydrolysis, between untreated and pretreated biomass [16,22,24,28,30], or the increase in enzymatic delignification (Fig. 10.6) [31,32].

With a high carbohydrate content, LCB represents an important sugar source for the production of high valued products. These sugars are not easily accessible because of LCB recalcitrant

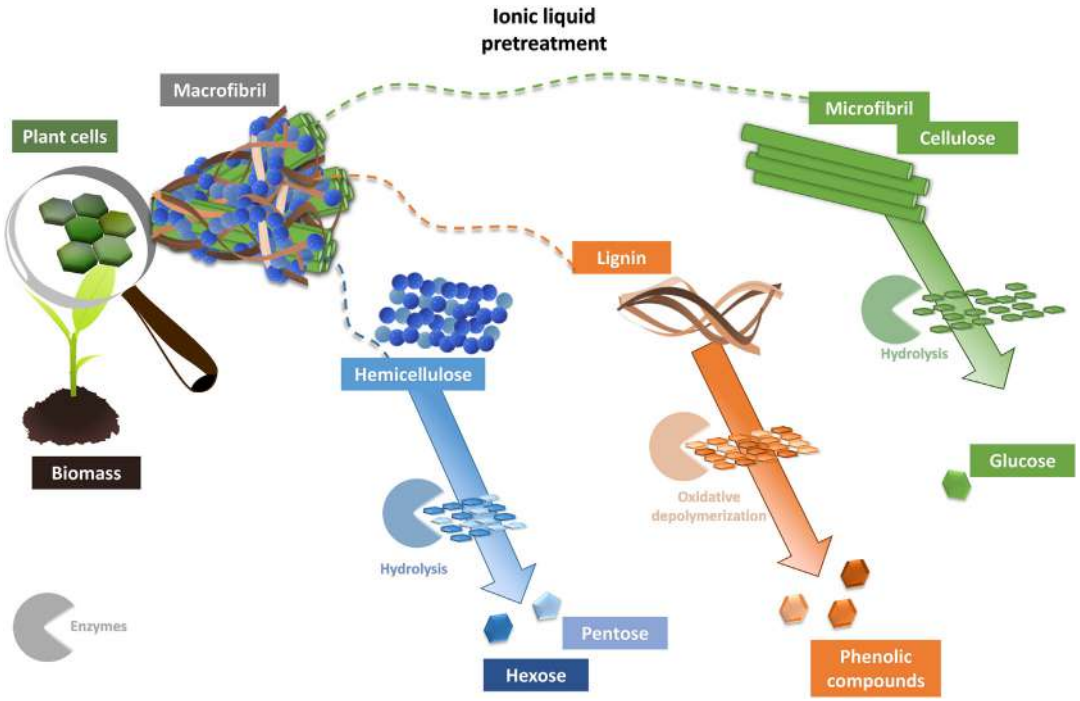

FIGURE 10.6

ILs pretreatment to decrease LCB recalcitrance to perform enzymatic reactions.

structure but this complex structure can be disorganized by ILs pretreatment. The enzymes used for the subsequent hydrolysis of the polysaccharide fraction are cellulases and hemicellulases. Cellulases include *endo*-(1,4)-β-D-glucanase (EC 3.2.1.4), *exo*-(1,4)-β-D-glucanase (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) acting in synergy. They hydrolyze cellulose into glucose monomers, while hemicellulases depolymerize hemicellulose and hydrolyze its components that are galactans, xylans, mannans, and arabans. Hemicellulolytic enzymes include endoxylanase (EC 3.2.1.8, 1,4-β-D xylan xylanohydrolase, which hydrolyzes β-D xylano pyranosyl linkages of xylan to form xylo-oligosaccharides) and β-D xylosidase (EC 3.2.1.3, xylobiase, which catalyzes the hydrolysis of xylobiose or xylo-oligosaccharides, producing D-xylose sugar in the hydrolysates) [31,58]. Numerous studies used the 1-ethyl-3-methylimidazolium acetate which is highly effective to disorganize LCB polymers and to improve enzymatic hydrolysis. However, the results differ slightly and depend on the biomass and the conditions of pretreatment like temperature, process time, and biomass loading [7,22,28,36,41,50,59–68]. Alayoubi and collaborators used [C2mim][OAc] to carry out the pretreatment of different biomass (a model cellulose and two industrial forest residues, spruce and oak sawdusts) at low temperature (45°C). Then, the saccharification step was performed with cellulases from *Trichoderma reesei*. The enzymatic hydrolysis of the untreated biomass provided glucose yields of 24.9% for long fiber cellulose, 25.5% for spruce sawdust and 11.6% for oak sawdust, after 80 h of catalytic reaction. After IL pretreatment of the same substrates, the glucose yields were significantly improved, even at 45°C. Indeed, after 80 h of enzymatic hydrolysis, a glucose conversion yield of 68.2% was reached for long fiber cellulose, 49.3% for spruce sawdust and 59.3% for oak sawdust. Also, the ethanolic fermentation performances of the obtained glucose by the *Saccharomyces cerevisiae* were improved by 2 to 3 times with this IL pretreatment.

The same IL was used in the study carried out by Auxenfans and collaborators. They showed that enzymatic saccharification was significantly improved by [C2mim][OAc] pretreatment of both, softwood and hardwood sawdust. They investigated the impact of different anti-solvents for the IL regeneration after pretreatment on enzymatic saccharification performances. Regardless of the regeneration solvent used, the IL pretreatment increased the conversion yield by 3 to 4 times for spruce substrate and by 7 to 8 times for oak substrate in comparison with the conversion of untreated biomass after 72 h of reaction. All the anti-solvents tested led to high glucose yields after enzymatic hydrolysis. In addition, they correlated the improvement in enzymatic saccharification to a diminution of cellulose crystallinity index after pretreatment (from 30%–40% in untreated biomass to less than 5% in pretreated substrates) [30].

The study carried out by Araya-Farias and collaborators proposed to perform a design of experiment [a Partial Least Square (PLS)—Second Order Design] to optimize the main operating conditions of pretreatment with 1-ethyl-3-methylimidazolium acetate IL which are pretreatment temperature (25°C–150°C), process time (40–180 min), biomass loading (2%–5% w/v) and concentration of IL in water [10%–100% (v/v)] [28]. They performed the optimization on two different biomasses: wheat bran (WB) and destarched wheat bran and the pretreatment efficiency was evaluated through glucose and xylose yields after hydrolysis catalyzed by the Cellic CTec2 hemicellulolytic cocktail. A strong quadratic PLS model to predict optimal pretreatment conditions was obtained, reaching high glucose and xylose yields for the enzymatic hydrolysis of WB with maximum glucose (83%) and xylose (95%) yields at 150°C for 40 min with 10% of IL in water and 5% of biomass loading (Fig. 10.7).

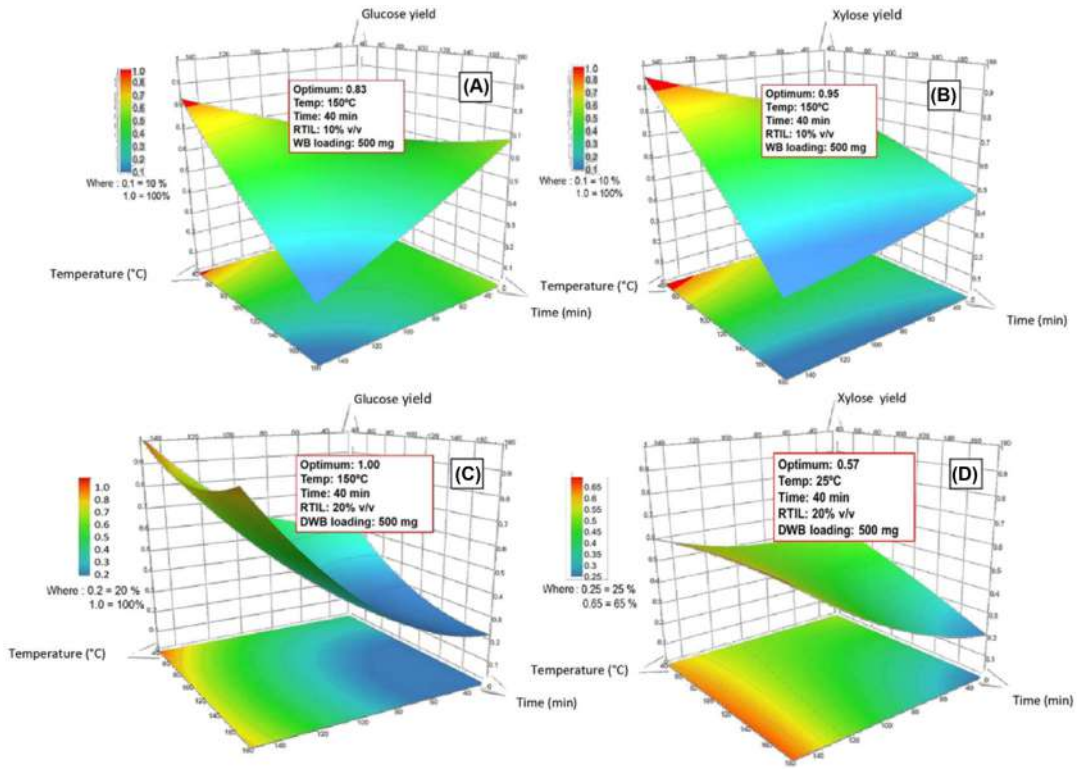

FIGURE 10.7

Response surface plots of sugar release after enzymatic hydrolysis for WB (A,B) and DWB (C,D) as a function of time (40–180 min) and temperature (25°C–150°C).

Reproduced with permission from M. Araya-Farias, E. Husson, J. Saavedra-Torrico, D. Gérard, R. Roulard, I. Gosselin, et al., Wheat bran pretreatment by room temperature ionic liquid-water mixture: optimization of process conditions by PLS-surface response design, Front. Chem. 7 (2019). https://doi.org/10.3389/fchem.2019.00585.

Similar results were obtained with destarched WB, but the optimum temperature was found at 25°C. Their conclusions pave the way to optimize pretreatment to obtain the best enzymatic hydrolysis yields; however, they also point out that such strategies should be optimized independently for all biomass.

The lignin polymer that remains in pretreated LCB still affects the polysaccharides conversion by limiting hydrolysis, either by acting as a physical barrier or by adsorbing hydrolytic enzymes [69]. Laccases (EC 1.10.3.2) have been evaluated as effective tools to delignify pretreated LCB to boost the subsequent enzymatic hydrolysis (Fig. 10.8) [31]. Laccases catalyze the oxidation of phenolic compounds generating unstable phenoxy radicals that can polymerize into aromatic compounds that have less inhibitory activity [70,71]. They can also oxidize the phenolic subunits of lignin, but the non-phenolic subunits have high redox potentials, making them resistant to oxidation by laccase alone. However, when combined with the appropriate mediator, laccase oxidation capacity can be extended to lignin non-phenolic units, thus increasing the delignification process [72].

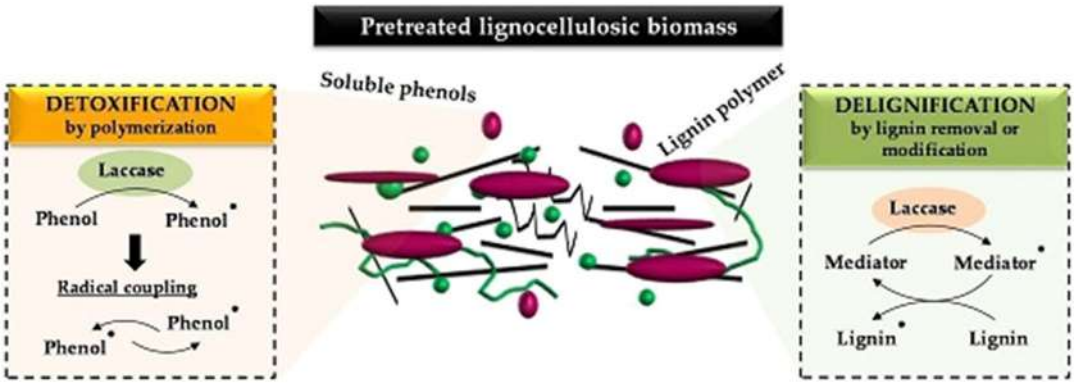

FIGURE 10.8

Laccases as a powerful tool for delignification and detoxification of pretreated LCB.

Reproduced with permission from Ú. Fillat, D. Ibarra, M.E. Eugenio, A.D. Moreno, E. Tomás-Pejó, R. Martín-Sampedro, Laccases as a potential tool for the efficient conversion of lignocellulosic biomass: a review, Fermentation 3 (2017) 17. https://doi.org/10.3390/fermentation3020017.

Hilgers and collaborators have shed some light on the delignification process using laccase/mediator systems (LMS). They showed that the β-O-4′ linkages of lignin undergo four types of reactions (the majors ones being β-O cleavage and Cα oxidation of intact linkages and the minor's ones being $C_\alpha-C_\beta$ cleavage and O-4′ cleavage) yielding in different types of residues [72]. A range of delignification between 80 to 90% have been reported in literature, thus increasing the sugar production through enzymatic hydrolysis by 2 to sevenfold [73–76]. Financie and collaborators pretreated oil palm frond biomass using the hydrophilic 1-ethyl-3-methylimidazolium diethyl phosphate IL [C2mim][DEP], prior to delignification. The authors found a lignin content of 24.0 wt.% in the raw biomass, 19.6 wt.% after enzymatic delignification by laccase, and 8.5 wt.% after pretreating the biomass with the IL prior to delignification. These results showed a significantly delignification of biomass when pretreatment with the [C2mim][DEP] IL is performed [32]. Using laccases after pretreatment represents a powerful tool toward higher sugar yields after enzymatic hydrolysis.

Furthermore, studies have been conducted based on the possibility to use the aromatic aldehydes obtained from lignin depolymerization to synthesize ternary amine-based ILs [77]. These biomass derived ILs demonstrated excellent pretreatment efficiency, since 90%–95% glucose and 70%–75% xylose yields were obtained for samples after enzymatic hydrolysis. Using ILs to pretreat biomass to reach high sugars yields after enzymatic hydrolysis and in parallel, using laccases to depolymerize lignin to create bio-based ILs that could be used to pretreat biomass as well, is a promising perspective toward a circular bioeconomy.

The enzymatic hydrolysis of chitin by applying the chitinase produces *N*-acetylglucosamine, *N,N′*-diacetylchitobiose or glucosamine. Those molecules have interesting properties and can be used as platform molecules for different applications in food, cosmetic or pharmaceutic industries as skin moisturizer, joint-pain reliever, antimicrobial or antitumoral agent and as elicitor or pesticide [16,21,78–84]. It has been reported that pretreatment with 1-ethyl-3-methylimidazolium acetate prior to hydrolysis with chitinases from *Trichoderma viride* and *Streptomyces griseus*

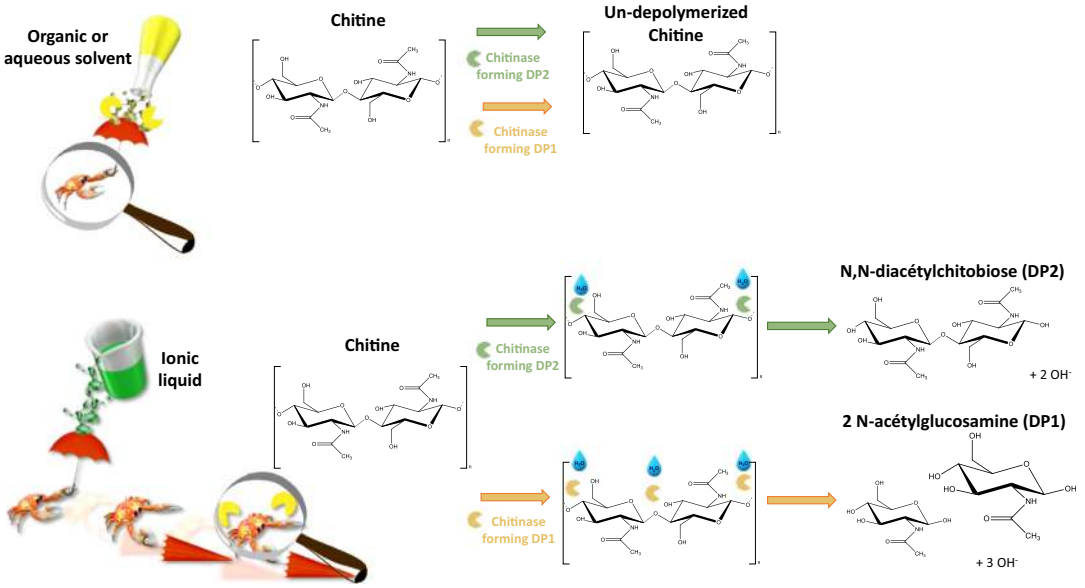

FIGURE 10.9

IL pretreatment to decrease chitin recalcitrance to perform enzymatic hydrolysis.

significantly improved enzymatic hydrolysis by increasing enzymes accessibility to chitin (Fig. 10.9) [85].

Other enzymes have been used to hydrolyze chitin after ILs pretreatment, like chitinase from *Streptomyces albolongus* [57]. High dimers and monomers yields were reached and the better performances were correlated with chitin structural changes after pretreatment.

However, as is the case for LCB, several parameters also impact pretreatment and enzymatic hydrolysis efficiency, such as the chitin source and extraction process, or the IL type and chitinase used. Though the research in this area is fairly recent, there are some studies that have begun to investigate this field. Xu and collaborators used two different chitinases cloned from *Paenibacillus pasadenensis*, expressed in *Escherichia coli* and followed by a purification and characterization steps. Eight ILs were also tested to pretreat chitin from crab shell particles, prior to enzymatic hydrolysis. The 1-butyl-3-methylimidazolium acetate IL showed the best pretreatment efficiency by weakening the structure of chitin and by decreasing its crystallinity [86]. Huet and collaborators also studied the pretreatment with 1-ethyl-3-methylimidazolium acetate IL on different chitin sources from insects and shrimps (commercial chitin, unpurified chitin-0 step, deproteinized and partially demineralized chitin-2 steps). Differences were observed in the yield of *N*-acetylglucosamine and *N,N*-diacetylchitobiose after hydrolysis of untreated chitins while significant improvement and similar values were reached for the three pretreated chitins Fig. 10.10 [16]. Furthermore, they correlated the obtained yield of *N*-acetylglucosamine with chitin's crystallinity index (Fig. 10.10D). It was evident that the pretreatment with IL led to a disruption of the highly ordered intra- and inter-chain hydrogen bond network of chitin, that is a diminution of the crystallinity index, allowing it to produce mono-oligosaccharides more efficiently by enzymatic hydrolysis.

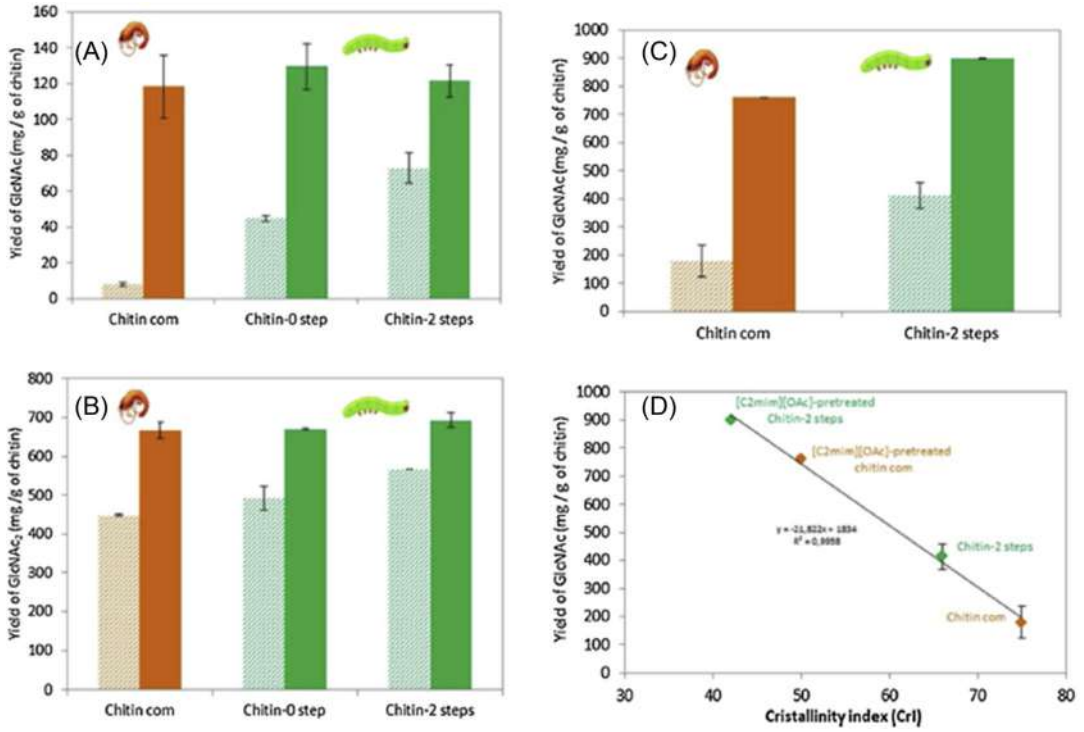

FIGURE 10.10

(A) *N*-acetylglucosamine or (B) *N,N*-diacetylchitobiose yield (mg per g of chitin) at 24 h of enzymatic hydrolysis of chitins, before (stripped bars) and after (full bars) IL-pretreatments, catalyzed by the respective chitinase from *T. viride* (*top*) or *S. griseus* (*bottom*); (C) *N*-acetylglucosamine yields obtained by the enzymatic hydrolysis of extracted and commercial chitins in aqueous buffer before (*stripped bars*) and after (*full bars*) [C2mim][OAc]-pretreatment (these reactions were catalyzed by the chitinase from S. *griseus* for 24 h then supplemented with the chitinase from *T. viride* for 12 h) and (D) correlation of *N*-acetylglucosamine yields and initial Crystallinity index of chitins before enzymatic hydrolysis.

Reproduced with permission from G. Huet, C. Hadad, E. Husson, S. Laclef, V. Lambertyn, M. Araya Farias, et al., Straightforward extraction and selective bioconversion of high purity chitin from Bombyx eri *larva: toward an integrated insect biorefinery, Carbohydr. Polym. 228 (2020) 115382.* https://doi.org/10.1016/j.carbpol.2019.115382.

10.3 In situ enzymatic transformation of biomass in ionic liquid-aqueous media

The literature promoting RTILs for disorganizing/fractioning LCB and thus improving accessibility of constitutive polymers for their subsequent enzymatic transformation is particularly flourishing. These ILs often are hydrophilic imidazolium-based ILs [7,14,22,30,47,49,87]. However, these multi-steps sequential strategies, though effective and promising, may lead to technological, scientific, and economical drawbacks for considering a transposition at larger scale. Even though some

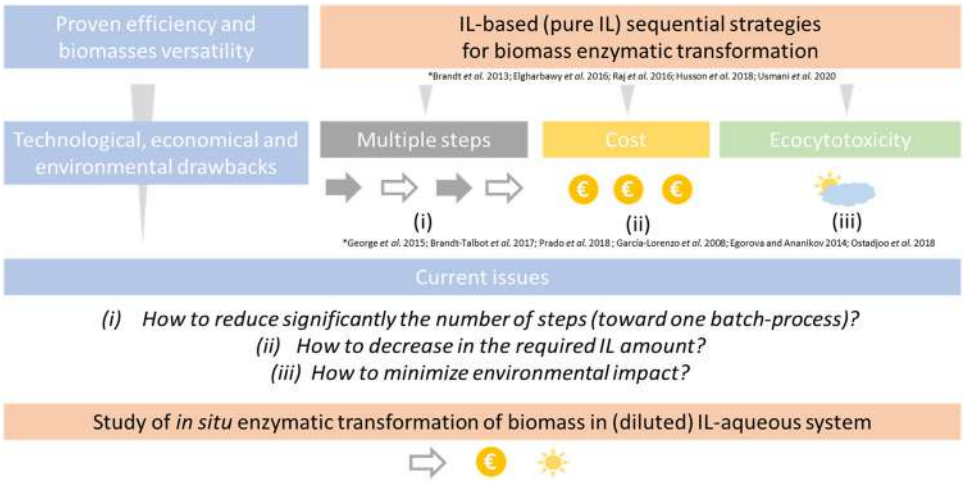

FIGURE 10.11

Simplified view of the thinking leading to study in situ enzymatic transformation in IL-aqueous systems in biomass research area. *Non-exhaustive list of papers from the well documented literature.

of these solvents present thermal stability, low vapor pressure and recycling facility in comparison with other organic solvents, their eco-friendly properties remain questionable and require still deeper ecotoxicological studies to conclude if an industrial transposition could be considered or not [88–90]. In addition, the high cost of the IL (e.g., the estimated price for 1-ethyl-3-methyl imidazolium acetate range from \$20–101 kg^{-1}) feeds inevitably the industrials reluctance to scale-up these ILs-based technologies [91–93]. In this context, several issues have been raised for attempting to accommodate these environmental, technological and economic considerations. The corresponding thinking, which has motivated to study in situ enzymatic transformation of biomass in IL-aqueous systems, is presented in Fig. 10.11.

10.3.1 Concept and scientific challenge

The investigation of enzymatic transformation in nonconventional solvents was pioneered by Klibanov in the 1980s [94]. Then, numerous studies demonstrated the feasibility to orientate hydrolases (lipases, proteases) activity toward synthesis rather than hydrolysis in anhydrous organic media, opening a large field of new applications [95–98]. In this medium engineering approach, some ILs, particularly hydrophobic ILs known for stabilizing enzymes, progressively emerged as greener alternative to the usual organic solvents and their relevance is now recognized in the literature [99]. However, the implementation of enzymatic reaction in ILs do not always aim to favor synthetic activity of hydrolases in the biomass biotransformation area. Indeed, an important part of studies concerns the development of efficient enzymatic hydrolysis/depolymerization reactions of constitutive biopolymers from LCB in diluted hydrophilic IL-aqueous systems [59,65,87,100–107]. These strategies were referred as simultaneous strategy in opposition with the sequential strategy described before. The scientific challenge consists thus to maintain enzymatic activities in these

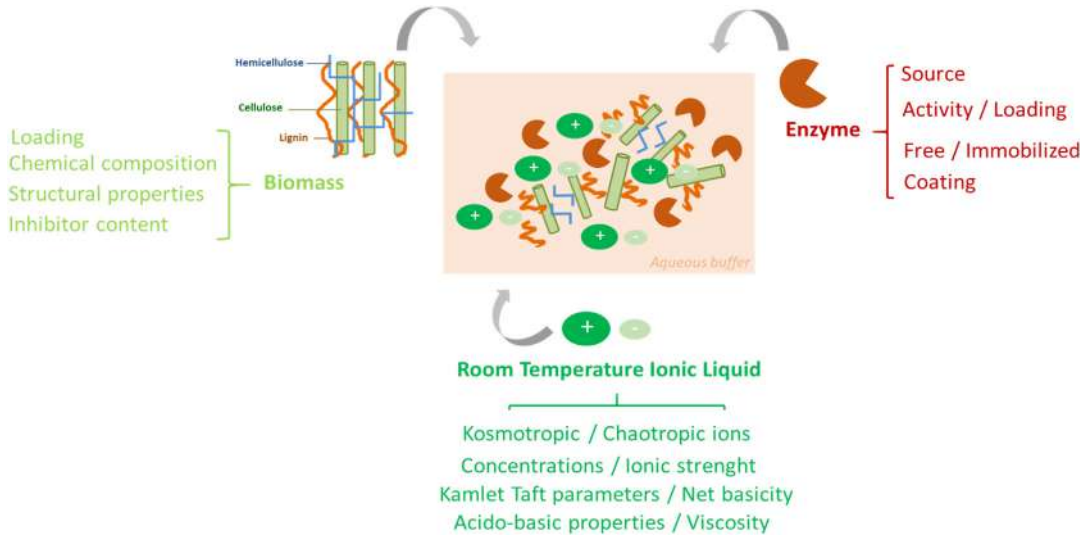

FIGURE 10.12

Multiple factors influencing the performances of in situ enzymatic transformation of biomass in IL aqueous system.

systems while hydrophilic ILs are known for destabilizing enzymes [46]. Numerous studies about factors affecting enzyme activity and/or stability in IL-aqueous solutions (Fig. 10.12) are reported in the literature leading to admit that the deep understanding of the mechanisms is particularly complex [46,108–112].

For instance, kosmotropic anions [such as acetate anion (OAc)] and chaotropic cations [such as *N*-butylpyridinium (C4Py) cation and 1-ethyl-3-methylimidazolium (C2mim) cation] seem to stabilize enzyme in opposition with chaotropic anions [such as tosylate and tetrafluoroborate anion (BF4)] and kosmotropic cations [such as 1-butyl-3-methylimidazolium (C4mim) cation] which could destabilize it [109]. Another factor could be the basicity of the IL. Indeed, the classically used ILs for LCB transformation, depending on their acido-basic properties and concentration in the medium, could induce a pH deviation from optimal pH affecting its tridimensional active structure [46,108]. Other studies showed that the increase in viscosity and ionic strength in the medium due to IL presence can induce deleterious effect on enzyme activity [111,112]. Halo-tolerance, thermo-tolerance, acido-tolerance and surface charges of enzymes appear also to be protecting factors against the detrimental effects of biomass-dissolving ILs leading to retain at least partial activity in diluted-IL aqueous media [102,108,110]. Other approaches for enzymatic activity saving in theses reaction media consist for instance to immobilize enzymes by inclusion in alginate or enzyme coating with hydrophobic IL [101,106]. Obviously, the performances of these enzymatic systems are also dependent on the accessibility of the substrate for enzyme. It is important to notice that in biomass transformation field, the substrate is a recalcitrant matrix, often insoluble whatever the IL-amount in the aqueous medium, limiting, by its very nature, the accessibility to its constitutive polymers [59,65]. Some studies suggested the pertinence of additional parameters such as

β and α KT parameters of the selected IL for better understanding enzymatic performances in these reaction systems related to the solvation/availability of the (ligno)cellulosic substrate [7,113]. Indeed, β and α KT parameters describe the hydrogen-bond basicity (essentially influenced by the IL-anion) and the hydrogen-bond acidity of the IL, respectively. A high value of β KT parameter or more of the difference between β and α KT parameters would be favorable to a suitable availability of the substrate in these diluted-Il aqueous systems. However, the same idea can be applied to enzymes resulting in detrimental effect due to protein unfolding [59,106].

10.3.2 Focus on enzymatic hydrolysis of polysaccharidic fractions from representative biomass in presence of ionic liquids

This part focuses on some examples about enzymatic hydrolyses of model (hemi)cellulosic substrates and representative LCB (forest, agricultural and dedicated crops) in diluted-IL aqueous systems. Enzymatic hydrolysis refers to selective depolymerization of cellulosic and/or hemicellulosic fractions with cellulases and/or hemicellulases (mainly xylanases) as biocatalysts, respectively. Table 10.1 presents a non-exhaustive list of papers in this specific area and their respective outstanding results (Table 10.1). Some of them will be described below.

In 2008, Kamiya and collaborators described for the first time in the literature *in situ* enzymatic saccharification of cellulose in hydrophilic IL-aqueous solutions. This study demonstrated the feasibility to implement efficient cellulase-catalyzed hydrolysis of model cellulose in presence of 25% v/v of an imidazolium-type IL with an alkylphosphate anion in aqueous medium. These promising results initiated then numerous investigations about in situ saccharification of cellulosic materials potentially useful in integrated biorefinery processes [104].

Thereafter, literature data reported for instance a study about in situ saccharification of industrial sawdusts from softwood and hardwood (forest residues) and *Miscanthus x giganteus* (dedicated crops), catalyzed by the cellulase from *T. reesei* in presence of various 1-ethyl-3-methylimidazolium acetate [C2mim][OAc] or 1-ethyl-3-methylimidazolium methylphosphonate [C2mim][MeO(H)PO_2] concentrations (% v/v) [59]. Here, the selected cellulose cocktail was a mixture of endoglucanases (EC 3.2.1.4), cellobiohydrolases, (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21) acting in synergy to convert cellulosic fraction into glucose. The enzymatic performances were expressed as glucan conversion yield into glucose after 72 h of reaction (Fig. 10.13).

For forest residues, [C2mim][OAc] or [C2mim][MeO(H)PO_2] influenced differently the reaction system performances according to wood species (Fig. 10.13A and B). Indeed, [C2mim][MeO(H)PO_2] would be more suitable for softwood with significant yields of glucose up to 30% v/v of IL in the aqueous media while [C2mim][OAc] led to significant yields on oak for the same ratio of IL, as compared to IL-free aqueous media. Beyond IL concentration of 10% (v/v), yields of glucose decreased progressively until a total enzymatic deactivation at 50% (v/v) whatever sawdust and IL. For these two sawdust residues, the key IL concentration for the implementation of efficient in situ saccharification was 10% (v/v) whatever the IL used. The implementation of enzymatic saccharification in presence of [C2mim][OAc] in similar conditions for wheat straw conversion led also to similar conclusion [87].

Surprisingly, the performances trend of these reaction systems applied to *Miscanthus x giganteus* was significantly different (Fig. 10.13C). Indeed, the presence of [C2mim][MeO(H)PO_2] just

Table 10.1 In situ enzymatic hydrolysis of (hemi)cellulosic model substrates or lignocellulosic biomass.

Substrates	Ionic liquids	Enzymes	Main results	References
Model cellulosic substrate	1-ethyl-3-methylimidazolium diethylphosphate	Celllulase from *Trichoderma reesei*	The maximum tolerated IL-concentration to maintain competitive performances was 25% v/v	[104]
Model cellulose, com stover	1-ethyl-3-methylimidazolium acetate	Cellulase from *Trichoderma viride*, hyperthermophilic endoglucanases	Hyperthermophilic enzymes showed significantly higher 1−3-methylimidazolium acetate tolerance than *T. viride* cellulose	[110]
Carboxymethyl cellulose, pNP-cellobioside (soluble cellulosic substrates), α-cellulose (insoluble)	1,3-dimethylimidazolium dimethylphosphate, 1-allyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium chloride, 1-ethyl-3-methylimidazolium acetate	Celluclast preparation	100% (v/v) of 1,3-dimethylimidazolium dimethylphosphate was the best IL concentration retaining activity of 30% on the α-cellulose substrate.	[111]
Microcrystalline cellulose	Various imidazolium- and ammonium-based IL	Celluclast preparation immobilized onto a polymeric support (Amberlite XAD4)	Coating of celluclast preparation with butyltrimethyl-ammonium bis (trifluoromethylsulfonyl)imide maintained significant hydrolytic activity in pure 1-butyl-3−3methylimidazolium chloride.	[106]
Cotton cellulose	1-ethyl-3-methylimidazolium acetate, 1-ethyl-3-methylimidazolium phosphonate	Cellulase from *T. reesei*	An interesting compatibility of *T. reesai* cellulose was demonstrated for IL concentration in the medium from 10 to 40% v/v	[65]
Microcrystalline cellulose and hardwood dissolving pulp	1-(2-hydroxyethyl)-3-methylimidazolium acetate	Commercial cellulose (Shanghai Source Leaf Biological Technology Co., LTD)	The cellulases retained high activity in this IL for concentration of 15% v/v. At this concentration, the saccharification of straw, cotton and filter paper was significantly improved	[105]
Microcrystalline cellulose and hardwood dissolving pulp	Super base 1,1,3,3-tetramethylguanidine (TMG) and 1,5-diazabicyclo [4.3.0] non-5-ene (DBN)-based Ils and 1-ethyl-3-methylimidazolium acetate	Commercial thermos- and alkaline stabile cellulose and endoglucanase (cel5A) from *Trichoderma reesai*	Enzyme thermostability correlated with higher hydrolysis yields in IL-containing matrices, whereas activity at high pH values did not offer benefits in terms of IL tolerance.	[108]
PNP-xylose (soluble), birchwood xylan (insoluble)	1-ethyl-3-methylimidazolium acetate	GH10 xylanase from *Thermoascus aurantiacus* strain SL16W	The IL more affected the activity of the xylanase when pNP-xylose instead of xylan was used as the substrate. For pNP-xylose, enzymatic activity was reduced by 84% in medium containing 25% of IL against 37% with high loading of xylan.	[102]

Whatman No. 1 filter paper strip	1-ethyl-3-methylimidazolium acetate, 1-butyl-3-methylimidazolium acetate, 1-butyl-3-methylimidazolium chloride	Halophilic cellulases from *Aspergillus terreus* UniMAP AA-6	Halophilic cellulases produced from *A. terreus* UniMAP AA-6 exhibited higher tolerance to ILs in comparison with commercial cellulases.	[112]
Bagasse powder	Choline acetate, 1-ethyl-3-methylimidazolium acetate	Commercial cellulose Cellic CTec2 (cellulases and hemicellulases cocktail)	Choline acetate induced lower deleterious effect on cellulose compared with 1-ethyl-3-methylimidazolium acetate.	[100]
Carboxymethyl cellulose, rice straw	1-ethyl-3-methylimidazolium dimethylphosphate, 1-allyl-3-methylmidazolium chloride, 1-ethyl-3-methylimidazolium acetate	Cellulase from *Trichoderma aureoviride* strain HS	Alginate encapsulation of enzyme enhanced IL-tolerance of *T. aureoviride* cellulose. Encapsulated cellulose retained 76% of its activity at 40% (v/v) of IL concentration versus 5% for the free cellulose.	[101]
Birchwood xylan, Kraft pulp	1-ethyl-3-methylimidazolium acetate, 1-ethyl-3-methylimidazolium dimethylphosphate, 1,5-diaza-bicyclo [4.3.0] non-5-enium acetate	Thermophilic *Thermopolyspora flexuosa* GH10 xylanase	In a given reaction system, IL tolerance of the xylanase would enhance with the enzyme affinity for the substrate.	[103]
Carboxymethyl cellulose, cellulose, cellobiose, and *p*-nitrophenyl β-d-glucopyranoside, rice straw	1-allyl-3-methylimidazolium chloride, 1,3-dimethylimidazolium dimethyl phosphate	Cellulase from halophilic, alkalithermophilic *Alkalilimnicola* species	Higher performance of saccharification of cellulosic model substrate with this cellulose were achieved in presence of 10%−20% v/v of IL. Transposition to rice straw led to competitive sugar yields in this one-batch-process.	[107]
α-cellulose, cotton cellulose, cellobiose, oak and spruce sawdusts, *Miscanthus*	1-ethyl-3-methylimidazolium acetate, 1-ethyl-3-methylimidazolium methylphosphonate	Cellulose from *T. reesai*	10% (v/v) of IL in the reaction medium led to the highest yields of glucose without fractionation from sawdust. Constitutive enzymes of cellulose cocktail were differently affected by the presence of IL influencing thus their synergistic action. β and α Kamlet-Taft parameters constituted more physicochemical pertinent indicators than apparent pH value to investigate effects of IL on cellulose performances.	[59]
Wheat straw	1-ethyl-3-methylimidazolium acetate, 1-ethyl-3-methylimidazolium methylphosphonate	Cellulose from *T. reesai*, xylanases from *Thermobacillus xylanilyticus*	Significative activity of xylanase from *T. xylanilyticus* activity was retained for 1-ethyl-3-methylimidazolium acetate concentrations up to 50% v/v. Higher enzymatic saccharification performances were obtained with IL concentrations.	[87]

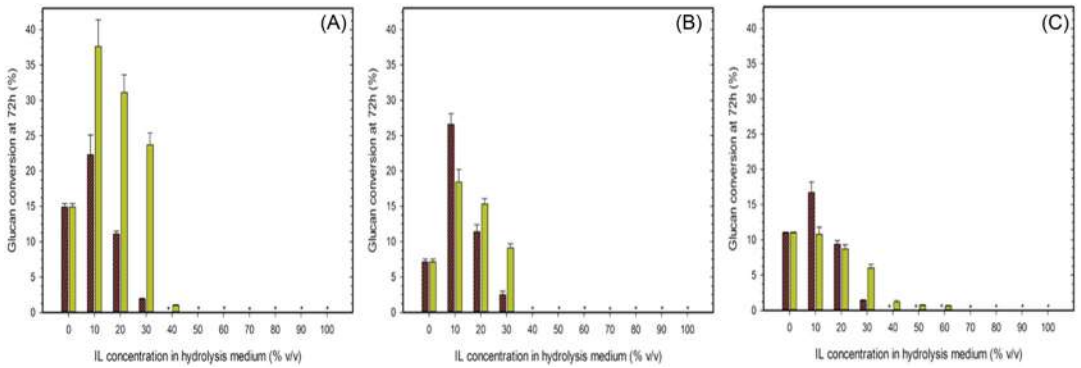

FIGURE 10.13

Glucan conversion yield (% at 72 h) obtained by saccharification of distinct LCBs catalyzed by the cellulase from *T. reesei* (5 U/mL) in aqueous-IL media for different IL concentrations. [C2mim][OAc] (*red dash bars*) and [C2mim][MeO(H)PO_2] (*green bars*). (*) No glucose concentration was detectable. (A) Spruce residues, (B) oak residues, (C) *Miscanthus x giganteus*.

Reproduced with permission from T. Auxenfans, E. Husson, C. Sarazin, Simultaneous pretreatment and enzymatic saccharification of (ligno) celluloses in aqueous-ionic liquid media: a compromise, Biochem. Eng. J. 117 (2017) 77–86. https://doi.org/10.1016/j.bej.2016.10.004.

highlighted enzymatic deactivation from the weakest IL concentration in the medium. Although 10% (v/v) of [C2mim][OAc] increased glucan conversion, the performances improvement remained very minimal compared to net aqueous media. In addition to the physicochemical properties of the IL, the nature of biomass seems thus influence considerably the efficiency of these reaction systems. Chemical composition of biomass could restrict enzymatic performances in these reaction systems, particularly for rich-xylan biomass as *Miscanthus*. A high solvation by IL could both induce a better accessibility of cellulosic fraction (disorganisation of the lignocellulosic matrix, decrease in cellulose crystallinity) but also a release of free xylan in the medium affecting cellulase adsorption [114].

In addition to the chemical composition of LCB, supramolecular structure of constitutive cellulose seems to influence performances of these enzymatic systems. Indeed, cellulase-catalyzed hydrolysis on three model cellulosic substrates in presence of ILs led to different performances depending to the respective crystallinity indexes (CrI) of the substrates [29,59]. For α-cellulose (CrI = 25%), whatever the studied IL, ([C2mim][OAc] or [C2mim][MeO(H)PO_2]), their presence in the reaction medium led to a progressive decrease in glucose yield with IL concentration increase, although the enzymatic deactivation was less marked with [C2mim][MeO(H)PO_2]. Similar behavior was also observed with a highly digestible cellulose (CrI < 5%). Only enzymatic system containing long fibers of cellulose from cotton (CrI = 73%) in presence of various concentrations of [C2mim][MeO(H)PO_2] evidenced a key IL concentration (10% v/v) allowing the improvement of performances in comparison with those obtained in a net aqueous medium.

The synergistic action of cellulolytic enzymes in a cellulase mix would be thus influenced by (i) the lignocellulosic matrix, (ii) the supramolecular structure of constitutive cellulose and (iii) the constitutive anion of the selected IL. The performances of in situ enzymatic hydrolysis of (hemi)

cellulosic substrates in presence of IL may result from a compromise between a better accessibility of the substrate and deactivation effects on enzyme, both induced by IL solvation (Fig. 10.14). It could be suggested that the implementation of cellulase-catalyzed hydrolysis in aqueous-IL solution would be more suitable for recalcitrant substrates. A highly digestible substrate in this kind of system would therefore only evidence deleterious effects of IL on the enzyme.

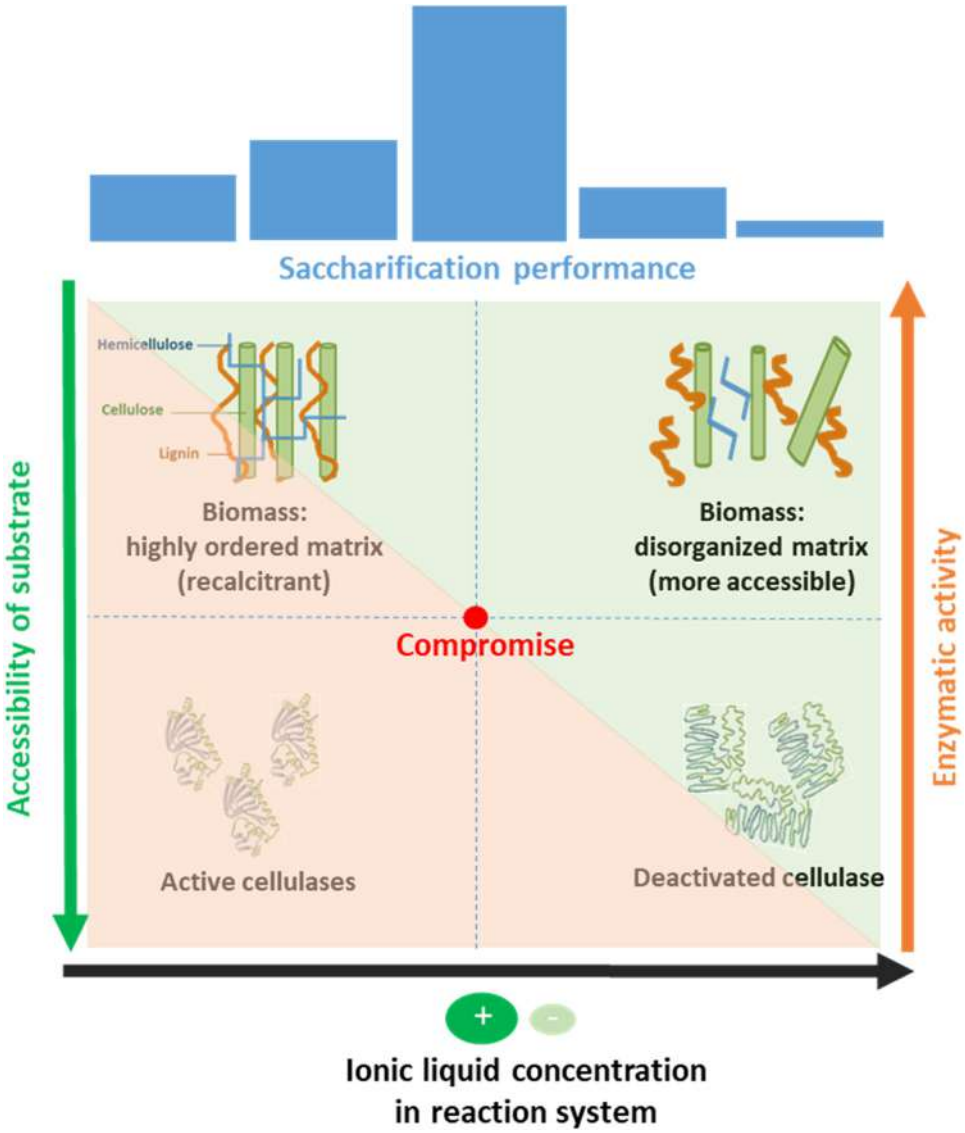

FIGURE 10.14

Simplified view of the proposed compromise governing the performances of in situ cellulase-catalyzed hydrolysis of lignocellulosic biomass in aqueous-IL system.

It is important to point out that individual cellulolytic enzymes contained in a cellulase mix displayed different IL-tolerance, respectively. For example, β-glucosidases activity would be particularly sensitive to the presence of [C2mim][OAc] in opposite to endoglucanases or cellobiohydrolases [59]. IL-deactivation of cellulases could be thus circumvented by modifying individual enzymes proportions in a given cocktail.

Endo-xylanases (EC 3.2.1.8) act on β-1,4 glycosidic bonds between xylopyranoside residues in xylans (main hemicellulose constituent) and liberated mainly xylooligosides [115]. Xylanases from GH11 family preferentially act between two unsubstituted xylose residues whereas xylanases from GH10 family can hydrolyze linkages between two xylose residues with one harboring a substituent [116]. Literature reports also studies about the selective depolymerization of hemicellulosic fraction from LCB catalyzed by xylanases in presence of ILs.

For instance, the tolerance of an *endo*-xylanase (GH11) from *Thermobacillus xylanilyticus* was investigated in reaction systems containing wheat straw as substrate and different [C2mim][OAc] concentrations [87]. From 10% v/v to 50% v/v of [C2mim][OAc], enzymatic activity were significantly higher than those obtained in the net aqueous medium with a gradual increase until 30% v/v. Above 50% v/v, enzyme deactivation due to larger amount of IL occurred. This promising compatibility, greater for *endo*-xylanase than for cellulases, could be related to the hyperthermophilic properties of enzyme source (*T. xylanilyticus*). Similar tolerance to [C2mim][OAc] was also described in the literature for xylanase produced by other thermophilic microorganisms such as *Dictyoglomus thermophilum* or *Sporotrichum thermophile* [117,118]. Although thermostability appears to be a pertinent indicator to predict the compatibility with hydrophilic ILs, it alone does not guarantee a high tolerance. The mechanisms describing how ILs could affect xylanase activity are not yet well defined. Comprehensive studies based on molecular dynamics simulations provided nevertheless some pertinent hypotheses about it. In a previous study, higher concentrations of IL may correlate with slight conformational changes from the initial crystallographic structure of xylanase GH11 from *Trichoderma longibrachiatum*. This suggested that the decrease in enzymatic activity in presence of IL would thus not be due to denaturation. A consensus seems to be established around both drastic effects induced by IL on enzyme dynamic motion even at low IL concentrations, and constitutive cations from IL acting as competitive inhibitor (Fig. 10.15) [119]. This explanation correlates

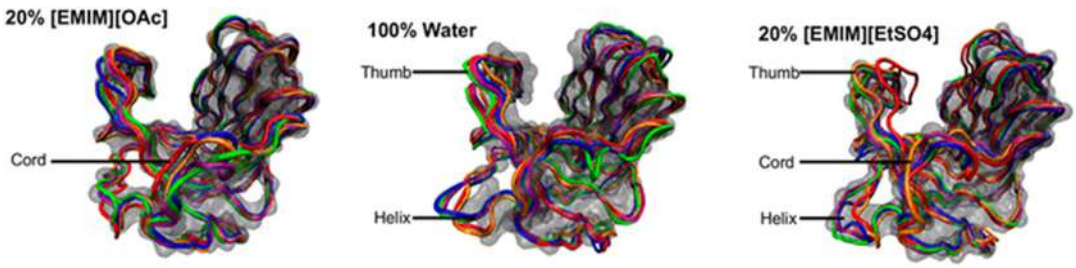

FIGURE 10.15

Snapshots of the molecular dynamic trajectories (310K) of xylanase GH11 from *Trichoderma longibrachiatum*. Areas of interest are indicated. Gray cloud (minimized structure). Red (100 ns). Orange (200 ns). Green (300 ns). Blue (400 ns). Violet (500 ns).

Reproduced with permission from V.W. Jaeger, J. Pfaendtner, Structure, dynamics, and activity of xylanase solvated in binary mixtures of ionic liquid and water, ACS Chem. Biol. 8 (2013) 1179–1186. https://doi.org/10.1021/cb3006837.

with the idea that substrate affinity and loading would be potential factors participating to the preservation even the improvement of enzymatic activity of xylanases in presence of hydrophilic ILs [102,103].

10.3.3 Focus on enzymatic depolymerization of lignin in aqueous-ionic liquid solutions

Phenolic compounds issued from oxidative enzymatic depolymerization of lignin constitute precious building-blocks for various applications such as food, plastic, or pharmaceuticals [120]. Among the large panel of lignin degrading enzymes (peroxidases, oxidases), the laccases (E.C. 1.10.3.2) seem to be the more suitable oxidoreductases for the development of lignin degradation processes. The main reasons would be that laccases constitute multi-copper-containing enzymes requiring molecular oxygen as electron acceptor rather than H_2O_2 as well the other lignolytic enzymes and exhibits a wide range of phenolic and non-phenolic substrates [121,122]. However, the performances of laccase-catalyzed degradation of lignin remains governed by the respective redox potentials of enzyme and substrate. Although laccases, in aqueous medium, directly oxidize the phenolic subunits of lignin (minor part in lignin), the non-phenolic units (major part of lignin) are particularly recalcitrant due to their high redox potentials (>1500 mV) as compared to those of laccases [123–125]. Indeed, laccases can be classified according to their T1 site (a region of the active site containing Cu coordinated by two histidines, one cysteine and an axial ligand) as low ($<+500$ mV), medium (from $+500$ to around $+700$ mV) and high (from $+720$ to $+800$ mV) redox potential laccases [126]. To overcome this redox potential difference not conducive to spontaneous oxidoreduction reaction, enzymatic oxidative degradation of lignin can be implemented in presence of mediators for example, additional small aromatic molecules acting as electron shuttles such as 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS), phenolic lignin dimer (GBG) or 1-hydroxybenzotriazole, with intermediate redox potential [127]. The scheme of the reaction catalyzed by laccase or LMS is presented (Fig. 10.16).

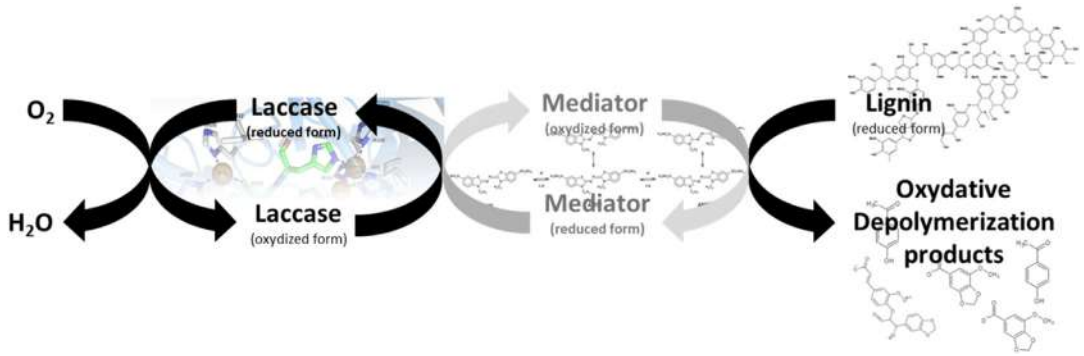

FIGURE 10.16

Simplified view of the oxidative depolymerization scheme of lignin catalyzed by either single laccase (in black) or laccase-mediator system (in black and gray).

However, the efficiency of laccase-catalyzed depolymerization of lignin is limited by the very low lignin solubility in the aqueous medium preserving laccase activity [128]. One alternative to this scientific and technological constraint would be to add organic co-solvents for solubilizing lignin. However, the conventional solvents that dissolve lignin (dimethylsulfoxide, *N,N*-dimethylformamide, acetone, dioxane) can induce laccase deactivation for some of them and do not answer to the current environmental requirements [129,130]. In this context, the substitution of organic co-solvent in LMS by IL is emerging more and more in the literature [131–134]. For oxidative lignin depolymerization, the challenge consists thus to rationally select an appropriate IL improving lignin solubility in the LMS while maintaining enzymatic activity of laccase [125,132]. A study reported that kinetic performances of the laccase–mediator system for the oxidation of lignin model compounds were higher in the net aqueous solution in comparison with reaction media containing 5, 15, or 30% (v/v) of 1-ethyl-3-methylimidazolium ethylsulfate ([C2mim][$EtSO_4$]) and [C2mim][OAc] [134]. In other paper, the laccase from *Trametes versicolor* was demonstrated to be 10-fold more tolerant to [C2mim][$EtSO_4$] than to 1-butyl-3-methylimidazolium chloride ([C4mim][Cl]) [135]. To better understand IL-laccase interactions, Stevens et al., proposes a comprehensive study of IL-laccase interactions in LMS for developing efficient lignin degradation routes [132]. The effects of three distinct ILs: diethylamine hydrogensulfate ([DEA][HSO_4]), [C2mim][OAc] and choline lysinate ([Ch][Lys]) on the performances of laccase from *T. versicolor* in a LMS were finely investigated. Fig. 10.17

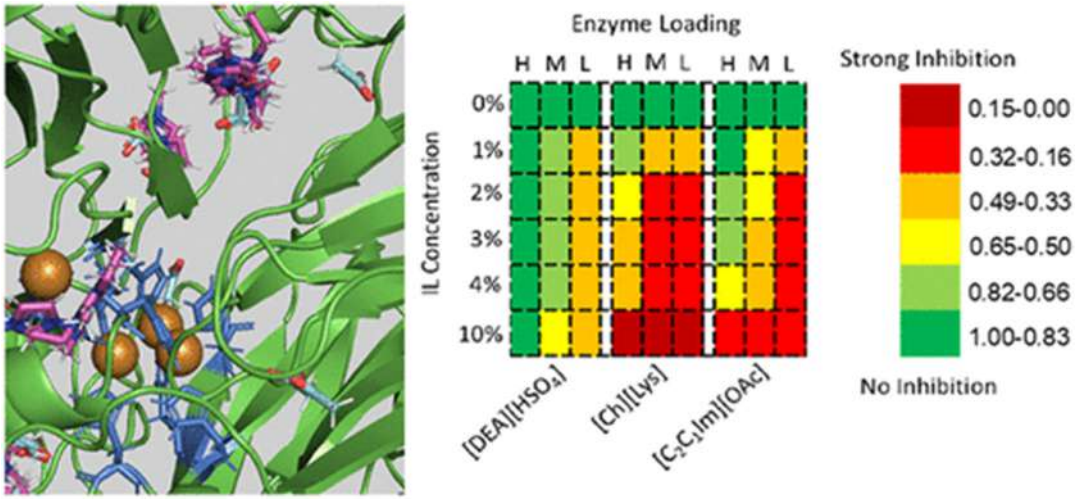

FIGURE 10.17

Activity of laccase from *Trametes versicolor* in IL solutions. *H*, high laccase concentration (3.0 μg mL^{-1}), *M*, medium laccase concentration (1.2 μg mL^{-1}), L = low laccase concentration (0.6 μg mL^{-1}). Values indicate the ratio of TvL activity in ILs relative to TvL activity in net aqueous buffer. Inhibition effect is also represented in function of IL-concentration.

Reproduced with permission from J.C. Stevens, L. Das, J.K. Mobley, S.O. Asare, B.C. Lynn, D.W. Rodgers, et al., Understanding laccase–ionic liquid interactions toward biocatalytic lignin conversion in aqueous ionic liquids, ACS Sustain. Chem. Eng. 7 (2019) 15928–15938. https://doi.org/10.1021/acssuschemeng.9b02151.

presents the activity of this enzyme depending to the IL concentration in LMS. Among these three distinct ILs, only [DEA][HSO_4] did not affect the oxidation of mediator by the laccase. [C2mim][OAc] and [Ch][Lys] were suggested to be inhibitor from the lowest IL concentration. Docking simulation and kinetic studies suggested that these two ILs may bind close to the active site limiting both suitable interaction with substrates due to steric hindrance and effective progress of the catalysis.

Thermostability of laccases would not be exclusively the pertinent indicator to predict enzyme tolerance in hydrophilic ILs. Indeed, a recent study has shown that 2% (w/v) of [C2mim][OAc] in an aqueous medium induced a drastic decrease ($>50\%$) in activity of the laccase from *Thermus thermophilus*, enzyme exhibiting an optimal temperature around 90°C in native conditions [136]. Overall, the development of efficient laccase-catalyzed depolymerization of lignin in presence of ILs still requires prospecting of new sources of enzymes, medium engineering approaches for a rational selection of suitable ILs and/or direct evolution to provide better tolerance of laccase to ILs [121,128]. To date, literature data reveals a consideration lack of the thermophysical properties of IL-aqueous solution (density, viscosity, electrical conductivity, thermal conductivity) to achieve a better understanding of lignin depolymerization in these nonconventional LMS [137].

10.3.4 Expanding enzymatic transformation in ionic liquid-aqueous media to chitinous biomass

The promising performances of in situ enzymatic transformation of constitutive polymers from LCB in the presence of IL led to the transposition of this strategy for valorizing chitinous biomass. One way of valorization consists of the hydrolysis of chitin catalyzed by chitinases for generating chito-mono/oligosaccharides such as *N*-acetylglucosamine (DP1), *N,N′*-diacetylchitobiose (DP2) or larger chito-oligomers (COS) as platform molecules or bioactive oligomers [138]. Chitinases cocktail are composed of endochitinases (E.C. 3.2.1.14) which act randomly in the internal regions of chitin and generate DP2 and the soluble low molecular mass oligomer of *N*-acetylglucosamine such as chitotriose and chitotetraose; chitobiosidases (E.C. 3.2.1.29) which hydrolyze non-reducing chain extremities of chitin microfibrils, releasing DP2 and β-1,4-glucosaminidases (E.C. 3.2.1.30) which cleave the oligomeric products of endochitinases and chitobiosidases into DP1 [139]. Similarly, to LCB transformation, the proof of concept to produce selectively DP1, DP2, or COS with very competitive yields from chitin by enzymatic way after IL-pretreatment has been recently demonstrated [16,85]. In addition, ILs were showed as interesting and sustainable alternative for chitin extraction from crustaceous and insect biomasses and for pretreatment of chitin prior to its deacetylation for producing chitosan production [54,140]. Therefore, it seemed relevant to investigate the feasibility to implement chitinase-catalyzed depolymerization of chitin in the presence of IL. To our knowledge, only one paper from the literature reports such a study [85]. This one described one-pot enzymatic hydrolysis of chitin catalyzed by either chitinase from *T. viride* or *S. griseus* in [C2mim][OAc]-aqueous medium. Yields of DP1 or DP2 obtained after 24 h of reaction in these nonconventional media are presented in Fig. 10.18.

The presence of IL in aqueous media did not modulate the strong selectivity of each enzymatic cocktail: chitinases from *T. viride* generated mainly DP1 and the main product obtained with chitinases from *S. griseus* was DP2 as in net aqueous medium. From 5 to 20% v/v of IL, the achieved yields were always superior to those obtained without IL. The increase in yield could be due to a compromise between beneficial effect of IL on structural disorganization of chitin (loss of fiber

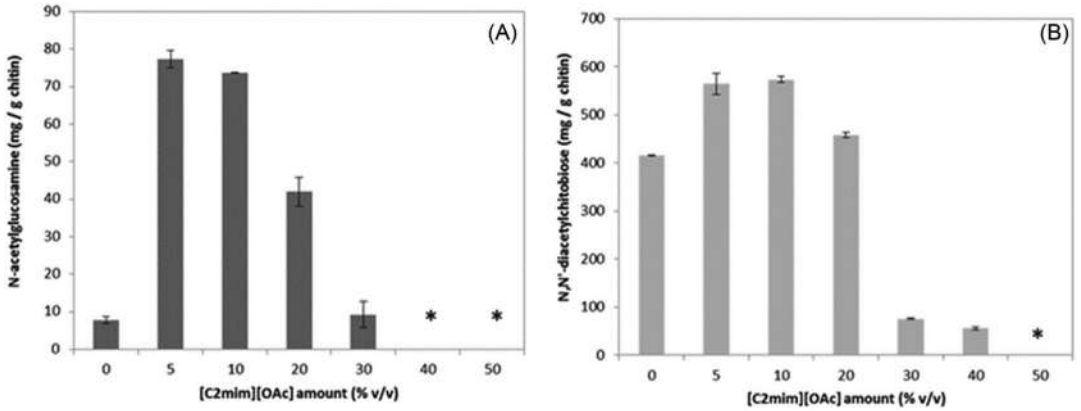

FIGURE 10.18

N-acetylglucosamine or *N,N*′-diacetylchitobiose yield (mg/g chitin) at 24 h of enzymatic hydrolysis of chitin catalyzed by the respective chitinase from *T. viride* (A) or *S. griseus* (B) in [C2mim][OAc]-aqueous media for different concentrations. **N*-acetylglucosamine or *N,N*′-diacetylchitobiose concentration was not detectable.

Reproduced with permission from E. Husson, C. Hadad, G. Huet, S. Laclef, D. Lesur, V. Lambertyn, et al., The effect of room temperature ionic liquids on the selective biocatalytic hydrolysis of chitin via sequential or simultaneous strategies, Green Chem. 19 (2017) 4122–4131. https://doi.org/10.1039/C7GC01471F.

organization, intra-crystalline disruptions), leading to better accessibility to enzyme, and deactivation effect of chitinase as described for hemicellulolytic enzymes in similar reaction media. Therefore, it seems wise to consider the transposition of this simultaneous strategy in chitin biorefinery.

10.4 Biopolymer esterification: a promising alternative of lignocellulosic biomass valorization

As the most abundant and valuable biopolymer derived from LCB, cellulose has gained increased attention in industrial applications due to its low cost, chemical stability, environmental friendliness, and recyclability [141,142]. Indeed, cellulose serves as a versatile resource for derivatized materials such as fibers, films, composites, fuels, and chemicals [143]. As well, lignin holds enormous potential as a renewable feed- stock for upgrading to a mass of high-value compounds [144]. Nonetheless, this polymer is largely undervalued, and its applications are limited to low-added value products. Most of the lignin is burned as low-value fuel and only 2% is intended for the manufacture of low-value products, such as dispersing or binding agents [145,146].

Until now, we have focused on ILs pretreatment step of LCB to make its constituent polymers -cellulose-hemicellulose and lignin- more accessible for subsequent depolymerization reactions and valorization processes to produce platform molecules of high industrial value. But besides this approach, there are other strategies to valorize LCB polymers based on the functionalization of their reactive functional groups. The functionalization of polymers has gained much attention from different industrial sectors dedicated to the production of materials, and the esterification route is positioned

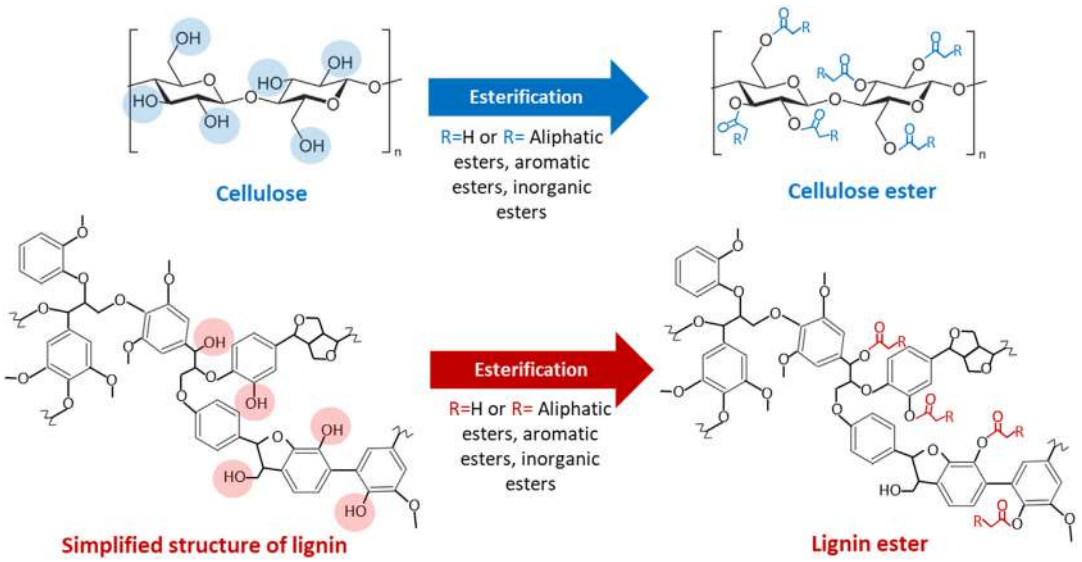

FIGURE 10.19

Schematic representation of potential reactive functional groups of cellulose and lignin to perform esterification reactions.

as an efficient approach to transform LCB into value-added bio-based materials [147]. Indeed, esterification is a 100-year-old technology applied to make cellulose processable and useful [141].

Cellulose and lignin can be esterified on hydroxyl functional groups as shown in Fig. 10.19. Cellulose is a homopolysaccharide of β (1→4) D-glucosyl residues, resulting in a linear chain polymer composed of anhydrous glucose units, each one containing three hydroxyl groups available for substitution [148,149]. The resulting cellulose esters are particularly attractive for their applications in the field of bioplastics such as fibers, films, and filters [141].

Lignin has phenolic hydroxyl groups and aliphatic hydroxyl groups at C-α and C-ϒ positions on the side chain. The abundance of the chemical sites offers different possibilities for chemical modification and suggests that lignin could play a central role as a new chemical feedstock, particularly in the formation of supramolecular architecture and aromatic chemicals [150,151]. Most of the applications based on esterified lignin are dedicated to the synthesis of polyesters, epoxy resins and elastomeric materials [151]. Esterification of lignin is a widely used method to improve the compatibility with polymers, for example, the miscibility in apolar polyolefin matrices, enhancing the production of bio-based composites [152,153].

Recently, the direct use of LCB or lignocellulosic agro-forestry wastes to produce LCB-based plastics has also established itself as an alternative to the conventional production of biomass-based plastics. Indeed, Chen and collaborators [147] as well as Suzuki and collaborators [141] reported a direct conversion of sugarcane bagasse into an injection-moldable cellulosic thermoplastic having effective mechanical properties. The homogeneous esterification of poplar wood enhanced the compatibility and improved the processability of wood with synthetic polymers [154].

10.4.1 Chemical esterification versus bio catalysis

Chemical esterification is the traditional method to produce polymer esters using organic solvents as the reaction media [142,148]. Cellulose can be acylated by fatty acid chlorides dispersed in organic solvents such as dimethylsulfoxide (DMSO), acetone, chloroform, toluene, DMF or DMSO/TBAF, in the presence of pyridine or sulfuric acid as catalyst [142,148]. For lignin esterification, tetrahydrofurane, 1,4-dioxane or *N*-methyl pyrrolidone dioxane are the most widely used solvents together with chemical reagents such as thionyl chloride ($SOCl_2$) [152].

Nevertheless, the use of organic solvents to carry out esterification are volatile and toxic, resulting in environmental and safety problems and thus limiting the commercial development of long chain cellulose and lignin esters. Not to mention the high reaction temperatures, the extreme pH, the production of by-products and salts and the uneven distribution of substituents during chemical esterification [142,148,155].

The drawbacks of the chemical route are not in agreement with the current environmental requirements and green chemistry framework [152], which have promoted the pursuit of greener and sustainable alternatives to perform esterification reactions. Herein, bio catalysis is a greener and simpler route to perform chemical transformations and it has been considered as an attractive pathway for organic synthesis [156].

Among biocatalysts, lipases (triacylglycerol hydrolases, EC 3.1.1.3) have been used to carry out esterification reactions [156]. The natural function of lipases is to catalyze hydrolysis of fatty acid esters in aqueous media. Particularly, in nonaqueous media, they are able to catalyze the reverse reaction of hydrolysis, including esterification and transesterification on various biomolecules such as alcohols, amino-alcohols, peptides, flavonoids or cyclodextrins [155,156].

Lipases prefer hydrophobic solvents in which they can preserve the active site conformation of their proteins and therefore maintain their catalytical performance. Hexane, t-amyl alcohol, and some ILs, have proven to maintain the activity of lipases [156]. Consequently, hydrophobic solvents are chosen for better lipase performance, in which, however, the polar or hydrophilic substrates as cellulose cannot be dissolved [157].

Two main constraints may govern the application of lipases in catalyzing the modification of LCB polymers. First, the substrate solubility which limits considerably its availability for enzyme reactivity/accessibility on the hydroxyl groups. Secondly, the stability of the catalytical activity of lipases in the reaction media. To overcome this problem, ILs have been widely used over the past two decades for enzymatic conversion of substrates [158]. A medium engineering approach coupled with a rational choice of biocatalysts have been found suitable for finding the appropriate solution allowing both substrate availability and high enzyme activity [155].

10.4.2 Nonconventional reaction media for enzymatic esterification of lignocellulosic biomass polymers: a double benefit

Besides the interesting properties of ILs such as low vapor pressure, recyclability, low toxicity, non-inflammable, and high thermal stability [144,155,156], one of the great advantages of using ILs for enzymatic reactions is their double functionality: the solubilization of different substrates—especially those substrates that are insoluble or sparingly soluble in water and common organic solvents—and the preservation of the catalytical activity of enzymes [142].

ILs are promising solvents to carry out cellulose and lignin esterification with lipases. Indeed, some hydrophobic or water-miscible imidazolium-based ILs have improved the activity of lipases and also the efficiency of enzymatic esterification of various alcohols [155]. ILs can play a similar role as an organic solvent in influencing enzyme function as follows: (1) the IL replaces the water surrounding the enzyme; (2) when entering the micro aqueous phase, the IL interacts with the enzyme by modifying the conformation, dynamics, or active site; and (3) the IL interacts with the products and substrates, by reacting with or changing their partitioning between the nonaqueous and aqueous phases [158].

There is wealth of research about chemical esterification of cellulose [148,159–161], hemicellulose (xylans) [162,163], industrial lignin [152,164] and direct LCB [141,147,154] in ILs. Even though ILs are positioned as powerful and promising media for homogeneous esterification of polymers, the enzymatic esterification of these substrates in IL reaction systems seems to be new and unexplored.

To our knowledge, the first direct enzymatic esterification of the cellulose polymer (Avicel cellulose) with long chain fatty acids dates back to 2011 [165]. Still, the use of IL as co-solvent for the enzymatic esterification was not evaluated during this research. Later, Hulin and collaborators [155] described for the first time the lipase-catalyzed transesterification of commercial Kraft lignin (Indulin AT) with ethyl oleate in a nonconventional IL medium. The synthesized Kraft lignin-oleate exhibited new interesting textural and thermal properties different from those of the original Kraft lignin, along with a softer and more porous structure. Subsequent, the first preparation of esterified cellulose nanocrystals (E-CNCs) with long-chain fatty acids from cellulose in a one-pot enzymatic method was reported by Zhao and collaborators [157]. The produced E-CNCs have an improved crystallinity and reduced thermal stability compared with native cellulose, showing wider application prospects. Then, Wang and collaborators [142] evaluated different key reaction parameters of enzymatic synthesis of long-chain cellulose esters in mixed ILs. The acylation modification significantly changed the morphology, solubility, crystallinity and thermal properties of cellulose. Table 10.2 summarizes the studies regarding the preparation of cellulose and lignin esters through enzymatic esterification in ILs.

The conditions affecting the performance of enzymatic esterification reactions of LCB polymers—cellulose and lignin—in ILs are highlighted in these studies: type of IL (hydrophilic or hydrophobic), mass ratio of hydrophilic IL and hydrophobic IL in a binary ILs system, biocatalyst, acylating agent length, mass ratio of biocatalyst and substrate, temperature, and reaction time. Fig. 10.20 shows a binary ILs system to carry out enzymatic esterification on LCB polymers.

First, lignocellulosic polymers are dissolved in hydrophilic ILs at high temperatures, polymers not only are dissolved in the liquid medium, but the pretreatment with the IL makes them more accessible for the subsequent esterification reaction. Then, temperature is decreased to the optimal set value for the catalytical reaction (generally at 50°C for lipases) and the co-solvent, usually a hydrophobic IL, is added at a given mass ratio of Hydrophilic IL: Hydrophobic IL. Biocatalysts and acylating agents are added and the enzymatic reaction takes place under set conditions.

The hydrophilic ILs 1-butyl-3-methylimidazolium chloride ([C4mim][Cl]), 1-butyl-3-methylimidazolium hydrogen sulfate ([C4mim][HSO_4]), 1-allyl-3-methylimidazolium chloride ([Amim][Cl]) and 1-butyl-3-methyl-imidazolium trifluoromethanesulfonate [C4mim][OTf], are shown as suitable media to dissolve different cellulosic substrates and lignin, and to make them more accessible and available for the enzymatic esterification by lipases [142,155,157,165]. Among

Table 10.2 Summarize of enzymatic esterification studies of cellulose and lignin in ILs.[a]

Substrate	Acylating agent	Enzymes	Ionic liquids	Main results	References
Avicel cellulose PH-101	Fatty acid vinyl esters: Vinyl propionate Vinyl laurate Vinyl stearate	Lipases from: *Candida antarctica* *Candida cylindracea* *niger* *antarctica* (Immobilized lipase) Immobilized esterase from hog liver and immobilized Cutinase from *Fusarium solani pisi*	To pretreat cellulose: [C4mim][Cl] Enzymatic esterification: Vinyl propionate Vinyl laurate Vinyl stearate	Immobilized esterase from hog and immobilized cutinase from *Fusarium solani* where the only enzymes capable of catalyzing the acylation reaction For any acyl donor, the degree of esterification is no superior than 1.9% (for cellulose propionate)	[165]
Kraft lignin (Indulin AT)	Ethyl oleate (C18:1)	Immobilized lipases from: *C. antarctica* (CALB) *P. cepacia* (PCL) *Mucor miehei* (MML)	Single ILs: [C4mim][$MeSO_4$] [C4mim][OTf] [C4mim][PF_6] Binary ILs system: [BMIm][OTf]: [BMIm][PF_6]	Higher esterification yield with the binary ILs system with CALB enzyme (27.3%) Improved esterification performance for all [C4mim][PF_6]-coated immobilized lipase in [C4mim][OTf] systems, 25% for CALB lipase, 15.8% for MML lipase and 22% for PCL lipase	[155]
Microcrystalline cellulose (MCC)	Methyl laurate (C12:0 methyl ester)	Lipase from:*Candida rugosa*, type VII	Single IL for CNCs production: [C4mim][HSO_4] Binary ILs system for E-CNCs production: [C4mim][HSO_4]: [C4mim][BF_4]	Higher DS values when: Mass ratio [C4mim][HSO4]:[C4mim][BF_4] = 3:5. Molar ratio of methyl laurate:AGU = 11:1 Mass ratio lipase:cellulose = 2:10. 78% of esterification yield of E-CNCs (esterified cellulose nanocrystals)	[157]
Mycrocrystalline cellulose (MCC)	Methyl laurate (C12:0 methyl ester) Methyl palmitate (C16:0 methyl ester) Methyl stearate (C18:0 methyl ester)	Lipase from:*C. rugosa*, type VII	Binary ILs system between: [C4mim][Cl] [Amim][Cl] [C4mim][BF_4] [C4mim][PF_6]	Higher DS values when: Mass ratio [C4mim][Cl]:[C4mim][BF_4] = 6:4 Acylating agent = methyl laurate Mass ratio lipase/cellulose = 4:20 DS values vary from 0.213 to 1.452 depending on the acyl donor	[142]

[a][Amim][Cl], *1-allyl-3-methylimidazolium chloride;* [C4mim][BF_4], *1-butyl-3-methyl-imidazolium tetrafluoroborate;* [C4mim][HSO_4], *1-butyl-3-methylimidazolium hydrogen sulfate;* [C4mim][$MeSO_4$], *1-butyl-3-methylimidazolium methylsulfate;* [C4mim][OTf], *1-butyl-3- methylimidazolium trifluoromethanesulfonate;* [C4mim][PF_6], *1-butyl-3-methylimidazolium hexafluorophosphate;* DS, *Degree of substitution;* [C4mim][Cl], *1-butyl-3-methyl-imidazolium chloride.*

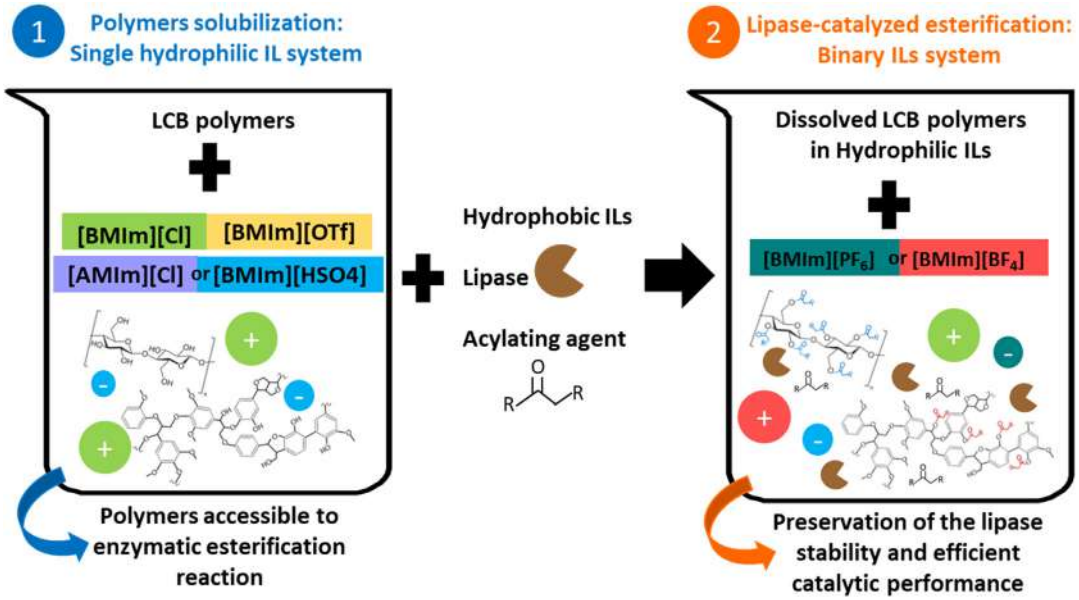

FIGURE 10.20

Binary ILs system to carry out enzymatic esterification reactions of polymers from LCB.

the known hydrophobic ILs, 1-butyl-3-methylimidazolium tetrafluoborate ([C4mim][BF_4]) and 1-butyl-3-methylimidazolium hexafluorofosfate ([C4mim][PF_6]), seem to be promising candidates as co-solvent due to the improved lipase performance in binary ILs system with cellulosic and lignin substrates [142,157].

The ratio of the hydrophilic IL and the hydrophobic IL may affect both, the substrate dissolution, and the enzyme activity, and therefore a compromise between these two crucial aspects is required. For example, the [C4mim][OTf] allowed the efficient solvation of lignin by increasing the nucleophilicity of its hydroxyl groups, and the [C4mim][PF_6] preserved or improved the activity of some lipases. This exposes the synergistic effect induced by two ILs in a binary system [155].

With cellulose derivate substrates, the hydrophilic [C4mim][HSO_4] can have double function. First, the production of nanocrystals by promoting the hydrolytic cleavage of the glycosidic bonds and releasing individual crystallites, and secondly, acting as co-solvent for the enzymatic esterification with the hydrophobic [C4mim][BF_4]. In this binary ILs system, cellulose had higher solubility and the catalytic activity of lipases were preserved [156]. Increasing the mass ratio of [C4mim][HSO_4]:[C4mim][BF_4] from 3:1 to 3:5 in this system, may increase the degree of substitution (DS) of cellulose nanocrystals, but higher concentrations of IL [C4mim][HSO_4] may deactivate enzymes by stripping essential water from the protein molecule [157].

Another binary ILs system composed by [Amim][Cl]:[C4mim][BF_4] allowed to dissolve rapidly the cellulose substrate, but the esterification reaction efficiency was lower compared to the binary ILs system constituted by [C4mim][Cl]:[C4mim][BF_4] ILs. Increasing the ratio of [C4mim][Cl] in this binary system improved the solubilization of cellulose, associated again to the destruction of the

semi-crystalline structure of cellulose or the reduction of the steric hindrance between the enzymes and the hydroxyl groups of cellulose, improving substrate access to the active site of lipases. However, mass ratio of [C4mim][Cl] greater than 6:4 led to a rapid decrease in cellulose esterification, showing the negative effect of higher mass ratio of hydrophilic IL on the enzymatic reaction [142].

In the case of lignin substrate, three imidazolium-based ILs differing in their constitutive anions—1-butyl-3-methylimidazolium methylsulfate ([C4mim][$MeSO_4$]), [C4mim][OTf] and [C4mim][PF_6]—were able to solubilize this polymer and improve the activity of immobilized lipases [155]. Nevertheless, the ester bond of the methylsulfate anion of [C4mim][$MeSO_4$] is hydrolytically unstable, releasing methanol CH_3OH and hydrogen sulfate anion [HSO_4], which is not suitable in the reaction medium [155]. The synergistic effect induced by the ILs in a binary system [C4mim][OTf]: [C4mim][PF_6] is also observed. The [C4mim][OTf] allowed the efficient solvation of lignin by increasing the nucleophilicity of its hydroxyl groups, and the [C4mim][PF_6] improved the activity of lipases [155]. In addition, a hydrophobic IL-coated lipase in hydrophilic IL system is proposed to reduce the use of hydrophobic IL. Indeed, the [C4mim][PF_6]-coated immobilized lipase in [C4mim][OTf] improved the esterification yield of immobilized lipases. This may be due to a better adsorption of [C4mim][PF_6] on their respective carriers leading to higher catalytic performance and lipase stability [155].

The dependence on the biocatalyst during enzymatic esterification is also a factor to consider when performing enzymatic esterification reactions. Oppositely to cutinase and esterase, lipases were not able to catalyze the esterification reaction of cellulose in a fatty vinyl ester reaction media [165]. The authors associated this finding to the ability of lipase to act preferentially on hydrophobic substrates. Indeed, immobilized CALB lipase provided superior efficiency in both binary and IL-coated systems for lignin esterification, among the evaluated lipases [155].

The type and length of acylating agent also play an important role on enzymatic esterification. It not only affects the reaction rate but also influences the DS and the properties of the synthetized products. In particular, Wang and collaborators [142] used methyl esters with different chain length (from 12 to 18 carbons) as acylating agents and the authors found that increasing the chain length of the acylating agents, decreases the DS value. This can be explained by the greater hindrance of the substituents with longer fatty acid chains and increased bulkiness. Even though the methyl palmitates provided a slightly lower DS value, the resulting cellulose palmitate possessed better properties than cellulose laurate, such as higher solubility in organic solvents and better melt processing [142].

If we explore other biomass apart from LCB, chitin is the second most abundant polysaccharides after cellulose, and it has received much attention because of its composition and reactivity to produce high-value compounds: chitio-oligosaccharides or chitosan-based materials [144]. The vast number of intermolecular and intramolecular hydrogen bonds make chitin a recalcitrant biopolymer hard to dissolve in traditionally aqueous solvents. In addition, the traditional chemical way to prepare chitin from shrimp/crab shells usually consumes plenty of water and is less eco-friendly [144]. Pretreatment of chitin with [C2mim][OAc] has exhibited great chitinase-catalyzed hydrolysis efficiency, presenting a promising route to decompose chitin [85].

Besides chitin fractionation, another study has focused on its modification to improve the physicochemical properties of chitosan. Zhao and collaborators [156] proposed for the first time a facile and efficient method to produce long-chain chitosan esters using CALB lipase in a binary ILs system composed by [C2mim][OAc]:[C4mim][BF_4]. The transesterification of chitosan was achieved

with high DS and selectivity in a homogeneous phase and the produced chitosan palmitate may be of interest for development of bioactive chitosan derivates, because of its unique structures (selectively acylated at 6-OH groups of chitosan). Namely, long-chain fatty acid chitosan esters are important chitosan products with attractive application prospects in biological and biomedical applications [156].

So far, the investigations concerning enzyme esterification of LCB polymers and chitosan have shown a mild enzymatic strategy in nonconventional reaction medium as a potential pathway to produce biomaterials. In addition, the advantages of using ILs as solvents for this biocatalytic reaction, fulfilling diverse functions, are underlined. These promising results provide leads for further research works using direct LCB or lignocellulosic agro-forestry as substrates for enzymatic esterification, with the aim of multiplying the current uses of LCB.

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CHAPTER

Biocatalysis in ionic liquids for a low carbon future

11

Andrew Craig Marr, Hasan Tanvir Imam and Patricia Catherine Marr

School of Chemistry and Chemical Engineering, Queen's University Belfast, Belfast, Northern Ireland

11.1 Introduction

The effects of atmospheric gases generated by human activity on the global climate are now well established and widely accepted. The origin of atmospheric CO_2 release lies in our dependence on carbon-based fossil fuels for energy and chemicals. Humans have become addicted to coal, oil, and gas extracted from beneath the Earth's surface, as they provide the heat, electricity, light, transportation fuel, and much of the consumer products, including medicines, upon which we rely.

Cutting our link to fossil fuels and forging a more sustainable human society that protects the planet will not be easy, but it has become vital. There will not be one dominant technology that will deliver a low carbon future, and many of the scientific and engineering innovations of the past decades must be developed in parallel. In this chapter, the potential roles for biocatalysis in ionic liquids (ILs) will be explored. The ability of biocatalysts used in conjunction with ILs to perform reactions of importance to the low carbon economy has been demonstrated in multiple areas, including the production of chemicals from biomass and the production of biodiesel. Examples of this will be discussed, and further possible applications are suggested.

11.2 Biocatalysis in ionic liquids

In the 21st century, whole-cell and isolated enzyme biocatalysts have a leading role in the drive for sustainability and a lower carbon future. Biocatalysis is inherently lower energy than chemocatalysis, as there are no metals to mine, and no requirement for the synthesis of catalysts, ligands, or modifiers from petrochemicals. A biocatalyst can be grown from natural building blocks. A relative disadvantage of biocatalysts is their comparative fragility, and this is one area in which ILs have been shown to be valuable. We have recently reviewed the applications of ILs to whole-cell and isolated enzyme biocatalysis [1].

ILs have been used in many ways to augment and improve biocatalysis, including their use as a second phase in biphasic whole-cell catalysis, an additive to the substrate solution, a solvent for isolated enzyme biocatalysis, and a hydrophobic coating. Benefits of ILs have included improved solubility and separations, greater enzyme stability, greater activity and better recycling.

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ILs are media that entirely comprise ions and are liquids under normal operating conditions. This unique structure affords many unique properties. One of the most important of these, from a biocatalytic point of view, is the ability of ILs to dissolve polar solutes and yet have tunable hydrophilicity ranging from highly water miscible to the totally insoluble. Examples of IL ions that have been used in biocatalysis are given in Fig. 11.1.

Issues that discourage industrial scientists and engineers from adopting IL media include high viscosities, lack of familiarity and suppliers, higher costs, and the potential for toxic effects and environmental damage. All these challenges can be overcome by judicious choice of the component ions. The viscosity can also be reduced by adding water as a cosolvent. Researchers are encouraged to work with relatively cheap and simple ions that have high biocompatibility and are built on bio-derived scaffolds, such as Choline [Ch] (**1**, Fig. 11.1). Other ions derived from natural products include amino acid and fatty acid derived anions. Biocompatibility and low toxicity are especially important in biocatalysis as the solvent is frequently water. Contamination of water should be avoided for economic and environmental reasons, and any reagents that could end up in an aqueous phase should be of known low toxicity. Highly functionalized ions should generally be avoided as they are expensive, and require a number of synthetic steps which increases the cost, carbon footprint and waste generated in the life cycle [2–6].

Biocatalysts have been used to promote a range of reactions in IL modified reactions. Transformations include hydrolysis, esterification, kinetic resolution, and oxidation. The IL can be added to improve the solubility of a substrate in water, as an alternative solvent for the reaction, or as a protein modifier to improve stability and recycle.

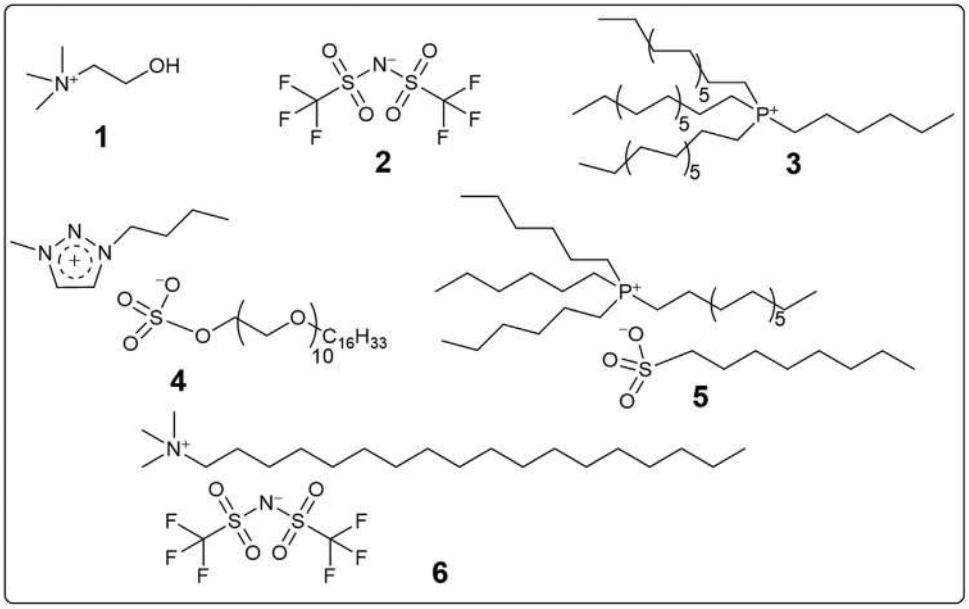

FIGURE 11.1

A selection of ions and ionic liquids that have been used in conjunction with biocatalysts.

Protein stability is key in isolated enzyme biocatalysis. Many enzymes must be operated in water, as organic solvents can cause detrimental structural changes, inhibition and/or loss of vital structural water. Some classes, such as the lipases, have high tolerance for certain organic solvents, but this tends to be in cases where the enzyme has evolved to transform a long chain "greasy" substrate and there are limited examples. In the cases where organic solvents are not tolerated ILs provide a possible alternative. In general, the mobility of water in IL phases is lower and the ability of an IL to diffuse into a protein is less. This means that the IL can form a protective layer around the enzyme, supporting structure and preventing the loss of vital water.

11.2.1 Ionic liquids as a biocatalyst modifier

ILs have unique solubilizing properties, including a strong tendency to dissolve polar molecules, and ILs are known that can disrupt extensive hydrogen bonding and dissolve carbohydrates.

ILs have been tested extensively as modifiers of both whole-cell and isolated enzyme biocatalysts. The way that IL modifiers are used depends on the hydrophilicity of the IL. The hydrophilicity of ILs is tunable, and ILs can vary from extremely water soluble, through water immiscible, to totally water insoluble. This provides a unique combination, media that dissolve hydrogen bonding and polar molecules, but are not miscible with water.

ILs with "traditional" ions such as halide and imidazolium tend to be water soluble. Water soluble ILs can be added to an aqueous phase bioprocess to increase the solubility of an organic substrate. In instances where the ions penetrate the protein and interact strongly with the amino acids, this tends to lead to a reduction in the efficiency of the enzyme. However, promotion effects have been observed where the interaction is fortuitous, or the IL forms a protective barrier around the protein and prevents loss of water from the structure, a common effect of exposing enzymes to organic solvents.

The creation of a hydrophobic IL starts with a hydrophobic ion. The longest established hydrophobic ions are fluorinated anions $[BF_4]^-$ and $[PF_6]^-$. These anions are of questionable stability and many researchers prefer to use bis[(trifluoromethyl)sulfonyl]amide $[NTf_2]^-$ (**2**, Fig. 11.1). $[NTf_2]^-$ confers lower viscosity upon ILs, however it is complex to make and therefore relatively expensive. More recently hydrophobic cations have emerged, these are commonly rendered hydrophobic using long alkyl chains. This is typified by long chain phosphonium cations such as tritetradecylhexylphosphonium $[P_{14,14,14,6}]^+$ (**3**, Fig. 11.1). Hydrophobic ILs can be layered onto or under (depending on density) an aqueous biocatalytic reaction to form a biphasic system. Advantages of this arrangement can include the removal of product and/or side-products as they are formed. This can drive the reaction and/or help to prevent inhibition and poisoning.

11.2.2 Ionic liquids as a solvent for isolated enzyme biocatalysis

It is difficult to dissolve a protein in a pure IL phase and the process can take days at elevated temperatures. In addition, the viscosity of ILs is significantly higher than that of water, and this can lead to mass transport limitations in reactions. These problems can be partly overcome by diluting the IL with water. Dissolution of an enzyme in an IL phase can be employed to increase the solubility of a water-insoluble substrate, as one would employ an organic solvent, or it can be done to

render the protein more stable and recyclable. Some of the benefits claimed include stabilizing effects, enhanced activity, and greater thermal stability.

11.2.3 Ionic liquids as an enzyme coating

Coating an enzyme in an IL can have a remarkable stabilizing effect, enabling long term storage and facilitating recycling. Lipase coated in a 1-butyl-3-methyl-1,2,3-triazolium cetyl-PEG10 sulfate IL (**4**, Fig. 11.1) could be stored for two years in an ammonium IL [7,8]. Phosphonium ILs were found to be an ideal reaction medium for transesterification catalyzed by the coated enzyme [9].

A related approach is the use of IL sponges introduced by Lozano and coworkers. In this case the IL serves as the coating and the reaction solvent. This system has been used extensively for biomass related reactions and is described in more detail in Section 11.3.3.2.

11.2.4 Enzymes coentrapped with ionic liquids

Immobilizing an enzyme improves its stability and recyclability. One form of immobilization is entrapment. Entrapment is the physical confinement of a catalyst within a support. This is achieved by performing a polymerization to form a supporting matrix in the presence of the biocatalyst. We have recently reviewed the entrapment of isolated enzymes [10].

The effects of ILs can also be exploited within entrapped enzymes. If the solvent for the polymerization is an IL, then the IL becomes confined with the gel. Factors that must be considered in entrapment include the effect on stability, the effect on activity, and the potential for leaching. The key to successful entrapment is to design a matrix that protects the enzyme, and supports the protein structure, but does not slow the rate of diffusion too much. This requires the formation of a highly porous matrix and so the pore size must be controlled to ensure the longevity of entrapment and avoid enzyme leaching. Coentrapment of an IL can help with all these variables. The IL has a templating effect on the material and helps to dictate pore size and extent. In addition, ILs can support the protein structure, and have a high affinity for the protein, helping to hold the enzyme inside the matrix and prevent leaching.

Many combinations of ILs and gel matrices remain to be discovered and explored. Recently, we have developed an IL supramolecular gel method for enzyme immobilization using amino acid derived low molecular weight gelators. These are small molecules that, when dissolved in a solution then cooled, form a gel matrix entrapping the solvent. The gel could be shaped into spherical beads. Lipase entrapped in IL gels were active and recyclable toward the hydrolysis of para-nitrophenyl butyrate. The enzyme activity was determined to be solely within the gel.

11.3 Chemicals and liquid fuels from biomass

The discovery of cost-efficient routes to commodity chemicals and fuels using sustainable building blocks and green chemistry, the “biorefinery” concept, is an important driver for carbon reduction.

The purpose of the combined biorefinery is to mirror the fossil fuel energy and petrochemical industries by producing chemicals for energy and synthesis from bio-renewable primarily

plant-based sources. This is important as fuels are high volume but low cost. Chemical intermediates are upwards of 10 times more valuable by weight. This means that the production and sale of chemicals offsets the low cost of fuels and contributes to the overall profitability of the industry. There are many routes to chemicals from biomass. Thus far the principal sources of chemicals and fuels have come from carbohydrates and fats & oils. The potential use of lignin has also generated a lot of interest. This chapter concentrates on the formation of chemical intermediates and liquid fuels and does not include technologies that generate gaseous products from biomass such as anaerobic digestion for methane nor hydrogen production.

Carbohydrates are a rich source of chemicals and can be converted into a myriad of bio-renewable intermediates, which can then be employed in downstream synthesis [11]. Carbohydrates are highly oxygenated, and therefore the utilization of carbohydrate-derived chemicals requires the removal of oxygen and reduction. This contrasts with petrochemical feedstocks, which tend to be alkenes and alkanes, and require oxidation as an early step. 1,3-propanediol is one of the largest scale intermediate chemicals prepared from carbohydrate-derived biomass by whole-cell biocatalysis. DuPont Tate & Lyle Bio Products employ an engineered strain of *Escherichia coli* to convert corn starch into 1,3-propanediol with production grossing over 63 000 tons per year. Much of the product is used in polymer synthesis. 1,3-propanediol has high water solubility and the removal of the product from the fermentation broth is challenging and requires multiple steps including columns and distillations. However, biocatalysis methods have a significant advantage where the product is highly oxygenated, as industrial oxidations can be difficult to perform with high selectivity using chemocatalysts, and overall the replacement of the petrochemical route leads to a significant reduction in energy usage and carbon footprint [12].

Natural fats and oils are esters and can be hydrolyzed to long chain fatty acids and glycerol. Transesterification of fats and oils with methanol produces fatty acid methyl esters (FAMEs, Fig. 11.2), a form of biodiesel that can be prepared on an industrial scale. Chemocatalytic routes to biodiesel can be homogeneous or heterogeneous. The oldest catalysts known were homogeneous acids or bases. Potassium hydroxide is a typical catalyst for conventional homogeneous production. These catalysts are cheap and simple to use, but they contaminate the aqueous phase and generate crude aqueous glycerol as a side product. More recently purpose designed industrial catalysts have emerged. If a heterogeneous catalyst is used on a pure oil feed the glycerol formed can be pure. For example, in Saipol's Esterfip-H process biodiesel is prepared from rapeseed oil over a zinc aluminate oxide catalyst. Pure glycerol can be sold to the consumer products market for use in, for example, personal care products. A disadvantage of this approach is that the oil used could also serve the food industry, an alternative is to use waste cooking oil as a feedstock. The use of

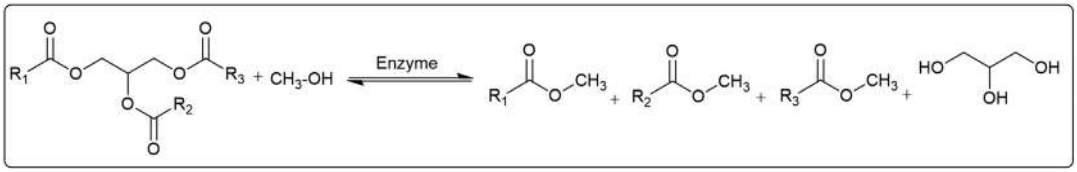

FIGURE 11.2

Transesterification of fats and oils to yield FAME and glycerol.

enzymes to catalyze the transterification of fats and oils to biodiesel is well established. In FAME production, the toxicity of methanol can significantly inhibit the reaction and must be careful monitored and minimized [13,14].

Lignin provides rigidity and support to many plants. The term refers to a class of complex and highly aromatic large molecules. Lignin is present in plant bound to cellulose (a carbohydrate) as lignocellulose. The cellulose can be dissolved, using an IL, for example, leaving relatively insoluble lignin biomass. A large amount of research has been dedicated to the utilization of lignin biomass, aimed at expanding the small number of chemicals, for example, vanillin, that are currently prepared from lignin. The interest stems from the structure of lignin, which contains many aromatic groups, including phenolic groups. Aromatics are an important class of chemical and are relatively hard to prepare from other high-volume biomass feeds [15].

11.3.1 Why use ionic liquids in biomass biocatalysis?

A key challenge in the conversion of renewable biomass into chemicals is the physical nature of the starting materials. Biomass can be very fibrous and insoluble and highly variable in its physical form. ILs have remarkable solubilizing power, and this can help to bring biomass into solution so that it can undergo chemical transformations, mediated by a biocatalyst [16]. For example, certain ILs have been shown to readily dissolve a wide range of carbohydrates. When dissolving biomass the IL must penetrate the material and disrupt the intramolecular forces. This means that small ions with strong interactions with the solute tend to be the most useful for dissolving biopolymers [17,18].

11.3.2 Whole cell biocatalysis for biomass conversion

The principal application of ILs in whole-cell biocatalysis is as a second phase for the operation of biphasic bioprocesses. In this application, the dual properties of high solubilizing power and low water solubility are exploited. A hydrophobic IL is layered either above or below the aqueous layer of a biocatalysis reaction and extracts the product as it is formed (Fig. 11.3). This mode of operation not only aids in separation but also prevents any poisoning or inhibitory effects that the product may have on the biocatalyst [19].

In one example Liu et al. demonstrated the application of phosphonium sulfonate IL $[P_{6,6,6,14}][C_8SO_3]$ (**5**, Fig. 11.1) in the conversion of glycerol to 1,3-propanediol by *Clostridium butyricum* (Fig. 11.4) [20,21].

The hydrophobicity of the phosphonium cation is a key component of the very low water solubility of $[P_{6,6,6,14}][C_8SO_3]$. Low water solubility is important in high volume whole-cell bioprocesses as the quantity of water involved makes water recycling key to the economics of the process. $[P_{6,6,6,14}][C_8SO_3]$ was found to have good biocompatibility and form a clean layer on top of the fermentation. Treatment with the IL increased the production of 1,3-propanediol and reduced the rate of cell growth. The IL exhibited a relatively high compatibility as maximal growth rates were over 50% of the control in 1:1 aqueous: $[P_{6,6,6,14}][C_8SO_3]$. In practice a much lower amount of IL than this would be layered on the fermentation broth. Extracting the product into an IL layer could facilitate a combined whole-cell biocatalysis- chemocatalysis route to value-added products from biomass. Whole-cell biocatalysts are perfectly tuned for biomass conversion as microbes evolved to live off biomass,

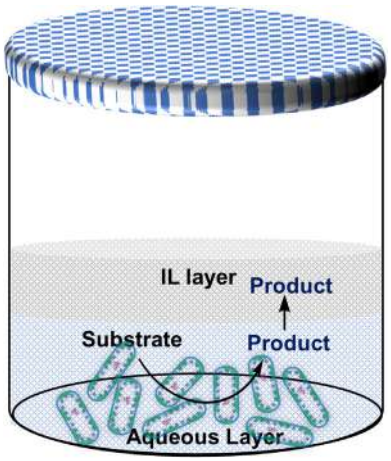

FIGURE 11.3

Biphasic whole-cell biocatalysis employing an immiscible ionic liquid.

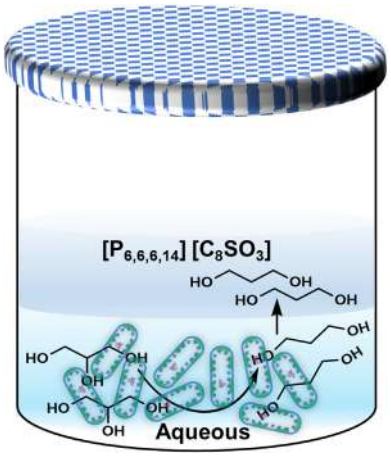

FIGURE 11.4

The conversion of glycerol to 1,3-propanediol using whole cell biocatalysis in an aqueous/ionic liquid biphasic solvent system.

and many chemocatalytic reactions operate well in IL solution. By operating a large-scale fermentation layered with IL, aliquots of product containing IL could be removed and used in different chemocatalytic reactions to prepare a range of chemical products [22,23].

The chemocatalytic conversion of 1,3-propanediol in ILs can be employed to generate amine and aldehyde products by hydrogen transfer (hydrogen borrowing) amination (*N*-alkylation) and hydrogen transfer initiated dehydration reactions (Fig. 11.5) [24–27]. Other products that are

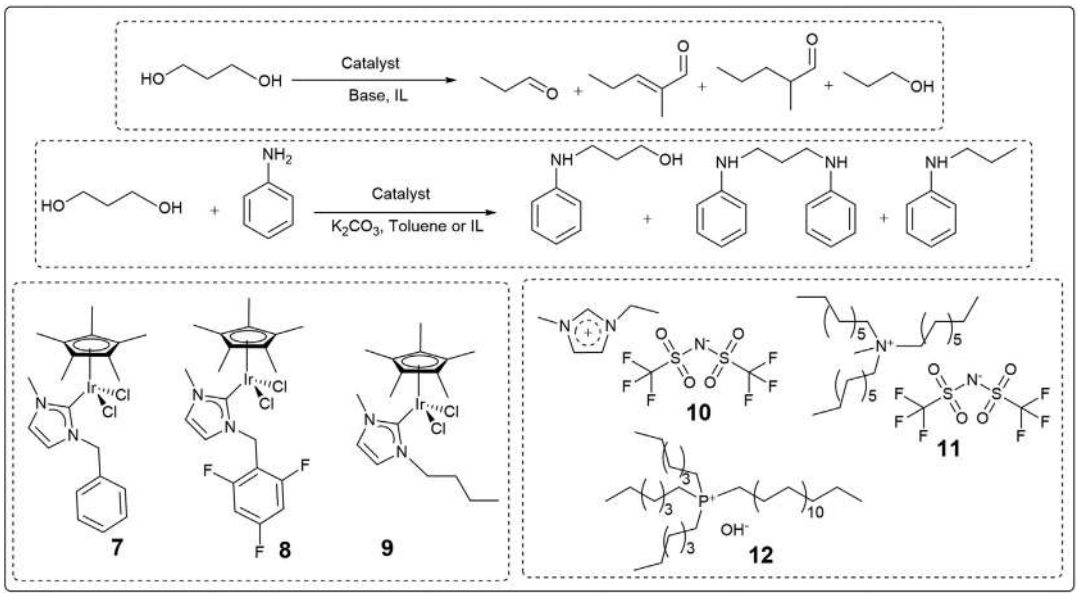

FIGURE 11.5

The conversion of glycerol to amines and aldehydes by combined whole-cell bio- and organometallic catalysis.

possible by similar methodology include higher aldehydes and nitrogen heterocycles. Considering these published procedures, and to generate commercial space time yields, improvements are required. In the whole-cell biocatalytic reaction the main problem is dilution. The production of a highly diluted product stream makes the extraction to the IL less effective and generates a product solution of low concentration. This could be overcome in two ways; firstly, the bioprocess could be operated at higher dilution, this could be achieved by genetic modification of the microbe, and/or by immobilization strategies. Secondly, and moving away from a biphasic bioprocess, the fermentation broth could be concentrated before IL extraction of the product from the aqueous solution. The main points for improvement of the chemocatalytic step are the reaction rate and the separation of the catalyst from the product. The separation problem was solved for aldehyde production, as aldehydes can be continuously removed from the reaction mixture by applying a modest (350−400 mBar) vacuum. The IL solvent is unaffected as ILs have extremely low vapor pressure.

11.3.3 Isolated enzyme biocatalysis for biomass conversion

The use of isolated enzymes for biomass conversion is a mature subject. ILs have been shown to confer desirable properties as solvents, modifiers and support media.

11.3.3.1 Ionic liquids as a modifier and/or solvent in isolated enzyme biocatalysis

A major application of transesterification in low carbon technologies is the preparation of FAME biodiesel by the transesterification of fatty acid esters with methanol (Fig. 11.2). The byproduct of

this reaction is glycerol, this can be sold for use in commodity products such as cosmetics, if it is produced in sufficient purity. Traditional homogeneous chemocatalytic routes to biodiesel, such as the use of soluble hydroxides, lead to contamination of glycerol and generate waste. The use of isolated enzymes that catalyze transesterification has been explored. Lipases are leading enzymes for this transformation, as they are designed to operate in an organic environment and convert substrates with long greasy organic chains.

Following on from literature studies employing the unstable hydrophobic anions $[BF_4]^-$ and $[PF_6]^-$ Fan et al. investigated the application of a hydroxy-functionalized pyrrolidinium cation in combination with the hydrophobic anion $[NTf_2]^-$ (**2**, Fig. 11.1) for the production of FAME esters of soya bean oil employing Lipase from *Candida ruosa*. Employing an IL containing water as the reaction solvent, the yield of FAME (82%) in 2 h was significantly higher than that achieved in organic solvents n-hexane (2%) and tert-butanol (13%), however the enzyme lost some activity upon recycling [28].

11.3.3.2 Ionic liquids in enzyme immobilization, recycling and separation

ILs can profoundly improve the stability and recycling of an isolated enzyme. Coating an enzyme in a hydrophobic IL has been shown to assist storage and recycling. Coating has been commonly employed on lipases, and many researchers use transesterification reactions to test the activity and recycling. Significant activity enhancements of up to a thousand times have been reported for transesterification.

Lozano and coworkers introduced an IL sponge concept and applied this to the transesterification of fats and oils [29–33]. This employed hydrophobic ILs that are solid and form sponge-like phases at room temperature, but are liquid at 60°C. These ILs were used as the reaction medium for biodiesel synthesis via the transesterification of triacylglycerides with methanol catalyzed by the lipase Novozym 435. The reaction was conducted at 60°C and yields were 95%–99% over 6 h. After the reaction water was added and the mixture was centrifuged to form three layers that could be separated: the sponge-like IL layer containing the enzyme, the aqueous glycerol layer, and the FAME layer. The enzyme and IL could be recycled repeatedly. The N-octadecyl-N,N,N-tri-methylammoniumbis(trifluoromethylsulfonyl)amide [C18tma][NTf_2] sponge (**6**, Fig. 11.1) had a half-life time of 1370 days. The strategy was also shown to work on waste cooking oils, and for FASEs, using the alcohol solketal instead of methanol.

11.4 Potential applications in a hydrogen economy

Electrocatalysis will be an important part of the energy mix in our low carbon future. Electricity networks and grids will play an even more central role in the delivery of energy as they can be easily connected to renewable power generating facilities and can collect geothermal, wave, wind and solar energy together to redistribute power where it is needed. The age of the electrical car has already begun, but the use of storage batteries that are powered from the mains is only one solution to low carbon transportation. Biomass derived fuels, and hydrogen powered cars are also likely to be prominent. Hydrogen can be utilized in vehicles in combustion engines, or in hydrogen fuel cells, within which hydrogen is oxidized and releases its electrons so that they can flow round a

circuit as electricity. H_2 can be used to store energy, cycling it to H_2O upon use and back to H_2 when there is excess energy in the grid / network.

When thinking of electrochemistry, biocatalysis doesn't necessarily come to mind, however enzymes have real potential in electrochemical reactions for the carbon economy. As an example, enzymes that may have a key role in the production and use of hydrogen are described (Section 11.4.1) [34–37].

Living things have evolved to harness energy from electrochemical reactions. In respiration, for example, a reduced carbon based chemical, such as a sugar or fat, is oxidized by oxygen in an enzyme-controlled reaction which harnesses the chemical energy stepwise and stores it for later use. A multitude of enzymes are designed to mediate redox reactions, with each enzyme controlling a half reaction. Examples include reductases, monooxygenases and dehydrogenases. In some bacteria hydrogen is employed as an important redox active chemical.

11.4.1 Hydrogen metabolism in nature

In some environments, such as beside an underwater hot spring, or inside the mammalian gut, there is not a ready supply of oxygen as an oxidant, but there are still abundant living organisms. Bacteria have evolved in anaerobic environments to depend upon the oxidation of hydrogen and the reduction of protons for their metabolism. The use of hydrogen in nature is extremely common, and not restricted to these bacteria. The enzymes that catalyze the reversible oxidation of hydrogen are called hydrogenases. These are the same reactions required for the generation and utilization of hydrogen for energy applications. Two classes of hydrogenase are the [NiFe] and [FeFe] hydrogenases (Fig. 11.6). The structure of the active sites is unusual as they resemble organometallic complexes, and as a result the chemistry of enzymes such as these has been termed bioorganometallic chemistry. [FeFe] hydrogenases active sites have a structure that closely resembles a class of known organometallic complexes, with a diiron center bridged by thiolates and coordinated to diatomic ligands carbon monoxide and cyanide. The structure of [NiFe] active sites is also centered on bridged thiolate donors, and the iron coordination is similar, but the second metal is Ni in an S_4 cysteine coordination environment. In contrast with many synthetic models, this Nickel has an unusual coordination geometry. The [NiFe] hydrogenase active site is of a rarer class of organometallic complex and attempts to copy them in the test tube frequently lead to highly labile complexes. However useful models have been prepared that give insight into the chemistry at the metal sites [38,39].

11.4.2 Enzymes for fuel cells

In nature the reducing potential of hydrogen is coupled to a myriad of reduction reactions, including the conversion of sulfate to sulfides in sulfate reducing bacteria and the generation of methane from CO/CO_2 in methanogenic bacteria. The redox couple that is most relevant to the provision of hydrogen energy is the coupling of hydrogen oxidation to oxygen reduction. This is the reaction required for a hydrogen fuel cell (Fig. 11.7). In a conventional hydrogen fuel cell containing a hydrogen proton exchange membrane (PEM) hydrogen is oxidized at the anode and the protons and electrons generated flow in different directions to get to the cathode, thus harnessing useful power. The protons flow through a PEM and the electrons flow through the circuit. PEMs are

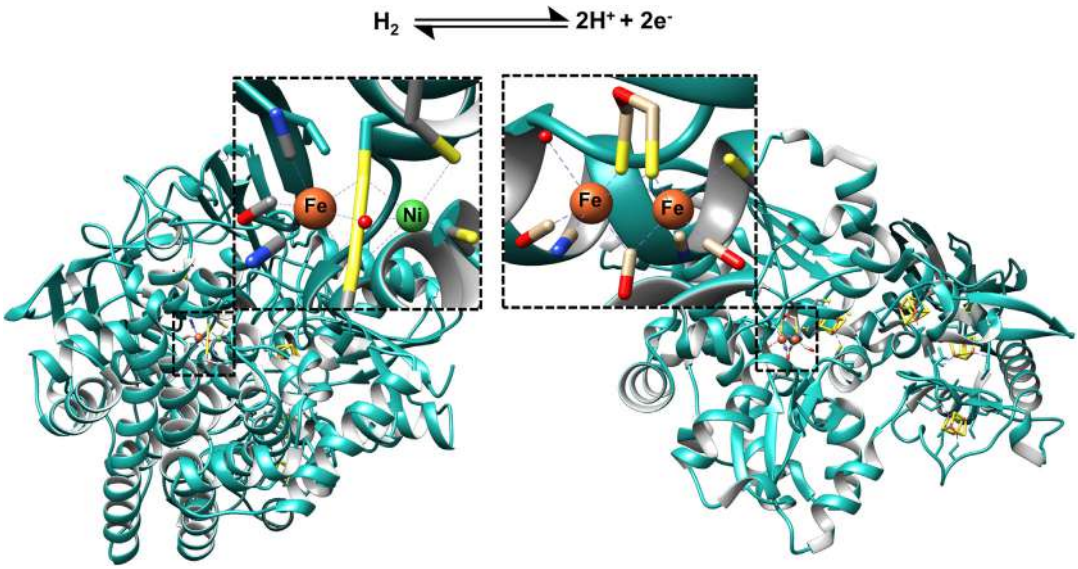

FIGURE 11.6

The structure of typical [NiFe] PDB 2FRV [40] (LHS) and [FeFe] hydrogenases PDB 3C8Y [41] (RHS) and the reaction they catalyze.

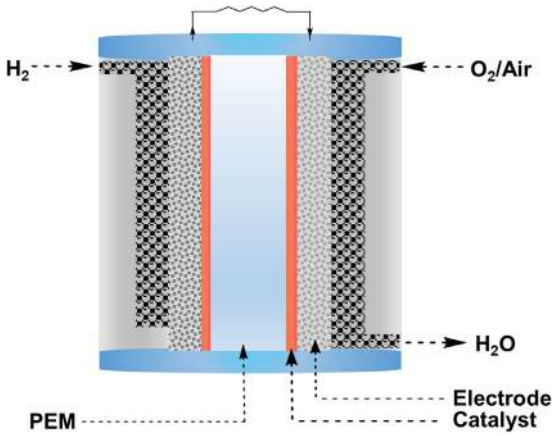

FIGURE 11.7

Schematic representation of a conventional hydrogen proton exchange membrane (PEM) fuel cell.

bespoke materials, there are several important chemical and physical properties required to perform as a PEM, and this can lead to them being costly. At the cathode the protons and electrons meet oxygen and combine to form water, the only byproduct of the fuel cell. To ensure that the fuel cell is sufficiently productive the electrodes must be constructed from catalytic materials. This reduces

the overpotential, which in turn reduces the amount of energy wasted as heat. The conventional hydrogen fuel cell employs platinum as the catalyst at both electrodes. In addition to Pt being an extremely rare and precious metal, it is also easily poisoned by gases that commonly pollute hydrogen, such as carbon monoxide, and these cells therefore require very pure hydrogen streams, significantly increasing costs. Despite these problems hydrogen fuels cells have been applied successfully in large vehicles such as buses and trucks, with multiple PEM fuel cells stacked together to increase the power output.

Replacement of the chemical electrocatalysts by enzymes has been demonstrated. These do not require a PEM, nor any precious metals, and do not tend to be as sensitive to contaminants in the gas streams. The protein provides separation of the electrocatalysts. These fuel cells are composed of enzymes adsorbed on electrodes such as graphite. The simple design makes miniaturization possible, and this would allow them to be used in applications for which conventional fuel cells are not suitable. In addition, enzyme fuel cells can be used directly on the body for medical applications [42,43].

Entrapment in supramolecular or polymer hydrogels can lead to high enzyme activity [44] and excellent enzyme recyclability [45]. A three-dimensional electrode could be constructed by entrapping the enzyme in an electron conducting redox polymer hydrogel [46]. This approach has been applied to increase the stability of [NiFe] hydrogenase against dioxygen and high potentials [47].

Several enzyme fuel cells have been constructed using [NiFe] hydrogenase enzymes. [NiFe] hydrogenases are the most efficient hydrogenases for hydrogen oxidation and more oxygen tolerant variants can be found. Materials methods can also be used to further enhance stability to O_2. In an early example Armstrong and coworkers compared a [NiFe] hydrogenase adsorbed on graphite, to a platinum electrode. The enzyme could oxidize hydrogen at rates comparable to Pt [48]. This was then developed into a fuel cell using an oxygen tolerant hydrogenase as the anode and laccase as the cathode enzyme. The cell worked "open" in air with 3% hydrogen [49].

A variety of enzymes catalyze the reaction at the cathode. The choice of the oxygen reduction catalyst is key as this half reaction still has a significant overpotential. Xu and Armstrong coupled an oxygen tolerant hydrogenase at the anode to bilirubin oxidase at the cathode to give an enzyme of reasonable power density and stability [50].

The power output of the enzyme fuel cell can be increased by stacking layers of enzyme, as for conventional PEM fuel cells. In the future the oxygen tolerance must be further increased, as must the ease of handling and stability of the enzyme fuel cells.

11.4.3 Other roles for enzyme electrodes

11.4.3.1 Hydrogen evolution

In 2022, most of the dihydrogen produced is grey or blue hydrogen from fossil fuels. The enzyme catalyzed electrosynthesis of H_2 is an important direction of investigation in the search for economic methods of green hydrogen production. For example [FeFe] hydrogenase from *Clostridium perfringens* was used with the artificial electron donor methyl viologen and using a bioelectrode at a reducing potential. HCl was incorporated into the buffer. Electrodes used were anatase TiO_2. Dihydrogen evolved with a current density of approximately 2 mA/cm. The Faradaic efficiency was $\sim$98% [51].

To truly qualify as a low carbon technology, the source of electrons/reducing potential must be considered. This can be rationalized as the consideration of the ultimate source of energy to drive dihydrogen synthesis. One possibility is to exploit solar energy. Reisner et al. demonstrated that a variant of [NiFe] hydrogenase containing selenium (a [NiFeSe] hydrogenase) could be integrated with a photoactive Ru bipyridine complex via a titania semiconductor [52]. In this system a sacrificial reducing agent or an overpotential is required. This problem can be solved by linking light harvesting to photocatalytic water splitting. In one example Reisner and coworkers employed [NiFeSe] hydrogenase from *Desulfomicrobium baculatum*. The enzyme was immobilized (adsorbed) on an electrode constructed from boron-doped p-type silicon wafer coated with a layer of amorphous TiO_2. The faradaic efficiency was essentially quantitative [53].

11.4.3.2 Ammonia production

Enzyme bioelectrocatalysis has been proposed as a replacement for the Haber–Bosch synthesis of ammonia. Currently ammonia for fertilizers is prepared from the reaction of dinitrogen and dihydrogen over a heterogenous catalyst. The hydrogen used is derived from fossil fuels and this provides a direct link between food and carbon fuels.

In nature nitrogen reduction is affected by nitrogenase enzymes. Minteer and coworkers employed the nitrogenase metalloenzyme from *Azotobacter vinelandii* to show that the enzyme could be used to generate ammonia and electrical energy from N_2 and H_2 [54]. Many groups are currently working to improve ammonia production catalyzed by enzymes.

11.4.3.3 Chemical synthesis

Fuels and ammonia are low value products. Chemical products and intermediates have much more value and improve the overall economics of renewables utilization. Chemical transformations that can be affected by enzymes such as the reduction of dinitrogen, or the synthesis of dihydrogen, can be important first steps in the synthesis of chemicals. One approach to this is to introduce a downstream enzymatic cascade to value added chemicals coupled to the hydrogenase or nitrogenase activity. This approach was demonstrated by Chen et al. for nitrogenase and ammonia to prepare chiral amines, important and valuable chemicals in the fine chemicals and pharmaceutical industries. The coupled enzymes included transaminases for enantioselective transfer of the "NH_2" functionality [55].

Formate dehydrogenase converts CO_2 to formic acid and formates, this could be combined with hydrogen oxidation to provide building blocks for organic synthesis. The natural enzymes use cofactors such as Nicotinamide adenine dinucleotide (NAD^+). Direct electron transfer was demonstrated by Reda et al. using a tungsten-containing formate dehydrogenase adsorbed on a pyrolytic graphite edge electrode. By integrating enzymatic reactions with processes that evolve carbon dioxide excess CO_2 could be recycled into chemicals [56].

Multienzyme systems can be integrated to achieve the synthesis of organic chemicals. In 1994 Kuwabata et al. demonstrated the conversion of CO_2 into methanol employing the combined activity of a formate dehydrogenase and a methanol dehydrogenase [57].

Another important chemical product that can be prepared from CO_2 is carbon monoxide. In petrochemicals this is a product of steam reforming of methane to yield dihydrogen and carbon monoxide. Therefore, conventional routes to CO are tied to fossil fuels. The CO is then employed to carbonylate organic substrates and introduce oxygen, and specifically the carbonyl functionality

into hydrocarbons for the synthesis of aldehydes, carboxylic acids and esters. Carbon monoxide dehydrogenase (CODH) enzymes catalyze the biological Water-Gas shift reaction. In this reaction CO_2 and H_2 are in equilibrium with CO and H_2O. Additional steps could be added to the synthesis by employing further classes of enzyme, thus utilizing CO as an organic carbonyl group. In nature, acetyl-CoA synthases are Ni-containing metalloenzymes which are closely related to NiFe-containing CODHs. These enzymes catalyze acetate synthesis converting methyl and CO to acetic acid and acetate esters.

11.4.4 Potential roles for ionic liquids

Hydrogenases have been described as examples of enzyme bioelectrocatalysts. One property that all enzyme electrocatalysts share is their fragility compared to chemical catalysts. Immobilization is generally accepted as a key method for enzyme stabilization, and entrapment in hydrogels/redox polymer gels has been proposed as a potential route to the synthesis of more viable and long-lived bioelectrochemical devices. These polymer matrices contain water and/or organic solvents. However, water and organic solvents have some inconvenient properties for electrical applications, namely low viscosities (fast flow), high vapor pressures and evaporation rates, and low boiling points. In addition, the structure of the polymers employed can be complex and their synthesis multistep. ILs have properties that can overcome these problems and IL gels could therefore provide viable alternative protective environments for enzyme activity.

ILs have a long history of electrochemical applications, and their conductivity has been investigated from the very start of the scientific field [58]. ILs are comprised entirely of ions and are therefore conducting. Many ILs have good electrical stability and can serve as electrolytes in a range of electrical applications, including energy storage in capacitors and batteries and energy conversion in solar cells and fuel cells [59,60].

Early examples of modern ILs were designed with electrochemical applications in mind and, for example, the [NTf_2] anion (**2**, Fig. 11.1) was introduced to the IL field from their use in polymer electrolytes [61]. The formation of a conducting gel makes an electrolyte easier to apply as it reduces problems associated with leaking and facilitates easier fabrication of devices. IL gels can be prepared in ways analogous to the formation of hydrogels. Many inorganic oxide, supramolecular and polymer gels of ILs have been prepared. IL gels have been shown to have a range of applications in energy and biocatalysis [10,62]. IL gels have been used in medical sensors. Leleux et al. demonstrated the application of an IL polymer gel in a gold electrode for measuring impedance under the skin of patients [63]. The gel was added by dropping the IL (1-ethyl-3-methylimidazolium ethyl sulfate), and the polymer precursor poly(ethylene glycol) diacrylate, with a photoinitiator, onto the electrode and polymerizing upon exposure to UV light. They concluded that the IL gel improved the longevity of the electrode compared to a commercial electrode. Electrodes can also be constructed from IL gels containing a biodegradable polymer [64]. IL polymer gels with supramolecular crosslinks could be 3D printed.

As ILs support biocatalytic activity and have electrical and physical properties that lend toward applications in energy, including the synthesis of electrodes, it is a natural progression to apply IL gels in bioelectrocatalysis. This will open many new opportunities for low carbon technologies containing biocatalysts and ILs.

11.5 Conclusion and future prospects

Biocatalysts and ILs have become useful partners, and the properties of ILs are frequently desired to improve bioprocesses employing whole-cell and isolated enzyme biocatalysts. As new technologies are being developed to feed the emerging sustainable chemical and energy industries, biocatalysis will take an increasingly important role, as these methods allow humans to achieve remarkable chemical transformations without requiring rare elements or lengthy synthetic methods. The application of biocatalysis in energy and chemicals requires the production of robust catalysts and devices, and ILs can have a profound impact in this area.

This chapter has provided a snapshot of the potential benefits of ILs in bioprocesses. These applications are all at different stages of research and development, with the application of isolated enzymes and ILs to the conversion of biomass being the most developed. All of the uses cited have the potential to contribute significantly to a lower carbon future. Much more work in this area is anticipated throughout the coming decades.

The fields of biocatalysis and ILs are both rapidly evolving, and we can only imagine what will be possible in the future, and how researchers will rise to the challenges of creating a greener future.

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CHAPTER

Application of ionic liquids in pharmaceutics and medicine

12

Belen Altava[1], Santiago V. Luis[1], Eduardo García-Verdugo[1] and Raul Porcar[1,2]

[1]Inorganic and Organic Chemistry Department, E.S.T.C.E., Universitat Jaume I, Castelló, Spain [2]Organic and Bio-Organic Department, Faculty of Science, UNED, Las Rozas-Madrid, Spain

12.1 Introduction

The term "ionic liquid" (IL) is widely accepted and used to describe salts with melting points below 100°C, which are typically composed by cations and anions with relatively low symmetry or delocalized charge [1]. ILs are highly modular, providing a large structural diversity that can be used to adjust their physicochemical properties as to fulfill the requirements for a given application [2,3].

In 2007, Hough et al. grouped ILs into three different generations according to their properties and applications [4]. First-generation ILs were developed mainly as new solvents and characterized by their unique tunable physical properties such as thermal stability, strong solvation ability and low vapor pressure [5]. Second generation ILs were developed as functional materials with tunable chemical and physical properties. The term "task specific ionic liquids" was coined and they were applied as electrolytes in electrochemical processes [6], as anticorrosion coatings [7], as stationary phases for chromatography [8], in solar cells and fuel cells [9] as supports for the immobilization of enzymes [10], or in separation technologies [11] among other fields. At the beginning of the 21st century, a strong focus was given to the biological activity of ILs, including their toxicity and eco-toxicity, and the third generation of ILs was developed [3,5,12]. This recent trend has led to different pharmacological and agrochemical applications that, according to some authors, will involve the appearance of a fourth generation of ILs [12–14]. An exponential growth has been observed in recent years in the number of publications related to pharmaceutical and medical applications of ILs, and this is illustrated by the approximately 350 papers published in 2020 in this area.

In the pharmaceutical realm, three main technological applications can be established for ILs: (1) the development of bioactive ILs either through the preparation of ILs being themselves bioactive or alternatively being part of their structure an active pharmaceutical ingredient (API) known as API-ILs [15]; (2) the use of ILs as media for the formulation of active principles [16,17] or drug purification [18,19] and (3) the development of ILs-based controlled release systems or related applications (Fig. 12.1) [20,21].

Different review articles related to medical and pharmaceutical applications of ILs have been published to date [3,12,13,22,23], and most of them are focused on drug delivery applications [24,25]. This chapter follows a broader approach, analyzing the different potential approaches reported in the last decade that involve the use of ILs for pharmaceutical applications. A particular

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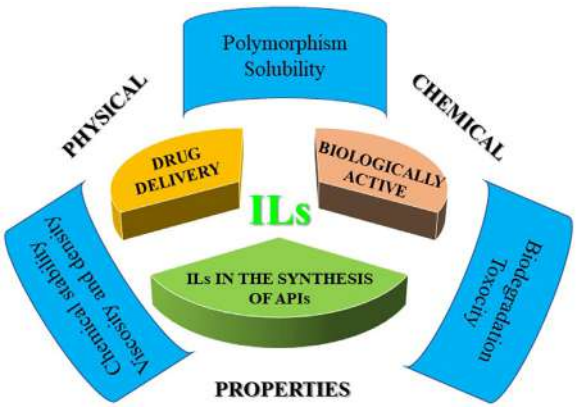


FIGURE 12.1

Main applications of ILs in the pharmaceutical field in connection with their physicochemical properties.

attention has been given to the design of ILs as therapeutic drugs. Only salts with Tm $<$ 100°C are considered here, not covering IL-related materials or organic salts with melting points $>$100°C [26].

The development of new active pharmaceutical principles, smart drug formulations, and administration systems of interest in the treatment of various diseases is a challenge that pharmaceutical industries must achieve without compromising safety and efficacy. The APIs market is expected to grow to nearly $ 1.2 trillion by 2022 [27]. In 2018, the Food and Drug Administration (FDA) approved 59 new drugs: 42 new chemicals and 17 biological entities [28]. However, 40% to 70% of the drugs under development do not reach the formulation stage due their low solubility in water and in most of the solvents pharmaceutically accepted, their limitations in bioavailability, dissolution rate or in their stability [29]. The formation of solid salts from the corresponding API is commonly used to overcome those limitations, and 70% of the new APIs are developed today following this strategy [30]. Furthermore, 50% of all commercial salt drugs have melting points above 100°C, which allows increasing physical stability during storage, as well as their thermal stability and capacity for manufacturing [31]. However, the irregular gastrointestinal absorption of solid formulations, together with the low therapeutic efficacy and the possible toxicity and side effects of their possible polymorphs are fundamental limitations of many of the APIs developed or under development [32]. Thus, there is a need to design new simple and complementary strategies to improve the solubility and bioavailability of drugs, as well as to solve polymorphism associated issues, especially for drugs based on oral administration [33].

12.1.1 Classification of ionic liquids in the context of pharmaceutics and medicine

ILs can be classified into two main groups. The first one involves the use of APIs as building blocks for the preparation of biologically active ILs through their transformation in the corresponding salt using an appropriate counterion (API-ILs). The second group does not encompass the use

of known APIs for the elaboration of the cation or anion component of the IL. This category includes ILs designed from natural building blocks and used as novel bioactive chemical compounds in which both cation and anion come from natural sources (BIO-ILs) [34].

Fig. 12.2 highlights how the high diversity in the nature of the anion and the cation in ILs can give place to multiple possibilities. It is worth mentioning that in API-ILs, although the IL is formed from an existing API, the counterion selected for the formation of the IL can determine the pharmacokinetic properties. Thus, the new IL derived from the registered API must be recognized as a new chemical entity by health authorities and subjected to the corresponding authorization and regulatory process. The first patent in the field of API-ILs was registered by Rogers et al. in 2007 [35].

12.1.1.1 Single or dual active ionic liquids derived from pharmaceutical ingredients (active pharmaceutical ingredient-ionic liquids)

Typically, APIs in their salt formulation contain small inorganic counterions such as sodium or potassium cations in the case of acidic APIs and chloride, sulfate, or phosphate anions for basic APIs. The approach for the development of bioactive ILs initially proposed in 2007 by Rogers and coworkers involved the replacement of those small simple counterion by an organic entity to produce an IL [4]. This can allow the conversion of solid active materials into liquid forms or low melting solids (API-ILs) [4,36]. Through the introduction of an specific organic counterion, the biological, chemical, and physical properties of the resulting API-IL salt can be tuned, maintaining its biological activity. Furthermore, the counterion of the API-ILs can be a second active ingredient allowing dual pharmacological activity and possible synergistic effects [37].

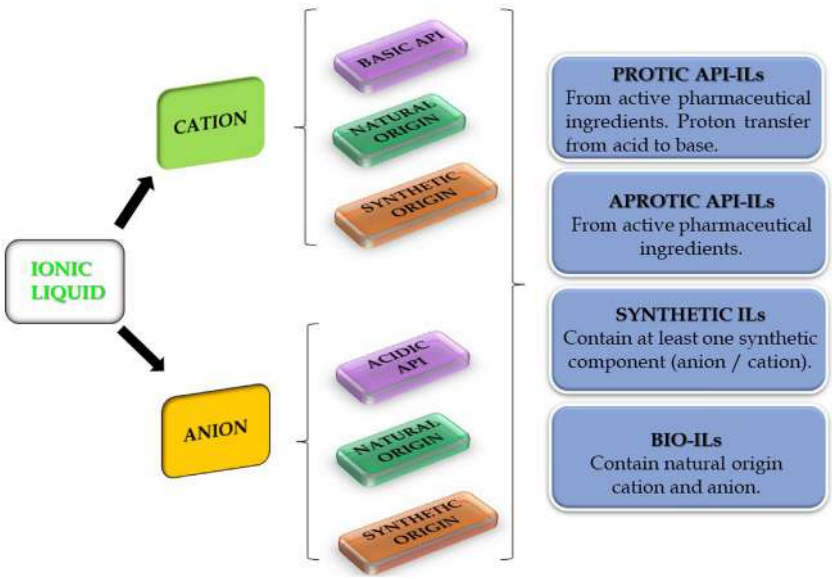


FIGURE 12.2

Classification of bioactive ILs as a function of anion/cation nature.

Fig. 12.3 summarizes some common APIs used for the design of API-ILs before their transformation into the corresponding cation or anion. It must be noted that the counterion in the API-IL can be a second API with a different pharmacological activity [15,38,39], or a biologically active

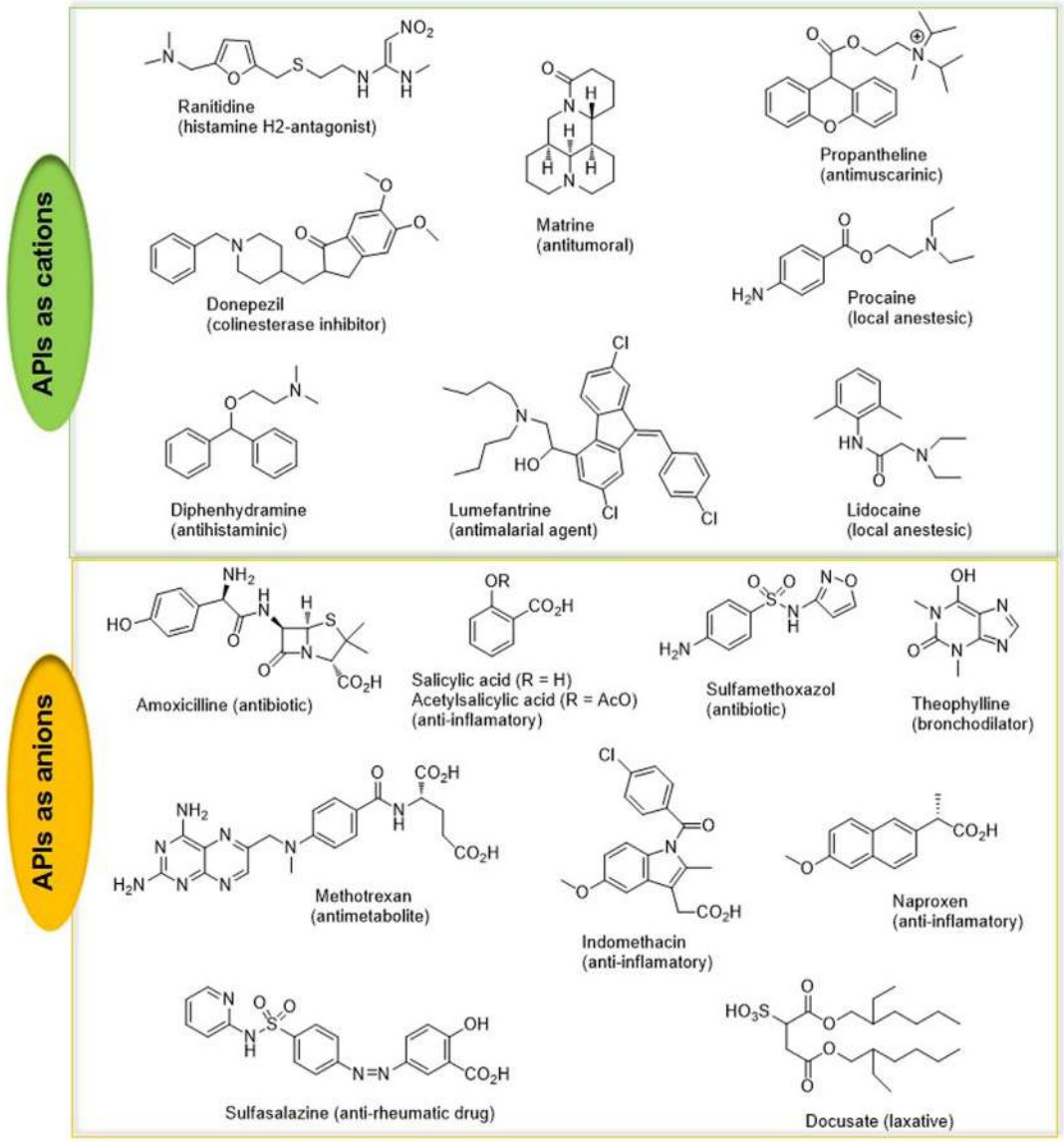


FIGURE 12.3

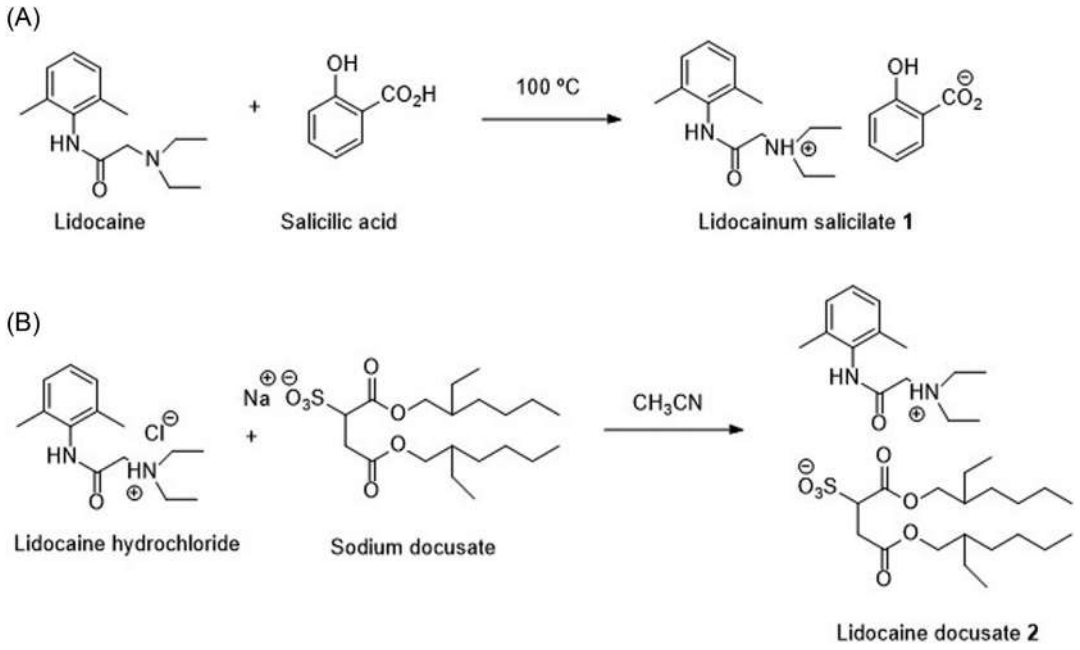
Selected APIs used for the design of API-ILs. References are cited along the chapter.

component like choline, cetylpyridinium or fatty acids [40–42]. Furthermore, uncommon counterions (i.e. imidazolium or phosphonium) can also be found in API-ILs [43,44].

12.1.1.1.1 Protic active pharmaceutical ingredient-ionic liquids from active pharmaceutical ingredients

Protic ILs are defined as those in which the cation is formed through protonation of a base. When an API contains an easily ionizable functional group (i.e. amine, sulfonic or carboxylic acid groups), the most common and simple way to prepare an API-IL is by proton transfer from a Brønsted acid to a Brønsted base. This can be achieved in solution using an appropriate solvent [14,45] or by melting the mixture. Thus, for example, lidocainium salicylate **1** was synthetized under solvent free conditions by melting a stoichiometric mixture of lidocaine and salicylic acid at 100°C to obtain a flowing liquid (Scheme 12.1A) [45]. Recently different protic API-ILs have been prepared by mechanochemistry, in the solid-state or in the presence of small amounts of solvent [46,47].

Anion metathesis using an alkaline salt and a hydrohalide salt has also been used to prepare protic API-ILs, especially when weak acids are involved. An example is the stoichiometric reaction between lidocaine hydrochloride and sodium docusate in acetonitrile used for the synthesis of the API-IL **2** (Scheme 12.1B) [38]. Protic bioinorganic API-ILs have also been synthetized, for instance, by reacting the organic hydrochloride of ranitidine or procainamide with two equiv. of the Lewis acid $ZnCl_2$ [48].



SCHEME 12.1

Synthetic approaches for the preparation of protic API-ILs [38,45].

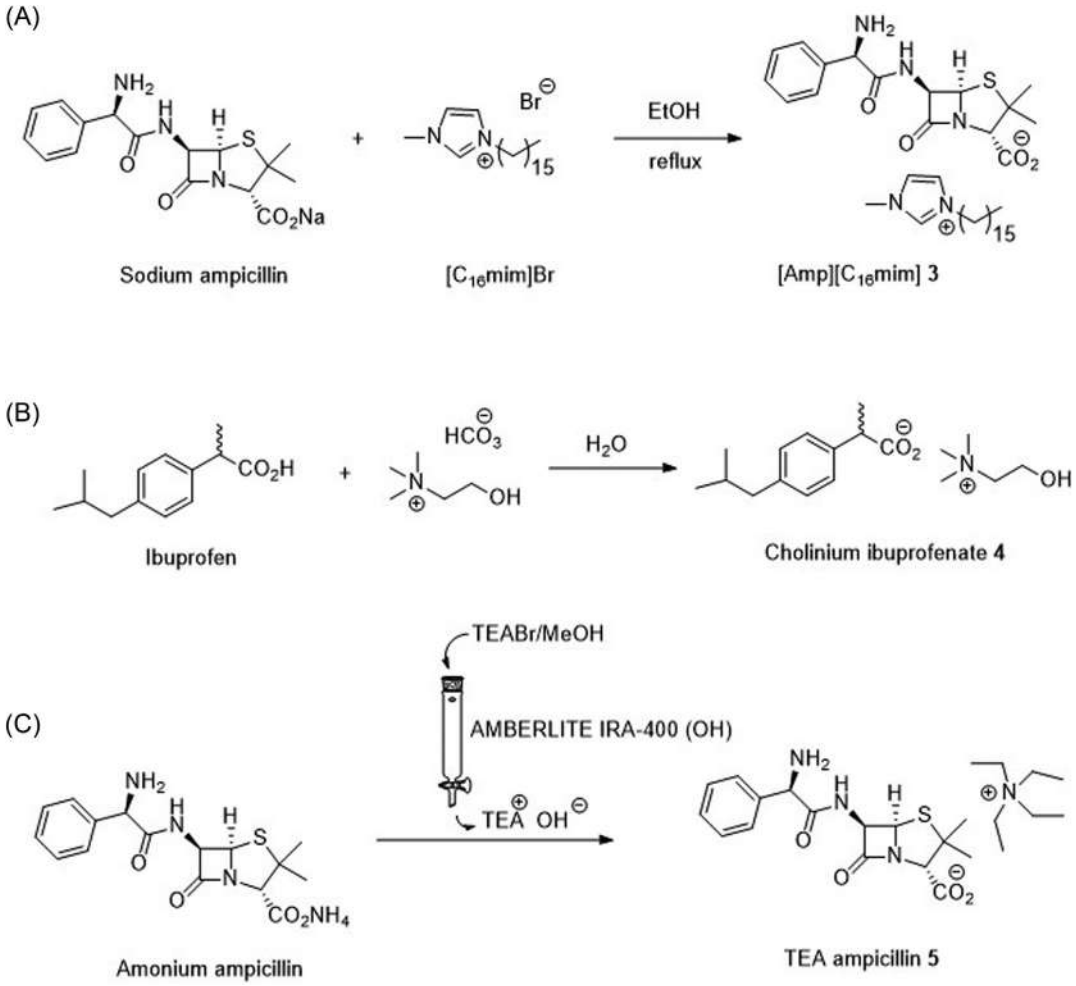
For an effective and complete proton transfer from the acid to the base in aqueous solution, a pKa difference >3 between the API and the counterion is required when primary amines are used, while ΔpKa >6 is needed for tertiary amines [49,50]. MacFarlane and Seddon proposed that the ionicity should be at least 99% for protic ILs to be considered as "pure" salts [14]. This parameter is important for protic API-ILs since the physicochemical properties and the mechanism of action of the resulting ILs depends on the involved hydrogen bond interactions [51]. A noncomplete proton transfer will lead to a mixture of the corresponding salt and the neutral acidic and basic species [52,53]. To determine the degree of association between the anion and the cation different experimental methods can be used [54], being FTIR-ATR, Walden plot and NMR methods the most used [14,38,39].

In pharmaceutical practice, it is well stablished that ionic species have difficulties crossing biomembranes while ion-pair formation enhances the transport of ionic drugs through the skin and across the absorbing membrane [55,56]. In this regard, MacFarlane and coworkers have studied the membrane transport activity of different protic salicylate-IL derivatives. Results demonstrated that the formation of ion-pairs or clusters involving hydrogen bonded species leads to more desirable drug delivery systems as they may cross the membrane barrier more easily than the more ionized species [14,57]. The mixture of lidocaine and ibuprofen, designed for topical formulations in the local treatment of acute pain and inflammation [58], formed a deep eutectic liquid (DES) through the partial formation of protic ILs species [59]. This DES was able to penetrate the skin more effectively than the corresponding lidocainium docusate IL **2** or the lidocainium chloride [15]. In this regard, it is often observed that the addition of an excess of the corresponding base or acid to protic ILs produces oligomeric hydrogen bonded species, leading to the liquefaction of the salt [51,60]. Overall, the degree of ionicity and the H-bonding interactions between the components of a protic API-IL is crucial for a proper design and application of the corresponding drug, as this can determine its final physical properties such as density and viscosity and the pharmaceutical performance, in particular membrane permeability [61].

12.1.1.1.2 Aprotic active pharmaceutical ingredient-ionic liquids from active pharmaceutical ingredients

The most straightforward methodology for the synthesis of aprotic ILs consists in the metathesis reaction between the halide of an organic cation and the alkaline salt of a less coordinating anion [45,62]. Thus, the reaction between excess sodium ampicillin and an imidazolium bromide yielded Amp-ILs such as [Amp][C_{16}mim] **3** (Scheme 12.2A) [63]. API-ILs can also be prepared from the corresponding acid and a basic salt, as in the synthesis of cholinium nonsteroidal antiinflammatory drugs (NSAIDS) performed by mixing cholinium bicarbonate and the corresponding NSAID as ibuprofen to give ILs **4** (Scheme 12.2B) [64].

When the formation of weaker acids than hydrogen halide is involved, the anion exchange cannot be efficiently performed. An alternative method has been described by Ferraz et al. using ion exchange resins (Amberlite IRA-400 OH) for the in situ synthesis of cationic hydroxide, in the synthesis of different ampicillin ILs such as **5** (Scheme 12.2C) [65,66]. In a related approach, a strongly acidic ion exchange resin (Amberlite IR-120) was used to obtain the corresponding acid from sodium valproate, while a strongly basic resin (Dowex 550A) was used to convert cetyltrimethylammonium bromide into the corresponding hydroxide. The mixture of both components allowed preparing easily the desired cetyltrimethylammonium valproate IL [67].



SCHEME 12.2

Synthetic approaches for the preparation of aprotic API-ILs [63–66].

Some APIs have been transformed into the cation part of an API-IL via their chemical modification. The first API-IL reported in 1998 was obtained by quaternization of the imidazole moiety of the antifungal drug miconazole, using different alkyl halides, and further anion metathesis [68]. More recently, the preparation of API-ILs where the drug structure is covalently linked to an imidazolium cation has also been reported [69].

12.1.1.2 Ionic liquids not derived from known active pharmaceutical ingredients

Imidazolium and ammonium are the most common cations present in ILs, although pyridinium and phosphonium have been also studied. In the case of anions, they can be small organic or inorganic entities but

FIGURE 12.4

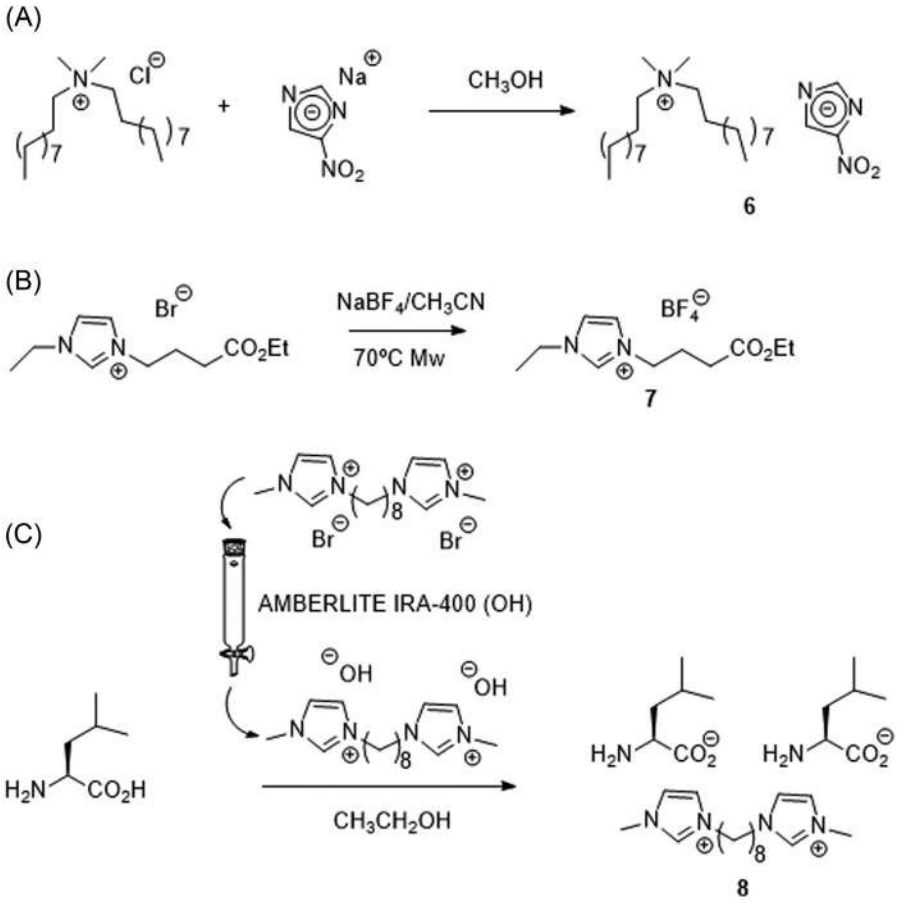
Most common representative cations and anions used in ILs.

also more complex structures (Fig. 12.4) [70]. Cations and anions of natural origin like those derived from choline or amino acids have also been used [71–73]. BIO-ILs are defined as those ILs whose anion and cation are derived from natural origin materials [34]. It must be noted that bioactive chiral ILs, displaying chirality in the anion or in the cation, can be prepared from accessible natural chiral substrates [74,75], or by chemoenzymatic synthesis through the resolution of the corresponding azole [76].

Most imidazolium or ammonium ILs have been prepared from imidazole or a tertiary amine after N-alkylation with a suitable alkyl or benzyl halide [77]. The imidazole ring itself can be synthetized from primary amines [78]. This allows obtaining imidazoles and then bioactive imidazolium ILs based, for instance, on amino acids [79]. For the preparation of the halide-free ILs, the most straightforward methodology is anion metathesis and this can involve a large variety of anions. Thus, IL **6** was synthetized by anion metathesis between didecyldimethyl ammonium bromide and sodium 4-nitro-imidazolate (Scheme 12.3A) [70]. Microwave or ultrasound irradiation has been used to facilitate anion exchange [80]. The synthesis under microwave irradiation of different imidazolium ILs, like **7**, afforded higher yields than the conventional method (Scheme 12.3B) [80]. For anions derived from amino acids, metathesis reactions are limited by the low availability of metal salts and by their ability to coordinate transition metal ions. This was solved by preparing the corresponding cation hydroxide using a ion exchange resin and reacting this with the free amino acid in a neutralization reaction [62]. The antimicrobial IL **8** was obtained by reaction between the corresponding bisimidazolium dihydroxide and a slight excess of amino acid (Scheme 12.3C) [73]. In a related approach, Earle and Seddon prepared carbenes by treatment of imidazoliums chlorides with a strong base and reacted them with a variety of acids to give the corresponding ILs [81]. As in the case of API-ILs, the reaction of a Brønsted acid and an imidazole base also affords the corresponding halide-free protic IL [82].

12.2 Physicochemical properties of ionic liquids of relevance for the pharmaceutical industry

Some of the main challenges the pharmaceutical industry must face, relate to overcoming the physicochemical problems many drugs present due to polymorphism, low solubility, and low bioavailability [3].



SCHEME 12.3

Synthetic approaches to halide-free imidazolium ILs [70,73,80].

12.2.1 Polymorphism

Polymorphism can be defined as the ability of a solid material to exist in two or more crystalline forms, leading to solids with different physicochemical properties (dissolution rate, solubility, stability and bioavailability) [83]. Polymorphism issues, result in significant economic losses in sales and in R&D to enable novel formulations back into the market [84]. It has been estimated that more than 50% of APIs present more than one polymorphic form [84,85]. Solutions to overcome this problem include the use of amorphous solid forms and cocrystals [61,86]. Alternatively, the delivery of the API as a liquid phase represents one of the most important advantages of the formulation of APIs as ILs [32]. The nature of the counterion plays a critical role in IL formulations. In general, large ions are preferred as they lead to charge dispersion, making difficult the formation of regular crystalline structures [87].

The lack of a melting point transition in ILs indicates that they do not crystallize and consequently will not exhibit polymorphism. It is generally accepted that ILs with melting temperatures lower than 50°C offer the advantage of polymorphism control. Thus, ranitidine hydrochloride can exist in two different polymorphic forms (Tm = 134°C−135°C and Tm = 140°C−144°C) [88], while the IL ranitidine docusate (**9**) has a glass transition temperature of −12°C [4]. In the same way, propantheline bromide is an API presenting different polymorphs, but the related API-ILs propantheline acesulfamate (**10**) and toluenesulfonate (**11**) avoid polymorphism problems (Fig. 12.5) [89].

However, polymorphism issues cannot be fully excluded with the formation of ILs. For example, the IL ethambutol dibenzoate **12** is a solid salt with Tm 90°C−96°C, presenting three polymorphic forms depending on the crystallization procedure [90]. Also the ibuprofen choline derivative **13** presents three different melting temperatures (19.3°C, 90.7°C, 118.6°C), indicating the presence of different polymorphs. However, when the cation is changed to tetraethylammonium, the resulted salt **14** did not exhibit polymorphism (Tm = 94.3°C) [91].

12.2.2 Solubility and dissolution rate

API-ILs were originally designed to obtain liquid forms at the temperature of application and later to increase water solubility by the selection of the appropriate counterion. High water solubility and high dissolution rate result in higher absorption rates, allowing lower doses of the drug to reach the therapeutic effect [29].

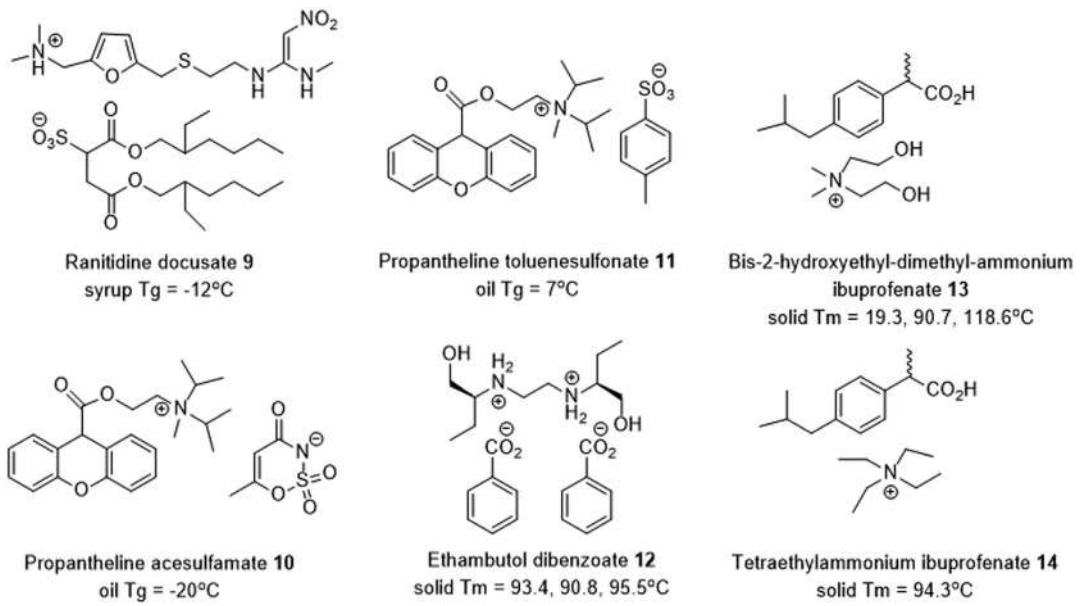


FIGURE 12.5

Physical appearance and thermal transition temperatures (Tm and Tg) of some API-ILs.

The formation of ILs, generally reduces the melting point, increasing both dissociation rates and solubility [67,91–94]. Table 12.1 compares water solubilities of different API-ILs and the original drugs. Choline sulfasalazine **15** exhibited about a 4000-times improvement in saline solubility over the neutral drug sulfasalazine, significantly enhancing its intravenous (I.V.) administration [92]. 1-Ethyl 2-methyl imidazolium methotrexate ([C_2mim][MTX], **16**), showed an aqueous solubility at least 3000 times higher than that of free MTX and higher than that of the MTX sodium salt in both water and simulated body fluids [95]. Choline niflumate **17** presented a water solubility five orders of magnitude higher than the parent drug [42]. Acyclovir (ACV) ILs **18** and **19**, showed a 450 times solubility improvement [96]. An increase of up to two orders of magnitude in the solubility of different NSAID ILs like choline ketophrenate ([Chol][Ket], **20**), choline naproxenate ([Chol][Nap], **21**), and choline ibuprofenate ([Chol][Ibu], **22**), was observed in comparison with the original NSAIDs, improving bioavailability and incorporation into hydrophilic matrices for controlled and enhanced drug release [64]. Furthermore, the ibuprofenate salt of the protonated DBU ([DBNH][Ibu], **23)** showed a 35×10^4-fold increases in water solubility over ibuprofen [97]. Indomethacin and alvarine ILs **24** and **25** presented six and five orders of magnitude higher solubilities than their parent drugs [98,99].

However, Hough et al. demonstrated that the combination of the relatively hydrophobic lidocaine cation with the hydrophobic anion docusate, afforded ILs **2** exhibiting reduced water solubility [4]. Tetraethylammonium ampicillin ILs also exhibited reduced water solubility, as compared to ampicillin, while the introduction of OH groups in the cation enhanced the polarity, leading to a 14 times solubility increase [94]. The selection of a suitable counterion allows controlling the balance between desired and undesired properties, in particular the kinetic solubility and/or the dissolution rate [94].

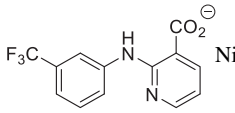
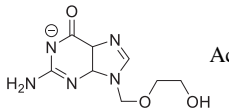
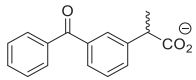
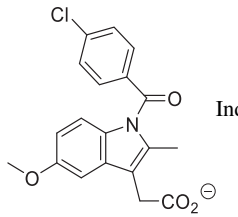
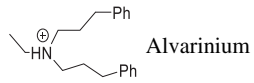
Absorption after oral administration is a requirement for many drugs. Thus, for poor water-soluble and poor permeable drugs a lipophilicity increase in the formulation is required for entering lipid absorption pathways [100]. In this regard, the lipophilic lumefantrine docusate IL has been developed to increase its lipidic solubility relative to lumefantrine free base (between 2 and 60 times higher in different lipid formulations) [92]. To study the hydrophilic/lipophilic balance of a molecule, determining the water-octanol partition coefficient is the most used method in drug design [101]. For ILs, a more accurate method has been reported based on liposome/aqueous phase partition coefficient [102].

The water solubility of synthetic ILs containing aromatic and nonaromatic cations with branched and linear alkyl side chains and bis(trifluoromethylsulfonyl)amide as anion, decreased in the order, imidazolium (im) > pyrrolidinium (pyr) > pyridinium (py) > piperidinium (pip). This was associated to changes in the water cavitation potential, influenced by the size of the cation and, to some degree, by its aromaticity. An increase from five to six carbon atoms in the alkyl side chain length, led to a reduction in water solubility, while the ILs with branched alkyl chains exhibited higher solubility in water than their linear isomers [103]. Regarding the anion nature, for a given cation, inorganic anions such as chloride, bromide, and tetrafluoroborate favored solubility, whereas the hydrophobic hexafluorophosphate anion formed no stable interactions with water [104].

12.2.3 Hygroscopicity

Most ILs are hygroscopic [105]. The water sorption affects not only the thermal and chemical stability of the ILs but also important pharmaceutical parameters such the powder flow, dosage

Table 12.1 Water solubilities of different API-ILs and their parent drugs.

Anion	Cation	API-IL	Water solubility mg/mL[a]	Refs.	API	Water solubility mg/mL[a]
Sulfasalazinate	[Chol]	15	39.32[e]	[92]	Sulfasalazine (rheumatoid arthritis)	0.01[e]
Methotrexate	[Chol]	16	530	[95]	Methotrexate (antireumatic)	0.15
					[MTX][Na]	330
 Niflumate	[Chol]	17	952.8 ± 46.1[b,d]	[42]	Niflumine (antiinflammatory)	0.0861 ± 0.0014[b,d]
 Aciclovirate	[Chol]	18	880	[96]	Aciclovir (antiviral)	1.574
	[P4,4,4,4]	19	800		AciclovirNa	100
 Ketophrenate	[Chol]	20	357 ± 18[c]	[64]	Ketoprophen (antiinflammatory)	
Naproxenate	[Chol]	21	121 ± 19[c]	[64]	Naproxen (antiinflammatory)	0.0209 ± 0.0004[c]
Ibuprofenate	[Chol]	22	309 ± 42[c]	[64]	Ibuprofen (antiinflammatory)	0.009 ± 0.001[c]
	[DBNH]	23	7000	[97]	Ibuprofen	0.02
 Indomethacinate	[TED]	24	710	[98]	Indometacin (antiinflamatory)	0.0009
Tosylate	 Alvarinium	25	38.10 ± 0.38	[99]	Alvarine (gastrointestinal disorders)	0.00096

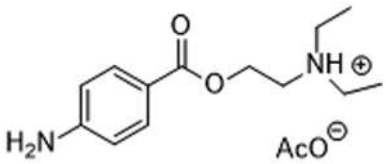
[a] *25° C.*
[b] *37° C.*
[c] *PBS aqueous solutions (pH 7.4, 0.1 M).*
[d] *isotonic ionic strength aqueous solution (0.15 M NaCl).*
[e] *In saline.*

accuracy or dissolution rate. Normally, the water content results to more liquefied salts. However, it has been reported how the anhydrous procainium-acetate **26** (Fig. 12.6), a room-temperature IL, when exposed to water transformed into a crystalline dihydrate salt (Tm = 52°C) [106].

On the other hand, it has been proposed that a hydration number greater than seven is necessary for the biological activity of ILs, these water molecules interacting rather strongly with the IL ions independently of the ion nature [107]. When the IL-in-water (IL/W) molecules ratio is <3, the solution retained the polar ionic network of IL molecules [103], while as the ratio increases, this network is weakened leading to final disruption [108]. The introduction of lipophilic groups in the cation or anion can reduce the absorption of water, modifying other parameters like the kinetic solubility [109]. Thus, for an optimal IL-drug formulation a balance between the different parameters is needed.

12.2.4 Chemical and thermal stability

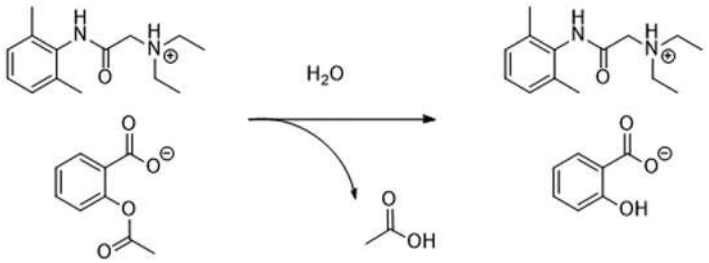
The solid crystalline state is expected to be more stable than the liquid formulation of a drug [110]. The physical and chemical stability of an amorphous or liquid drug is required prior to formulation and storage [111]. The hygroscopic character of ILs makes them more sensitive in terms of chemical stability, associated with their molecular mobility [112]. Lidocainium acetylsalicylate (**27**) (Fig. 12.7), when stored at room temperature and under air, slowly degraded to the corresponding



Procainium acetate **26**

FIGURE 12.6

Procainium-acetate structure.



27

FIGURE 12.7

Decomposition of lidocainium acetylsalicylate.

salicylate through a hydrolysis reaction (Fig. 12.7). Thus, for a therapeutic application, moisture needs to be rigorously excluded [45].

Furthermore, high thermal stability on ILs is required to allow applications such as their use in membranes—suitable for topical patches—that may require high temperature sterilization, or when used as solvent for drug synthesis or crystallization. Most ILs are thermally stable over 150°C [113].

12.2.5 Viscosity and density

The adequate viscosity of a fluid is crucial for effective chemical processes such as separation, mixing etc. In general, ILs are highly viscous fluids with viscosities even several orders of magnitude higher than those of water or widely used organic solvents [114]. Theoretical models to predict density and viscosity of ILs using quantitative structure property relationships have been described [115]. Their viscosities in general increase with the alkyl chain length or with the presence of functional groups, due to the increase in van der Waals interactions and hydrogen bonds [116]. Thus, as in other properties, the desired viscosity and density of an IL can be achieved by a proper design of the anion and cation.

12.2.6 Biodegradation

Many ILs can be classified as persistent pollutants being able to end in water classical treatment systems. Accordingly, biodegradation is an important parameter to be studied. The cation and anion nature plays a critical role in their degradation profile [117]. Thus, the presence of long unbranched alkyl chains, and hydroxyl, ether, aldehyde, carboxyl, aromatic, and other functional groups experiencing enzymatic hydrolysis such as ester or amide, promote biodegradability, whereas branched alkyl chains, quaternary carbon or tertiary nitrogen atoms, halides, and heterocycles hamper biodegradation [118,119].

For example, biodegradation assays for the theophylline ammonium API-ILs **28**, **29** and **30** determined that the anion was not biodegraded. Most of the ILs based on **28** only displayed a marginal biodegradation. For **28** with $R_1 = R_2 = CH_2CH_2OH$ and R_3 = cocoyl, the highest susceptibility to biodegradation was observed for the largest cocoalkyl chain (C18 as R_3), whereas the shortest cocoalkyl chain (C8) was characterized by the lowest biodegradability (Fig. 12.8) [120].

Cholinium-based ILs greatly improve their biodegradability [119]. Almost 50% of the biodegradable ILs synthesized to date contain cholinium as cation and an organic acid as the anion [119]. Recently, readily biodegradable BIO-ILs (**31**) have been synthesized combining amino acids and sugars. Biodegradability studies revealed that all ILs decomposed within 5–6 days in activated sludge [121].

12.2.7 Toxicity

Another important parameter concerning ILs for their pharmacological and medical application is toxicity. Bioassays performed in the last decade reveal that some ILs are less eco-friendly than originally considered [122]. Different biological tests are used in eco-toxicity studies, mostly regarding aquatic environments, involving bacteria or algae [123]. Bioassays on different healthy cell lines and carcinoma cell lines from rats or human origin have also been reported to estimate

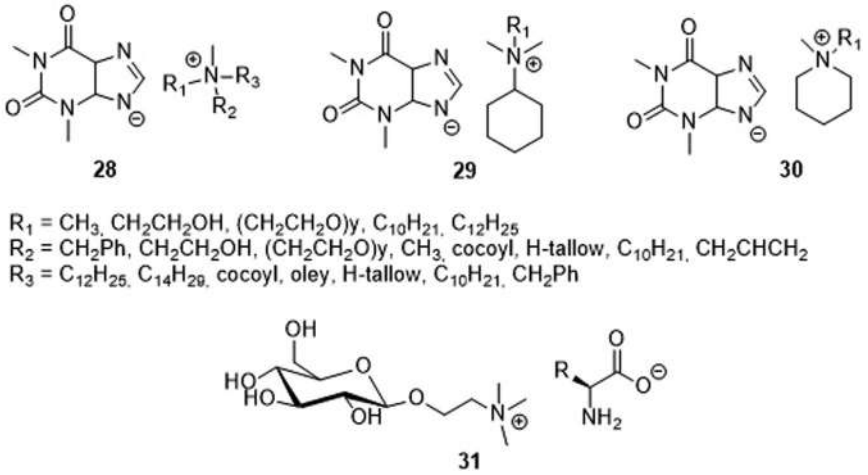


FIGURE 12.8

Structures for theophylline API-ILs and amino acid-based BIO-ILs.

the toxic potential of ILs [42,124,125]. Cancer cell lines have been often used in preference to normal cell lines to study toxicity of ILs because they have a highly unstable genetic load and undergo rapid cell divisions [126]. Furthermore, enzymatic bioassays have been used to estimate the impact of ILs on humans [127]. According to the toxicity ranking of Passino and Smith, a compound is characterized according to its EC_{50} value (half maximal effective concentration) as follows: 100−1000 mg/L, practically harmless; 10−100 mg/L, moderately toxic; 1−10 mg/L, slightly toxic; and 0.1−1 mg/L, highly toxic [128]. This section summarizes cytotoxicity studies against healthy cell lines such as MRC-5 and L929, while cytotoxicity bioassays against tumor cells will be discussed mainly in Section 12.3. It must be emphasized that "toxic" is not always equal to "bad" being the dosage of the drug the most important parameter. The selectivity index (SI) (ratio of the toxic concentration of a compound against its effective bioactive concentration) is an essential value defining the potential application of therapeutic drugs including ILs.

The main parameters that control the toxicity of ILs are the hydrophobicity of the cation induced by the presence of the different structural fragments and the nature of the anion [129,130]. The eco-toxicity of the imidazolium, pyridinium, ammonium, guanidinium and phosphonium ILs **32**−**41** containing different hydrophobic and hydrophilic anions was assayed toward the aquatic bacteria *Vibrio fischeri* (Fig. 12.9). Results showed that an increase in the alkyl side chain length in the cation always led to greater toxicity (Fig. 12.10). This can be rationalized in terms of the mechanisms of interaction of surfactants with the cell membrane (see Section 12.5) [131,132]. Regarding the nature of the cation, for ILs containing bis(trifluoromethylsulfonyl)amide (NTf_2) as the anion, the toxicity against *V. fischeri* was higher for aromatic (**32**, **33**) than for nonaromatic (**34**, **35**) ILs, increasing in the order, pyridinium > imidazolium > piperidinium > pyrrolidinium [131]. Furthermore, phosphonium-based ILs **38** were more toxic than imidazolium analogs (**32**). Regarding the nature of the anion, for imidazolium salts **32** the NTf_2 anion displayed the highest toxicity. The introduction of oxygen atoms in the alkyl side chain led to a toxicity decrease

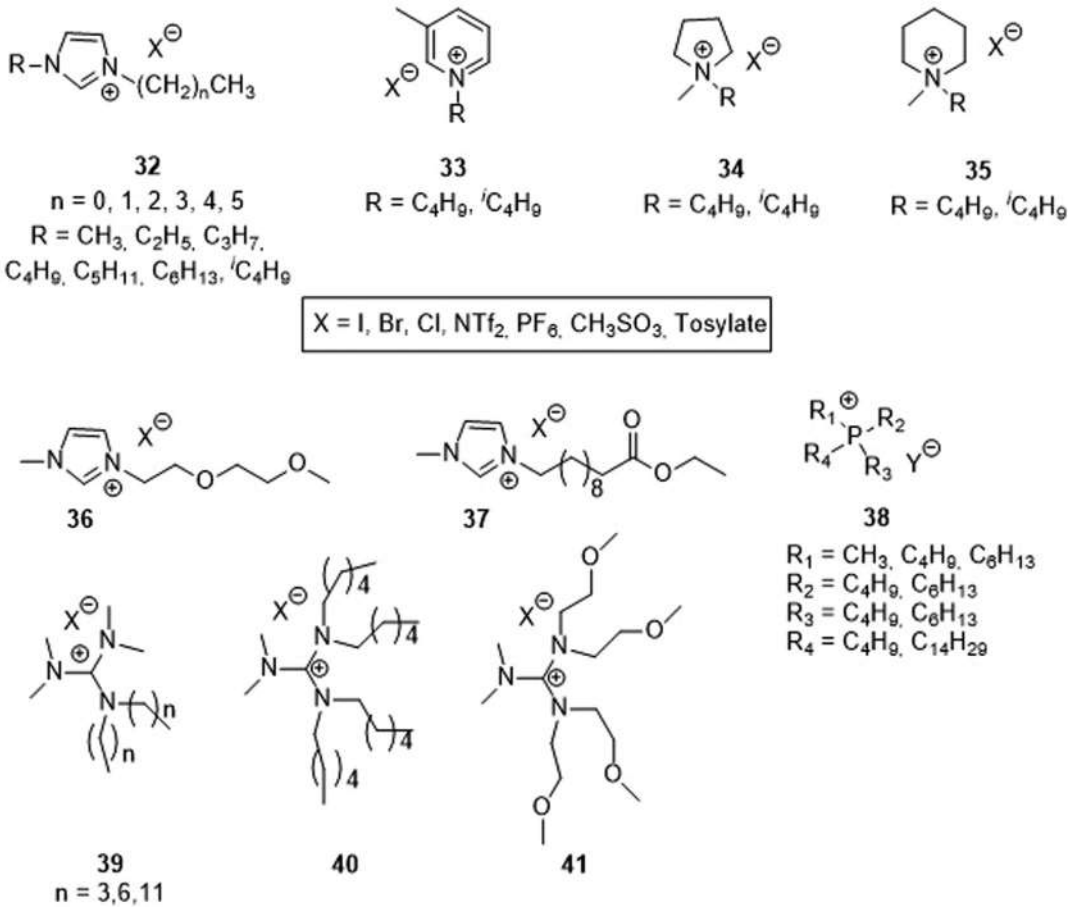


FIGURE 12.9

Structures of different aromatic and nonaromatic ILs with studied toxicity against *V. fischeri*.

independent of the cation nature (**36**, **37** and **41**) [132]. The toxicity of **32** having branched alkyl chains attached to the aromatic ring ($R_2 = {}^{i}C_4H_9$) was lower than when $R_2 = nC_4H_9$. However, for pyrrolidinium and piperidinium ILs (**34** and **35**), the opposite trend has been described with a lower toxicity for ILs with unbranched alkyl side chains [103].

Coutinho and coworkers classified most of the studied choline ILs **42** (Fig. 12.11) as "practically harmless" in terms of their toxicity for *V. fischeri*, although they presented higher ecotoxicities than some organic solvents and the imidazolium IL **32** (R = Me, $n = 1$, X = Cl) [130]. However, cholinium-based ILs are less cytotoxic than regular ILs toward hepatocyte carcinoma cell HepG2 [72,134]. The toxicity of salicylate-based imidazolium ILs **43** and **44** (Fig. 12.11) against *Artemia salina* (aquatic microcrustacean) and the healthy mammalian cell line MRC-5, showed that lipophobic ILs were significantly less toxic than nonfunctionalized analogs, with toxicities of the

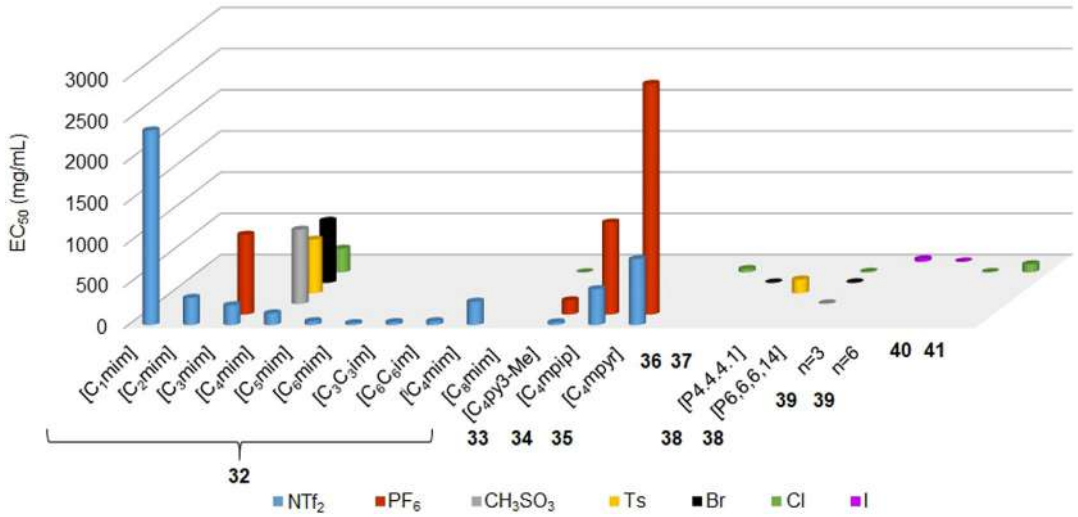


FIGURE 12.10

EC_{50} values (*V. fischeri*) estimated for different ILs after 15 min. Microtox® test was used to evaluate the inhibition of the luminescence. [133].

same order of magnitude than the reference standard, sodium salicylate. The introduction of an oxygenated alkyl substituent in the imidazolium cation (**44**) afforded a reduction in EC_{50} for MCR-5 of more than one order of magnitude. While the introduction of ether functionalities in **44** did not contribute to decrease toxicity against *A. salina*, the introduction of an hydroxyl group (Y = OH) afforded a reduction of EC_{50}, as compared to **43** [135]. These results suggest that the bioactivity of a given IL depends on the type of living organism.

The introduction of cations of natural origin can generate ILs with lower toxicities. Thus, the salicylates of the protonated amino acid esters **45** (Fig. 12.11), afforded low cytotoxicity against the L929 cell line (EC_{50} values in the mM range) [136]. Recently, Billeci et al. synthetized non-toxic gluconic-based ILs displaying ammonium, imidazolium and phosphonium cations, with IC_{50} values against different cancer cell lines within the mM range [137]. The covalent attachment of gluconic acid fragments to ammonium and imidazolium cations **46** ($n = 2$, R = But, X = Br) and **47** achieved EC_{50} values against HeLa cervical cancer cells of 2.18 ± 1.27 mM and not detected respectively. Gluconic acid was also used as the anion in ILs **48** (Fig. 12.11). The salt containing the phosphonium cation $PBut_4$ showed EC_{50} values of 5.02 ± 0.42 mM against HeLa cells [137].

Dicationic ILs (DILs) represent another class of ILs that have been investigated for medical and pharmacological applications. In this regard, some DILs derived from imidazolium **49**, pyridinium **50** and pyrrolidinium **51** (Fig. 12.11) have displayed a lower toxicity against *V. fischeri* than their related monocationic ILs [138].

Quantitative structure–activity relationship modeling (QSAR) of ILs has been also carried out [139,140]. Regarding aquatic organisms, it has been reported that toxicity increased slightly with the number of nitrogen atoms in the ring of aromatic cations. It decreased with ring methylation,

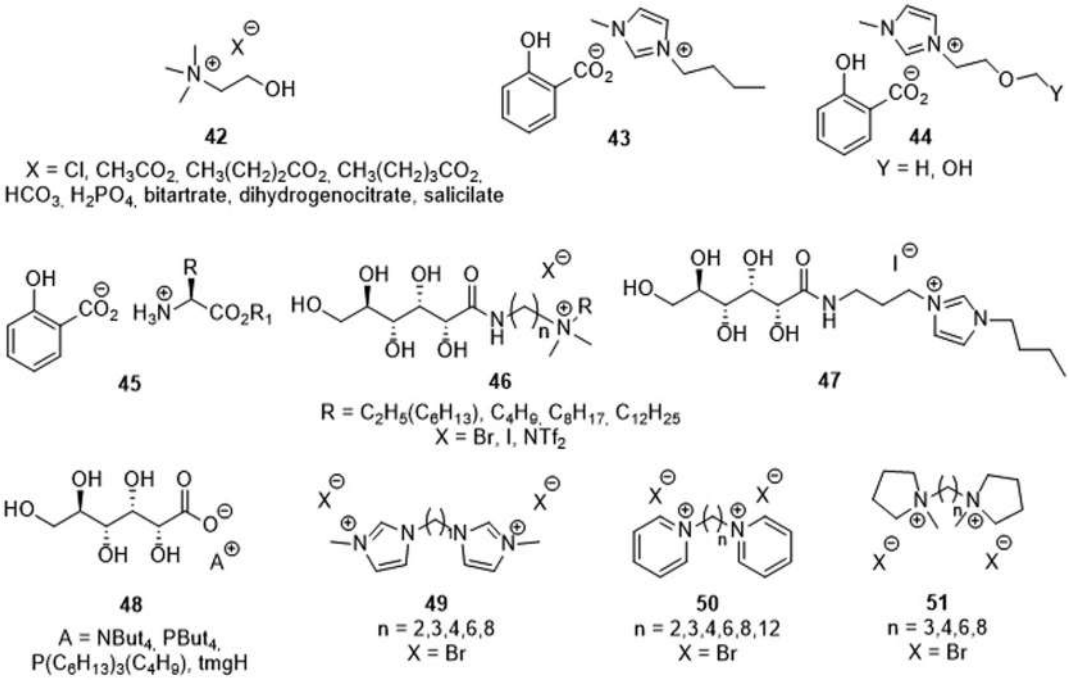


FIGURE 12.11

Structures of different choline, salicylic, gluconic, and dicationic-based ILs with studied toxicity.

and with an increase in the number of negatively charged atoms on the cation, consistent with the experimental finding that the choline cation (with a negatively charged oxygen atom) is relatively nontoxic [139]. An additional QSAR study was carried out on ILs with imidazolium, pyridinium, phosphonium and ammonium cations and salicylic, docusate and NTf_2 anions to identify their eco-toxicity. Results indicated that the cation head group and the alkyl side chain length have a significant effect on toxicity. The cetylpyridinium group was classified as slightly toxic and the imidazolium as the less toxic [140].

12.3 Bioactivity of ionic liquids

Initial data on the bioactivity of ILs, raised a significant interest in the research community for their study as agents against pathogen bacteria and tumor cells [129,141]. As some ILs-based pharmaceuticals have been approved by the FDA [30], ILs represent good candidates for the design of drug formulations with a balance between the desired pharmacological effect in the absence of adverse side effects, and the physical properties required for manufacturing, stability, solubility, transport and bioavailability [5].

Antibiotics are considered one of the great "miracles" of the 20th century. Now in the 21st century (post-antibiotic era), the miracle is turning into a nightmare, due to the growing problem of the resistance of microorganisms to classical antimicrobials and the lack of investment in the search for new antimicrobial agents. The abuse of antibiotics has increased bacterial resistance to available drugs which makes the treatment of infections caused by these microorganisms extremely difficult, and expensive; in many cases increasing morbidity and mortality [142]. The World Health Organization has developed a global action plan on antimicrobial resistance and a list of priority pathogens resistant to antibiotics [143]. It is estimated that 80% of microbial infections in humans are mediated by biofilms, including periodontitis, endocarditis, and chronic pulmonary cystic fibrosis. Biofilms are architectural colonies of microorganisms on a static surface (living or nonliving). Bacterial biofilms are usually pathogenic in nature and are prominent colonizers of permanent medical devices, such as central venous catheters, urinary catheters, and endotracheal tubes, often spreading hospital-acquired infections refractory to antibiotic treatment, requiring removal of infected devices [144]. Thus, there is a need to develop therapeutic strategies to inhibit biofilm formation by developing new compounds and/or methodologies with bacteriostatic and/or fungistatic or bactericidal and/or fungicidal capacity. Another important human disease of this century is cancer, which kills millions of people globally, being the second-leading cause of death in the world [145]. Innovative research has fueled the development of new medications and treatment technologies, being of great interest fighting against this illness using chemotherapy among other treatments [146]. However, selectivity of chemical compounds used in chemotherapy is an important issue as some of them show severe toxicities to normal cells. Survival rates are improving for many types of cancer, thanks to advances in cancer screening and treatment. In the present context, the solubility and polymorphism of the pharmacophore is an important problem, often restricting its use in chemotherapy [147].

12.3.1 Active pharmaceutical ingredient-ionic liquids

As mentioned above, most of the API-ILs described in the literature have been designed to improve solubility and avoid polymorphism of the registered API, while the specific activity inherent to the new API-IL has been less exploited.

12.3.1.1 Antimicrobial active pharmaceutical ingredient-ionic liquids

The active ions from an API-IL can be pharmacologically independent or act in a synergistic or antagonistic manner counteracting one ion the effects of the other. Some examples illustrating synergism between ions are shown with API-ILs based on antibiotics (Fig. 12.12) [63,148,149]. Seter et al. prepared the dual active ILs **52** and **53** from commercial antiseptic and antibiotic cations and anions and studied their activity against marine microbes. The results demonstrated that the new ILs were more active than the corresponding APIs independently [37]. Ampicillin ILs based on imidazolium and pyridinium were synthetized by Warner and coworkers [63]. Improvements of 25% and 10% in antibacterial activity were observed for the imidazolium ampicillin **54** relative to [Na][Amp] and halide imidazolium counterparts respectively. The enhanced activity was attributed to the increased lipophilicity, resulting in a more effective perturbation of the bacterial walls. Matrinium ILs **55**, based on fatty acid anions derived from vegetable oils, exhibited improved antibacterial and anticancer activities compared to matrine, as well as relatively lower cytotoxicity on

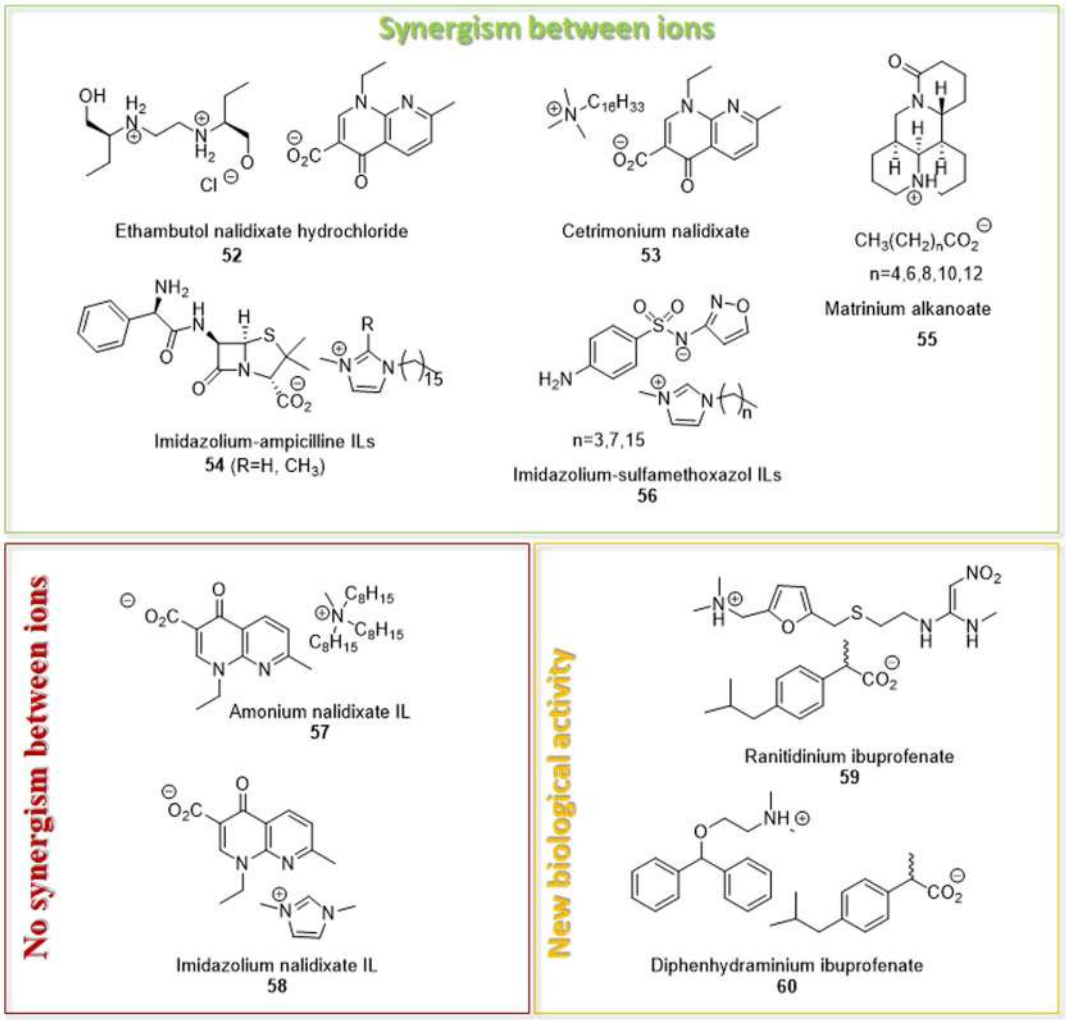


FIGURE 12.12

Structures of some antimicrobial API-ILs.

normal cells. Compound **55** ($n = 12$) showed a minimum inhibitory concentration (MIC) against *Escherichia coli* five times lower than matrine [149]. Giernoth and coworkers, synthetized different antibiotic ILs in combination with imidazolium-based cations. The resulting API-ILs containing hydrophobic cations were more efficient than the sodium salt of the API and the corresponding imidazolium bromide against different bacterial strains. IL **56** ($n = 16$) presented MIC values twelve and six times lower than sodium sulfamethoxazol and [C_{16}mim][Br] respectively, attributed to an efficient cumulative effect [148]. However, less hydrophobic cations as in **56** ($n = 4$) showed MIC

values two times lower than sodium sulfamethoxazol, confirming that the effects of API-ILs depend on the chemical structure of the cation.

An example illustrating the lack of synergism was observed by Rossmanith et al. for different nalidixate ILs against a *Salmonella* pathogen [150]. The activity of the nalidixate IL **57** was in the same range than the one for the corresponding precursors independently. The same was observed for the related IL **58**, with a nonactive cation, that was still active with MIC values in the same range as nalidixic acid [150].

The combination of two pharmacologically active ingredients in an API-IL can also result in a new biological activity not present in the precursors. Thus, antifungal activity was observed for ranitidinium ibuprofenate **59** that was not present in sodium ibuprofenate (analgesic) and ranitidine hydrochloride (H2 antagonist) [151]. Furthermore, sodium ibuprofenate [Ibu][Na] and diphenhydramine hydrochloride [Dip][Cl] did not present activity against different bacterial strains, while the corresponding [Dip][Ibu] **60** showed a good antimicrobial activity, indicating that the combination of these drugs results in a new biological activity [151].

Ammonium and piperidinium theophyllinate-based ILs were designed and studied as antimicrobial agents [120,152]. ILs **28**, **29** and **30** were less efficient antifungals than the commercial API at low concentrations, although at 1000 ppm they were very efficient [120].

12.3.1.2 Antitumor active pharmaceutical ingredient-ionic liquids

Fig. 12.13 shows the structure of some antitumoral API-ILs. The cytotoxic and antiproliferative activity of ampicillin-based ILs against several tumor cell lines have been studied, observing IC_{50} values in the nano or low micromolar range as well as very low toxicity toward normal cells [153,154]. One of the most promising antitumoral agent was the imidazolium-ampicillin salt **61** and the choline ampicillin **62** with IC_{50} values in the nanomolar range and a SI higher than 2000

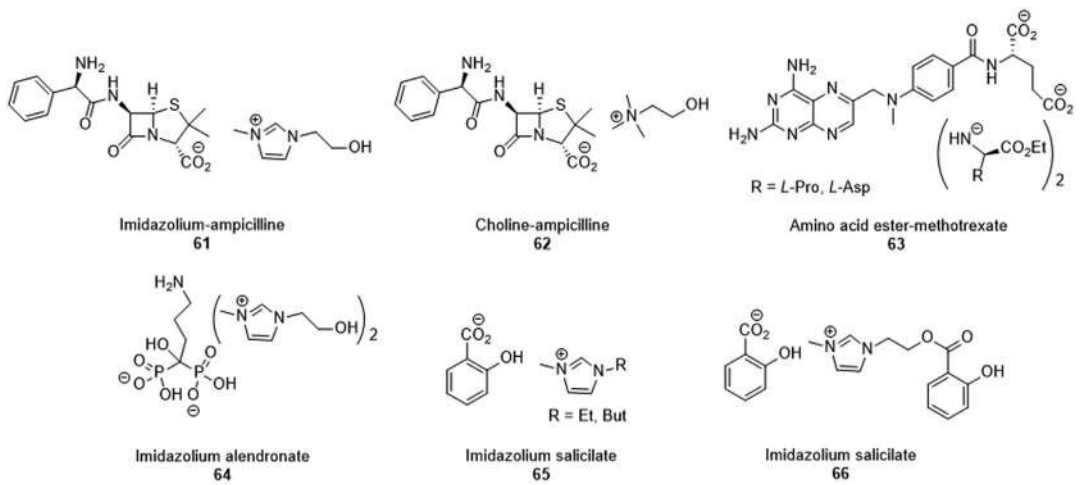


FIGURE 12.13

Structures of some antitumoral API-ILs.

for **62** [153]. Different methotrexate ILs have been developed and the corresponding amino acid- and imidazolium-based ILs showed higher antitumor activities than ammonium-based ILs [95]. The methotrexate IL **16** presented an enhanced in vitro antitumor activity as compared with the sodium salt of methotrexate. Interestingly, the amino acid-based methotrexate IL **63** presented higher toxicities than their precursors [95]. ILs based on alendronic acid (used to prevent osteoporosis) **64** displayed good anticancer activities against lung cancer and osteosarcoma cell lines, while showing very low toxicity toward healthy cells (SI > 100) [155]. The cytotoxicity of imidazolium salicylates has been studied by Egorova et al. [69]. The observed cytotoxicity was significantly higher than the one for the corresponding imidazolium salt and comparable to that of salicylic acid. For example, salicylate ILs **65** presented cytoxicities against normal fibroblasts cell line 3215 LS 30−50 times higher than [C_2mim][Cl] and [C_4mim][Cl]. The introduction of a salicylic acid fragment on the cation (**66**) led to an increase in activity.

12.3.1.3 Antioxidant agents

Antioxidants are substances that reduce the concentration of free radicals at skin, and thereby prevent and repair damages caused by endogenous and exogenous oxidative stress, being important as constituents in skin care products [156]. Phenolic compounds have been recognized by their antioxidant and antiinflammatory properties [157]. To overcome solubility problems of naturally available phenolic compounds, different phenolate ILs have been described. In vitro studies of bis(ammonium) ILs **67** (Fig. 12.14) derived from protocatechuic and gentisic acids as water-soluble antioxidants, indicated that their antioxidant activity was significantly higher than that of free acids or Trolox [158]. Cholinium salicylate **68** (Fig. 12.14) exhibited antioxidant and antiinflammatory activities comparable to those for salicylic acidic [159]. The antioxidant properties of ferulate-based protic ILs **69** (Fig. 12.14) were higher than for the parent acid [160].

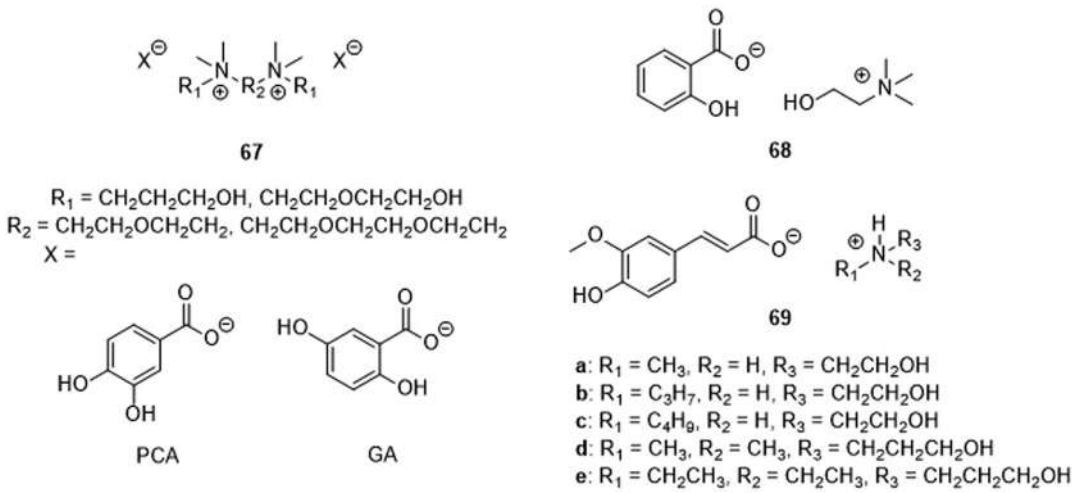


FIGURE 12.14

Structures of some antioxidant API-ILs.

12.3.2 Ionic liquids not derived from active pharmaceutical ingredients with biological activity

The inherent biological activities of many families of ILs not derived from APIs, including their (eco)-toxicological properties mentioned above, led to a growing interest for their study as new bioactive compounds with interesting properties for medical uses [161,162].

12.3.2.1 Antimicrobial agents

12.3.2.1.1 Ionic liquids derived from aromatic imidazolium and pyridinium cations

Cetylpyridinium chloride (CPC) **78** is used as an antimicrobial ingredient in commercial products such as mouthwashes and toothpastes, which are marketed for reducing plaque accumulation and gingival inflammation [163]. The antimicrobial activity of CPC was first described in 1939 [164]. Later, its bacteriostatic or bactericidal effects to bacteria in the oral cavity were demonstrated [165]. In 2004 Demberelnymba et al. reported the first imidazolium IL with antimicrobial properties [166]. Figs. 12.15 and 12.17 present the structures of some of the studied imidazolium and pyridinium antimicrobial ILs.

As discussed in Section 12.2.6, the nature of both cation and anion plays a critical role in the biological activity of ILs, being the hydrophobicity of the anion and cation the most significant vector.

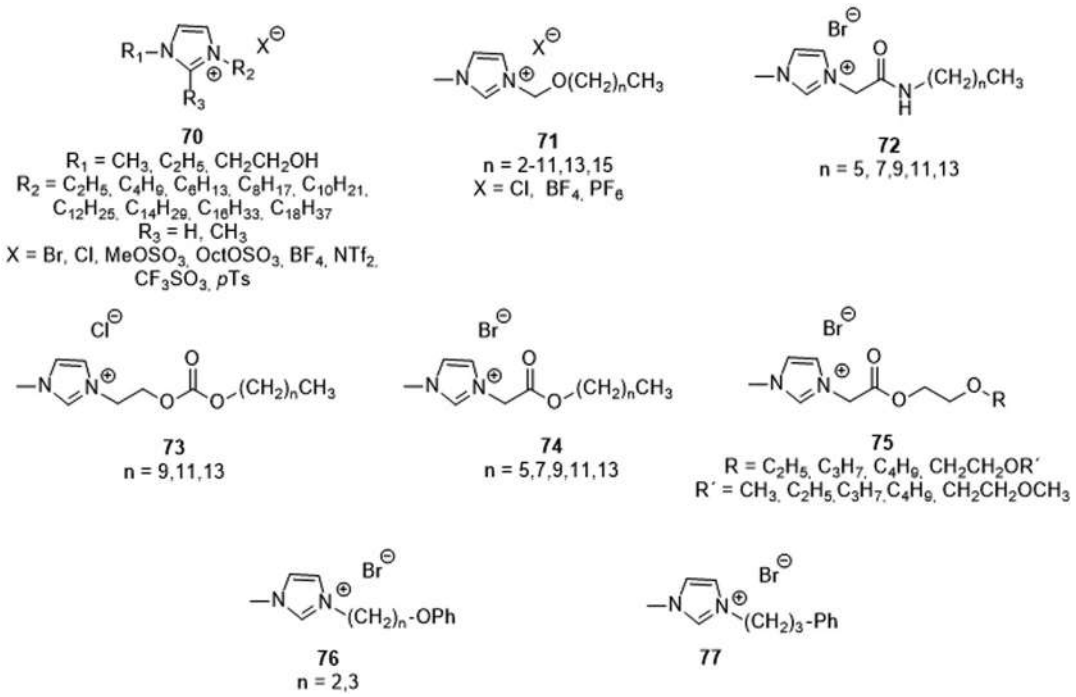


FIGURE 12.15

Structures of some antimicrobial imidazolium ILs.

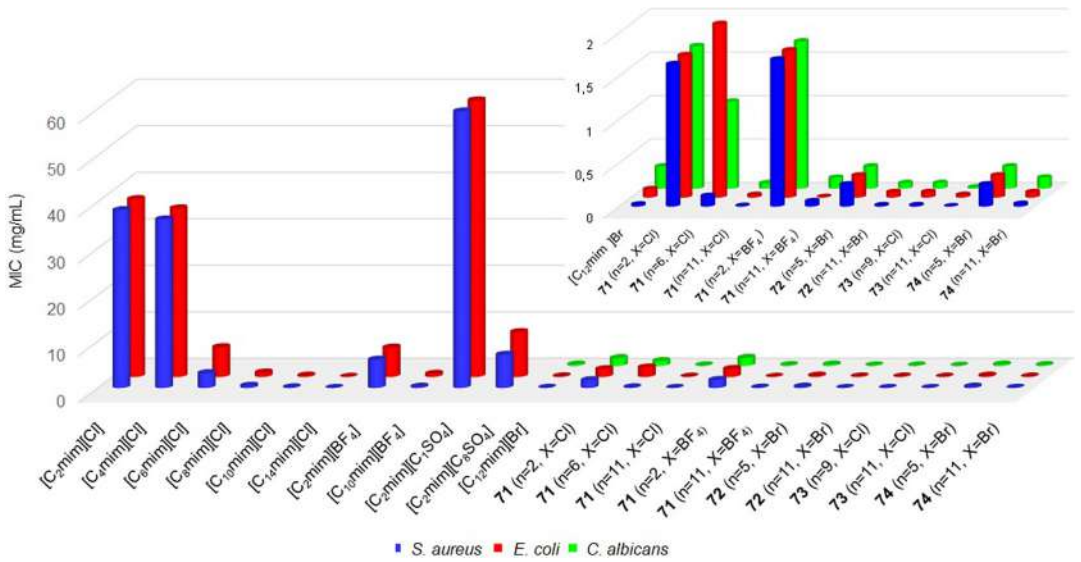


FIGURE 12.16

Minimum inhibitory concentration (MIC mg/mL) values for several imidazolium ILs against different bacteria strains. Antimicrobial activity assessment was performed by the microdilution method after 24 h of incubation at 37°C.

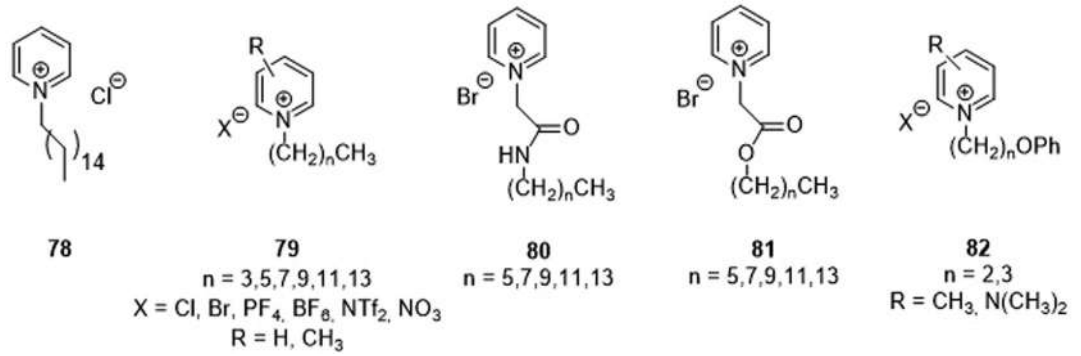


FIGURE 12.17

Structures of some antimicrobial pyridinium ILs.

The antimicrobial activity of different alkyl imidazolium ILs against pathogenic strains was reported, and their killing capacity increased with their hydrophobicity. Thus, the most active imidazolium ILs **70** presented C_8-C_{16} chain lengths, whereas salts with alkyl chains shorter than C_6 were not active (Fig. 12.16) [141,166–168]. The same behavior was observed for 3-methyl imidazolium salts with 1-alkoxymethyl chains (**71**), being C_{12} the optimal chain length showing the lowest MIC values

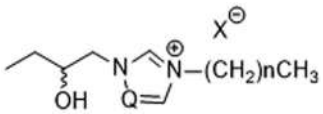
against Gram positive and Gram negative bacterial strains. For fungi strains, the optimal alkyl chain was C_{16} [77]. Furthermore, no significant differences in the antimicrobial activities were observed using different counter anions in the presence of long alkyl chains [77], while in the presence of short alkyl chains the anion became more relevant [167]. ILs with long alkyl chains can easily align with lipids and hence, accumulate within the bacterial cell wall, favoring its disruption (Section 12.5). Fig. 12.16 shown some representative EC_{50} values for different alkyl imidazolium ILs against Gram positive and Gram negative bacteria and fungi.

The introduction of other functional groups in the alkyl chain near the imidazolium ring, such as amide (**72**), carbonate (**73**) or ester (**74**) enhanced or maintained the bioactivity observed for the nonfunctionalized compound **70** (Fig. 12.16) [169–171]. In addition, the killing behavior of such functionalized imidazolium salts was dependent on the length of the alkyl chain length, being again C_{12} the optimal length. However, the introduction of ether or polyether functionalities led to a significant reduction in activity [80,172]. MIC values for *E. coli* and Candida albicans were higher than 6000 mg/mL for **75**. On the contrary, the introduction of aromatic groups in the alkyl chain increased the activity. MIC values for *E. coli* were 10 μg/mL and 6.25 μg/mL for **76** ($n = 2$) and **77** respectively [80,173].

Similar trends have been found for alkyl and functionalized pyridinium ILs **78**–**82** (Fig. 12.17). In general, pyridinium ILs were more bioactive (lower MIC values) than related imidazolium ILs [141,168,169,171,173].

Imidazolium and triazolium salts containing chiral alcohol fragments (**83**) in one of the N-substituents have also been designed as potent antimicrobial agents (Fig. 12.18). Imidazolium salts presented slightly stronger antibacterial activity than the triazolium salts. MIC values ranged between 0.005 and 0.5 mM for Gram positive bacteria strains for imidazolium **83** [76,174].

Different imidazolium ILs containing cations or anions of natural origin have been evaluated as antimicrobial agents against Gram positive, Gram negative and fungi strains (Fig. 12.19). Protic alkyl imidazolium and alkoxymethyl imidazolium salts containing lactate (**84** and **85**) manifested a pronounced bacteriostatic activity, with MIC values lower than the MBC (minimum bactericidal concentration) [74]. ILs **84** and **85** with short alkyl chains (C_1 to C_5) were inactive, while the L-lactates with C_{11} and C_{12} alkyl chains were the most active ones. The biological activity spectrum of the studied salts varied depending on the strain of microorganisms within the same group. Interestingly, lower MIC values were observed for L-lactate ILs than for racemic lactate ILs, highlighting the importance of chirality [74]. Chiral imidazolium and pyridinium ILs based on



83
n = 1,2,3,4,6,9,11,15
Q = CH, N
X = Br, I

FIGURE 12.18

Structures of antimicrobial chiral imidazolium and triazolium ILs.

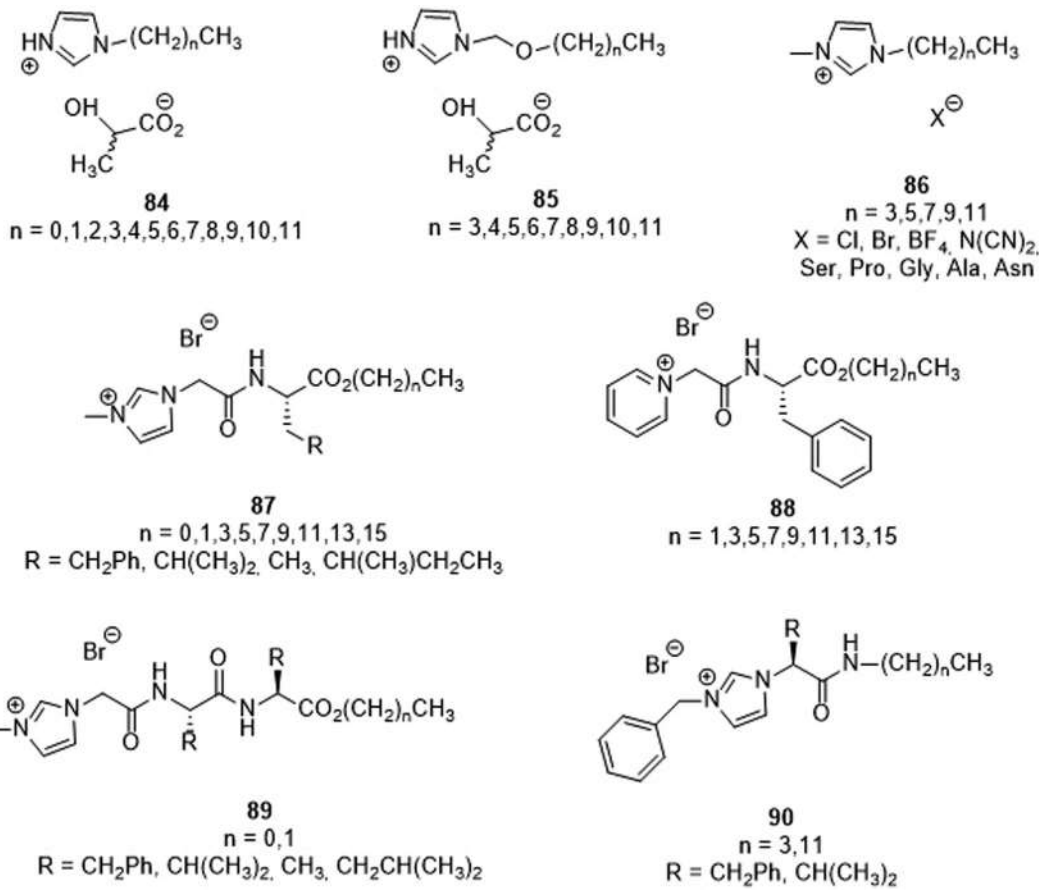


FIGURE 12.19

Structures for some imidazolium and pyridinium ILs containing amino acid derived fragments studied as antimicrobial agents.

amino acids have been reported as potent antimicrobial agents [75,79,175]. In the case of the [C_8mim] cation in 3-alkyl-1-methyl imidazolium ILs **86**, the five compounds with lower antimicrobial properties were those containing amino acid derived anions [175]. Imidazolium ILs N-substituted with chiral amino acid ester moieties (**87**) and dipeptidyl ester fragments (**89**) with short alcohol chain lengths ($n = 0,1,3$) presented moderate to low toxicities against different bacterial and fungal strains. Dipeptidyl compounds **89** displayed the highest toxicity toward the bacterial strains. A very low MIC value (125 μM after 24 h incubation) was obtained against the *Staphylococcus aureus* strain resistant to the antibiotic methicillin [176]. Biodegradable imidazolium and pyridinium salts **87** and **88** derived from L-phenylalanine showed MIC95 values decreasing with larger chain lengths until reaching the lowest values for C_{10} to C_{14} derivatives, regardless of the cationic head group nature [75]. In general MIC95 values were lower for Gram positive than for Gram

negative bacteria, which has been ascribed to their different cell wall structure [177]. Pyridinium derivatives **88** were, in general, more active than the related imidazolium derivatives **87** [75]. Monotopic and ditopic L-valine and L-phenylalanine imidazolium ILs containing different hydrophobic groups (**90** and **91**) were tested against Gram negative and Gram positive bacteria, with the more lipophilic ones showing higher toxic effects against Gram positive bacteria. Compound **90** ($R = CH_2Ph$, $n = 11$) presented lower MIC and MBC values against *E. coli* than the analogous valine compound and MBC and MIC values of 4 μg/mL toward *Bacillus subtilis*, being 15 times less active against human embryonic kidney cells HEK-293. Furthermore, monotopic salts **90** with longer alkyl chains were more active than the ditopic counterparts **91**.

Several authors have also designed politopic imidazolium and pyridinium ILs and evaluated their antimicrobial activity (Fig. 12.20) [80,178]. Study of dicationic imidazolium surfactants **92** revealed that the main structural factor affecting the antimicrobial activity was the nature of the hydrophobic tail, while the length of the spacer had no significant effect on the biological properties [178]. Messali and coworkers reported a pyridinium compound **93** ($n = 4$, $X = BF_4$) displaying a high inhibitory activity. The observed MIC values against methicillin resistant *S. aureus*, *E. coli* TM, *C. albicans*, and *Clostridium difficile* were 32, 64, 32 and 32 μg/mL respectively [179].

On the other hand, Gilmore and coworkers were the first to reveal the in vitro antibiofilm properties of a series of $[C_n mim][Cl]$ ILs against a range of clinically important bacterial and fungal pathogens [180]. The antibiofilm activity of these ILs was observed to be function of the length of alkyl chain. Gram negative microbial biofilms were less sensitive to $[C_n mim][Cl]$ than those from Gram positive bacteria [180]. The antibiofilm and antimicrobial activities of a series of alkyl quinolinium ILs (**94**, Fig. 12.21) against fungi and bacteria colonizers associated to nosocomial infections were also studied. Similar trends were observed, with a higher antibiofilm potency for the larger alkyl chains. For C_{16}, the MIC against *E. coli*, *S. aureus* were 5.8 and 11.6 μg/mL [181].

QSAR models for predicting the antimicrobial activities of ILs have been developed for a rational design of ILs to be included in pharmaceutical applications [175,182].

12.3.2.1.2 Ionic liquids derived from nonaromatic quaternary ammonium cations

Quaternary ammonium salts represent one of the most effective types of antiseptics in the last century (Fig. 12.22) [183]. In 1916, Jacobs was the first to find out the bactericidal properties of a series of quaternary salts of hexamethylenetetramine [184]. However, it was not until 1935, when Domagk synthetized benzalkonium chloride followed by its introduction in the market, that the

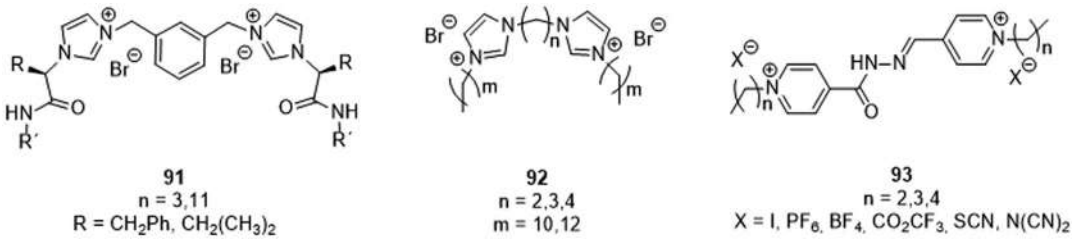
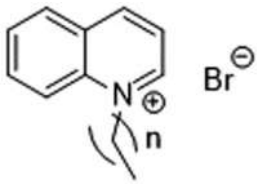


FIGURE 12.20

Structures for some antimicrobial ditopic imidazolium and pyridinium ILs.



94
n = 9,11,3,15,17

FIGURE 12.21

Quinolinium ILs studied as antimicrobial agents.

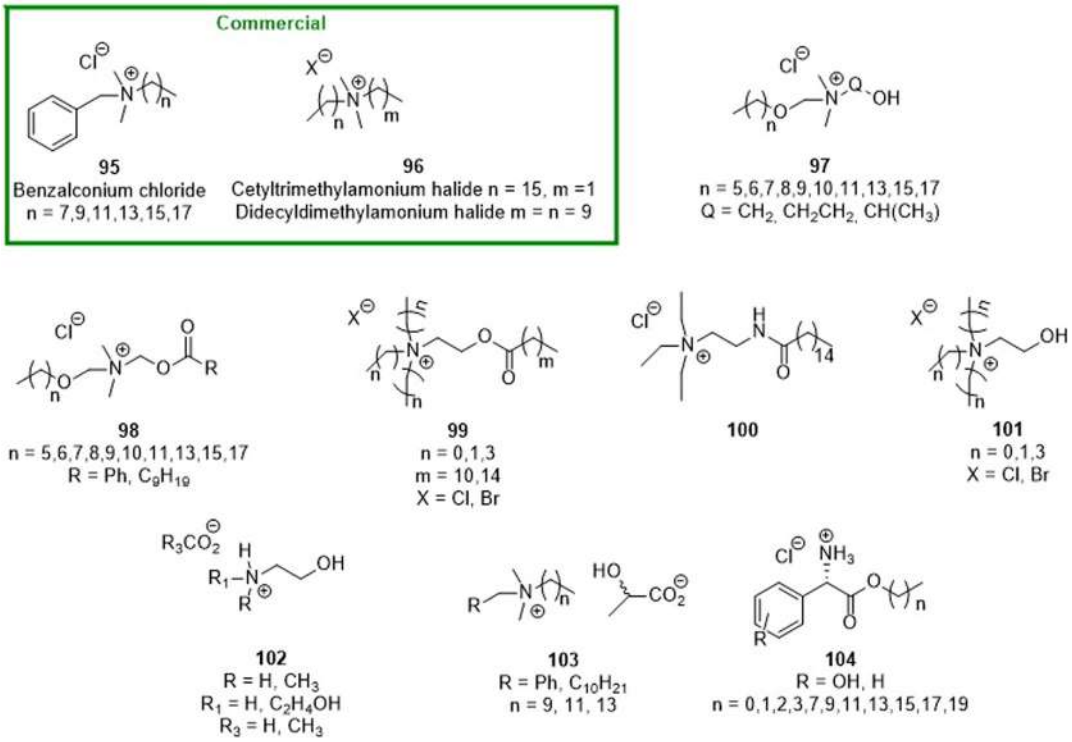


FIGURE 12.22

Structures of some antimicrobial alkylammonium ILs.

potential of quaternary ammonium salts as antibacterial agents was recognized [185]. The most common commercial monocationic alkyl ammoniums (Fig. 12.22) are benzalconium **95**, cetyltrimethylamonium **96** ($n = 15$, $m = 1$), and didecyldimethylamonium **96** ($n = m = 9$). Later, different

authors have modified the nature of the alkyl and aryl groups in ammonium ILs to enhance their toxicity profile (Fig. 12.22) [186]. J. Pernak et al. synthetized the choline-like quaternary ammonium chlorides **97** and **98**. Compounds containing 9/14 carbon atoms presented good antimicrobial properties. MIC values for the ester derivatives **98** were slightly lower than those for alcohols **97**. For **98** ($n = 7$; $R = C_9H_{19}$) MIC values against *E. coli* and *S. aureus* were 6.7 and 1.7 μM respectively [187]. Quaternary ammonium ILs containing labile ester and amide groups linked to long alkyl chains (**99** and **100**) have been reported as good antimicrobial and antiviral agents, presenting higher killing properties the compounds containing C_{10} to C_{16} hydrophobic tails. For **99** ($n = 1$, $m = 14$), MIC values against *Enterococcus faecalis, S. aureus, E. coli* and *Pseudomonas aeruginosa* were 8, 2, 64 and 250 μg/mL. However, the hydroxyalkyl derivatives **101** were not active with MIC values >1000 μg/mL [188,189]. The comparison of the antimicrobial activities of **99** and **81** suggested that ammonium ILs presented higher killing abilities than pyridinium analogs. Protic hydroxylammonium ILs (**102**) were highly active against human pathogens such as *S. aureus*, *Salmonella typhi*, and *Vibrio cholerae*, exhibiting inhibition effectiveness comparable to commercial gentamicin [190].

Ammonium ILs with lactate and azolate anions (**103** and **6**) are powerful antimicrobial substances. Compound **103** presented MIC values against Gram positive, Gram negative and fungi strains of <2, 250 and 8 mg/L respectively [70,71]. Amino acid derived protic ammonium ILs have been studied as antimicrobial agents by different groups [191,192]. Thus, protonated tyrosine esters (**104**, R = OH) are more active than the phenylalanine analogs [193]. The correlation between the aggregation capacity of the protonated phenylalanine and tyrosine esters **104** and their antibacterial activity showed that monomers, instead of micelles, were the responsible for this activity. Moreover, good antibacterial activity of bisalkyl ammonium ILs and pyrrolidinonium salts has also been reported [166,194].

12.3.2.1.3 Phosphonium ionic liquids

Tetraalkylphosphonium ILs have been the most studied in this group, Fig. 12.23 shows the structure of some antimicrobial phosphonium ILs. Their toxicity against several bacterial and fungi strains are in line with the cation lipophilicity [195,196]. For ILs **105**, the structure of both the cation and the anion affects their biological activity, being active those with alkyl chain lengths from C_5 to C_{14}. The exchange of the halide anions for other anions such as OTf, BF_4 or NTf_2 led to the lost of antimicrobial activity. For the cation [P6,6,6,14] and Cl, OTf, NTf_2 and BF_4 anions, the observed MIC values against *E. coli* were 31, >790, 644 and 876 μM respectively [195]. The introduction of triphenylamine moieties in one of the chains (**106**), provided an activity increase specially against Gram positive bacteria. Thus, for compounds **106** (X = Cl) and [P4,4,4,6][Cl] MIC values against *S. aureus* were 1 and 64 mg/mL respectively [197]. Furthermore, dicationic bis-phosphonium ILs **107** presented a broad spectrum of antimicrobial activity against ocular pathogens [198].

12.3.2.2 Ionic liquids as antitumoral agents

In 2003, the first report on naturally occurring imidazolium salts isolated from a root extract of *Lepidium meyenii* and their cytotoxicity against a panel of human cancer cell lines was published [199]. Later, the bioactivity of a variety of phosphonium-, tetralkylammonium-, pyridinium- and imidazolium-based ILs were investigated in vitro with different tumor cell lines [124,200–204] Kumar and coworkers studied the structure–activity relationship for 1-methyl imidazolium ILs **32** and **70** showing that the length of the

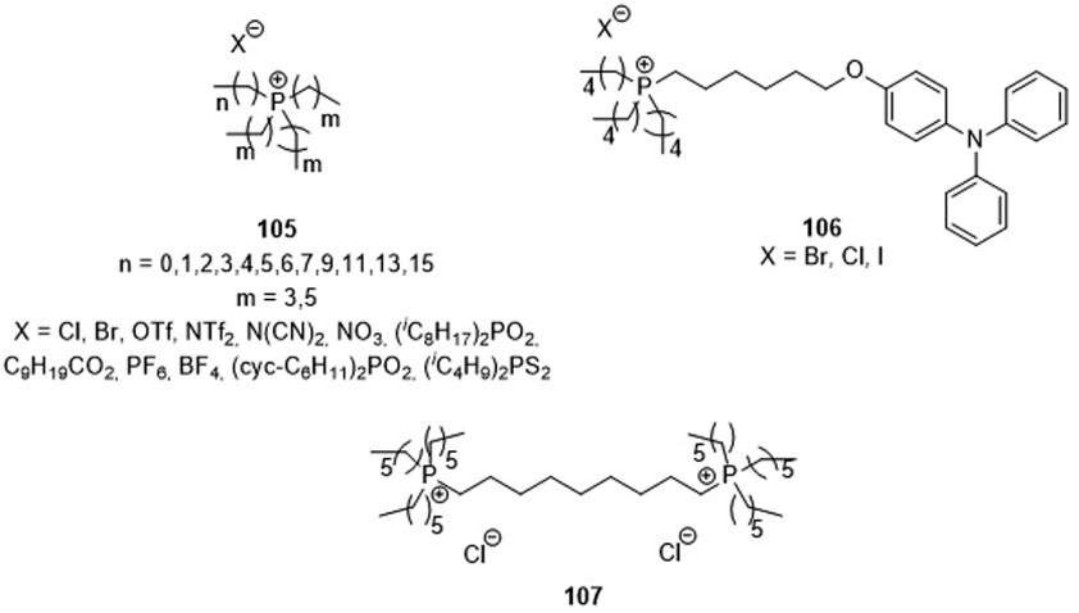


FIGURE 12.23

Structures for some antimicrobial phosphonium ILs.

3-alkyl substituent was very important for their antitumor and cytotoxic activity. Compounds with chain lengths up to C_8 were inactive against 60 different cancer cells, irrespective of the anions. The introduction of polar functional groups in these short chains did not show any improvement in the anticancer activity [200]. When the alkyl chain length increased was >10, a significant enhancement in the tumor growth inhibition was observed in multiple cell lines [200]. The same behavior was observed for tetraalkyl ammonium **96** and tetraalkylphosphonium **38** and **105** ILs [201]. In vitro cytotoxicities against the human breast cancer cell line IPC-81 of a series of alkyl pyridinium (**79**), alkyl imidazolium (**70**), pyrrolidinium (**34**), piperidinium (**35**), phosphonium (**105**), and quinolinium (**94**) confirmed that the alkyl chain length was the main vector controlling cytotoxicity. For salts having alkyl chains of comparable length, the antitumor activity followed the order tetrabuthylphosphonium > tetrabuthylamonium > quinazolinium > imidazolium > pyridinium > pyrrolidinium > piperidinium (Fig. 12.24) [125,204]. A different behavior was observed, however, against human breast cancer cells MCF-7, where cation cytotoxicity increased in the order pyridinium > pyrrolidinium > imidazolium > piperidinium for comparable chain lengths. ILs $[C_4mpip][Br]$, $[C_4mpyr][Br]$ and $[C_3mim][NTf_2]$ were nontoxic with EC_{50} values higher than 6000 μM, while EC_{50} values for $[C_8py][Cl]$, $[C_8mpyr][Br]$ and $[C_8mim][BF_4]$ were 9, 80 and 691 μM respectively [202]. Additionally, it has been observed that choline-derivatives and alkyltriethylammonium salts were less toxic than their pyridinium and imidazolium analogs against HeLa cell lines (Fig. 12.24) [203].

Regarding anion nature, the NTf_2 anion led to lower EC_{50} values against WST-1, IPC-8, HeLa and MCF-7 breast cancer cells than halide or BF_4 anions (Fig. 12.24) [200,201,205]. However,

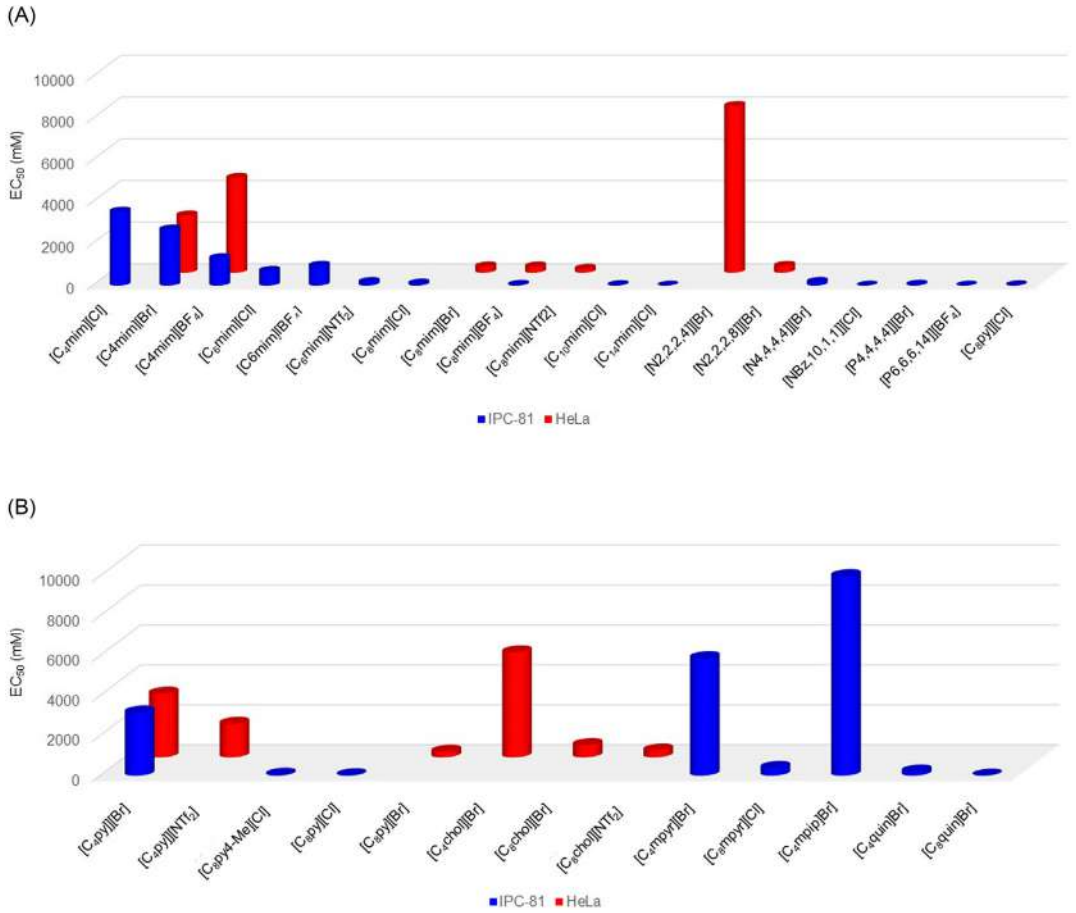


FIGURE 12.24

EC_{50} values for different ILs against IPC-81 and HeLa tumor cells. Cells viability were measured using an MTT assay or WST-1 reagent.

cytotoxicity assays against colon adenocarcinoma HT-29 and colorectal adenocarcinoma CaCo-2 cell lines using different cations revealed that the NTf_2 anion significantly decreased the activity, independently of the cation. Thus, for the tri-octylmethylammonium cation, acesulfame, saccharin and dicyanoamide anions induced higher toxicities than the NTf_2 anion [206]. These observations indicate that the structure−cytotoxicity relationships are clearly dependent on the specific cell line. Thus, the introduction of the salicylate anion in alkyl imidazolium-based ILs dramatically increased their cytotoxicity. The replacement of the chloride anion with salicylate for $[C_4mim]$ or $[C_3mim]$ cations led to a significant decrease in IC_{50} on carcinoma cell lines CaCo-2 and normal fibroblasts 3215 LS [69].

Imidazolium and pyridinium ILs containing fluorinated phenylacetamide chains (**108** and **109**) exhibited good anticancer activities against tumor lung H-1229 and A549 cell lines. The

percentages of inhibition for imidazolium **108** and pyridinium **109** were 48−59% and 50−99% respectively. DNA interaction studies showed a reasonably good binding affinity [207].

As mentioned earlier, one important parameter is the SI. In this regard, Kaushik et al. studied the activity of a series of ammonium and imidazolium ILs against human T98G brain cancer cells and compared their activities toward HEK nonmalignant cells [82]. Protic [Hmim]Cl (**110**) (Fig. 12.25) displayed a high activity against the T98G brain cancer cell line and low cytotoxicity on HEK cells (SI = 1.9) [82]. A series of 4,5-dialkylimidazolium surfactants (**111**) (Fig. 12.25), exhibited high antitumor activity against the tumor cell line C6 from rat glioma and the nontumor MDCK cell line respectively. Their antitumor and cytotoxic activity were negatively correlated with their surface activity. Compounds with C_7 chains exhibited higher antitumor activity than the analogous with C_{11} and C_{15} chains, being the SI higher than 12 for **111** ($n = 6$) [208].

The biological activity for a series of DILs **112** (Fig. 12.25) was the result of the combined action of the structures of both cation and anion. The cation seems to provide the main contribution with the anion becoming relevant only in the presence of short alkyl chains [209]. Furthermore, the antitumor activity was also affected by the isomeric substitution on the benzene ring, with the lowest IC_{50} obtained for *p*-isomers. Thus, $IC_{50} < 50\ \mu M$ for the *para* substituted **112** ($n = 10$, $X = BF_4$) while $>100\ \mu M$ for the *orto* substituted analog(Fig. 12.25) [209]. In vitro evaluation of pyridinium DILs **113** (Fig. 12.25) as potential anticancer scaffolds revealed that *meta* substituted compounds

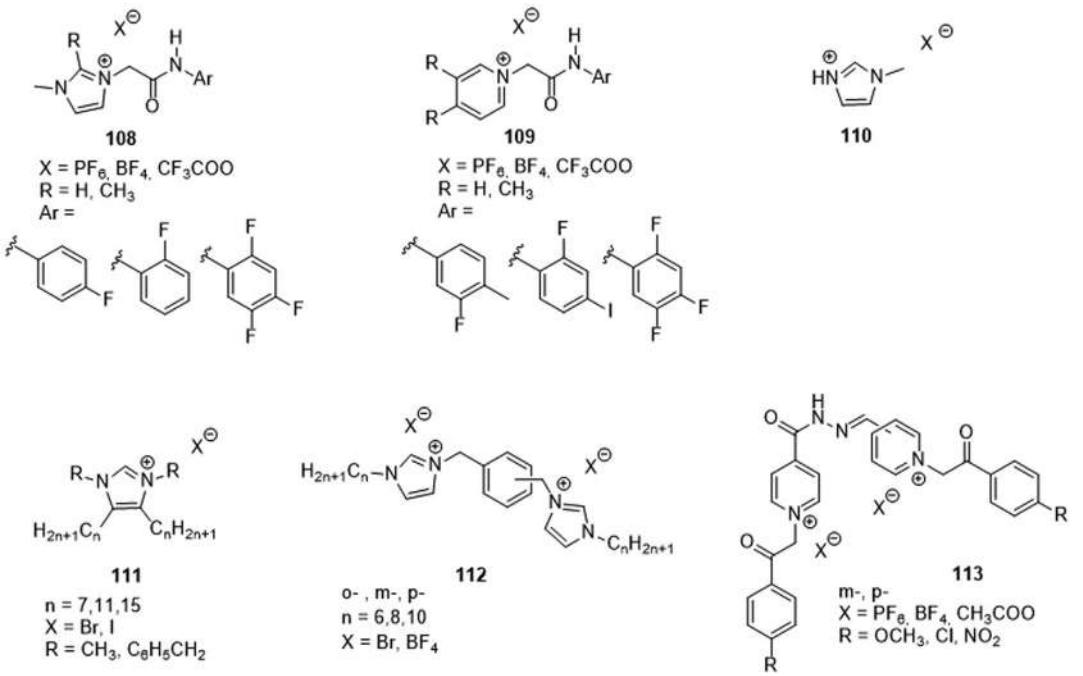


FIGURE 12.25

Structures of some ILs with antitumor activity.

with X = Cl and PF_6 as anions were the most active against all tested cell lines. Results were further supported by in silico studies revealing good docking score on the target protein phosphoinositide 3-kinase (PI3Kinase), with the highest binding energy score for ILs meta substituted **113** (X = Cl, PF_6) [210]. Different QSAR studies have been carried out to predict the cytotoxicity of ILs against different tumor cell lines [211].

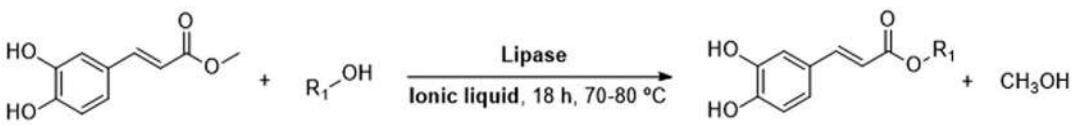
12.4 Ionic liquids as enhancers in the pharmaceutical industry

12.4.1 Ionic liquids in the synthesis of drugs

This section provides an overview of the application of ILs as media in the synthesis of drugs. Because of their excellent solvent characteristics, ILs have been investigated since 1990 as new reaction media and later in the synthesis and crystallization of pharmaceutical drugs or their precursors and as solvents for the extraction of natural compounds [212,213]. It is worth mentioning that the potential toxicity of ILs, as discussed in Section 12.2, could make them unsuitable for drug synthesis in case they remain as residual solvents in the final product. However, many ILs display similar toxicities than many pharmaceutical excipients, such as dimethylsulfoxide or polysorbate 80 and many organic solvents. Besides, as mentioned earlier, nontoxic ILs have been synthesized by selecting biocompatible organic cations and anions and displaying good physical properties like viscosity, to be applied as eco-friendly solvents [214]. Thus, the use of ILs in drug synthesis is a promising research area since ILs can facilitate the purification and isolation of the drug and control their polymorphism.

In many reactions, ILs can be employed as reaction media and catalyst. ILs containing the imidazolium cation in combination with soft anions are the most widely used for API synthesis [215]. One of the first examples using ILs as media for the synthesis of APIs was reported by Seddon for the preparation of the NSAID pravadoline [216]. Later, ILs have been used as solvent and/or catalyst for the synthesis of antitumor, antimicrobial, antimalarial or cholinesterase (ChE) inhibitor drugs among others, using a variety of synthetic approaches [217].

Thus, an efficient enzymatic synthesis of caffeic acid derivatives, via transesterification reaction, was developed using ILs as the solvent (Scheme 12.4) [218]. The basic [C_4mim][OH]



R_1 = $(CH_2)_2$Cyclohexyl, $(CH_2)_3$Cyclohexyl, $(CH_2)_4$Phenyl, $(CH_2)_5$Phenyl
Lipase = Novozyme 435, RMIM, PS-CI, TLIM
Ionic liquid = [C_4mim][NTf_2], [C_4mim][PF_6], [C_4mim][BF_4], [C_4mim][CF_3SO_3], [C_3mpra][NTf_2], [C_3mpyr][NTf_2], [C_3mpip][NTf_2], [N1,1,1,3][NTf_2]

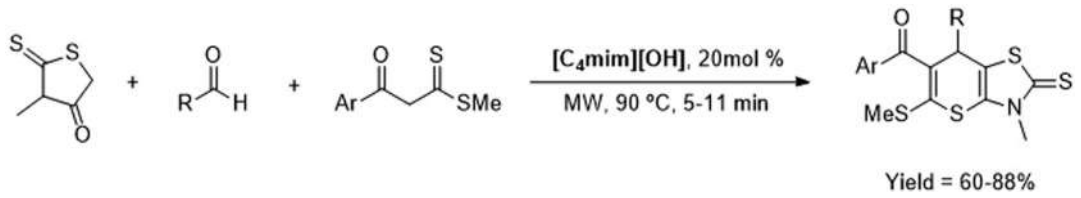
Yield = 84-98%

SCHEME 12.4

Enzimatic synthesis of caffeic acid derivatives using ILs as solvents [218].

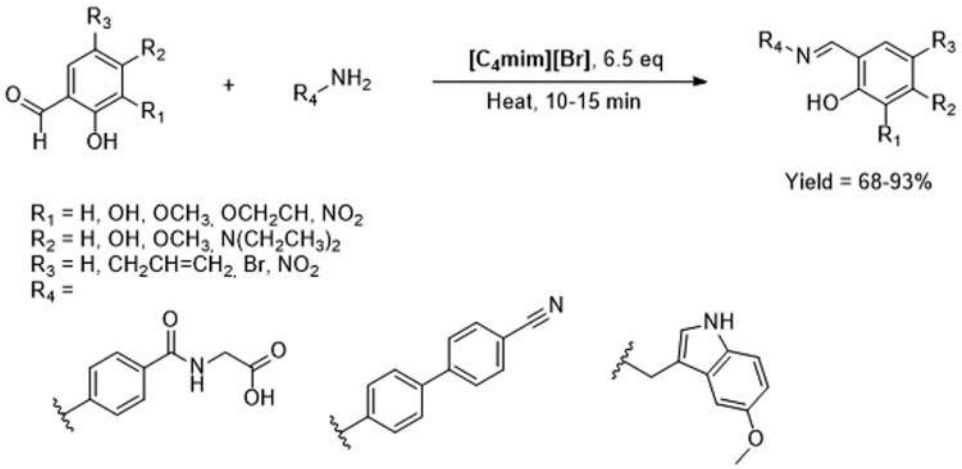
catalyzed the versatile three component one-pot synthesis of thiopyrano[2,3-d]thiazole-2-thiones under microwave irradiation, leading to high yields in short reaction times (Scheme 12.5) [219]. Different Schiff bases have been prepared as good ChE inhibitors through a condensation reaction in an IL (Scheme 12.6) [220].

Moreover, ILs provide promising results in the field of pharmaceutical crystallization, being a viable approach to manipulate crystal properties [18,221]. In this regard, various methods of crystallization using ILs have been reported [222]. Polymorphs are usually controlled using organic and/or aqueous solvents that allow to tune the solvent-solute interactions [223]. As liquids with designed properties, ILs represent excellent candidates as solvents for developing new polymorphs of APIs [224]. For instance, 1-ally-3-ethylimidazolium tetrafluoroborate was capable of producing new polymorphs of adefovir dipivoxil, which were not achievable with conventional organic solvents [224]. A crystal structural change of lysozyme was observed when using IL $[C_4mim][BF_4]$ [225]. Paracetamol was successfully crystallized from $[C_6mim][PF_6]$ and $[C_4mim][PF_6]$ using cooling crystallization, obtaining uncommon crystal patterns as compared to conventional solvents [226].



SCHEME 12.5

MW synthesis of thiazole derivatives catalyzed by $[C_4mim][OH]$ [219].



SCHEME 12.6

Synthesis of ChE inhibitors using ILs as the solvent [220].

12.4.2 Ionic liquids in drug formulation. Controlled release systems

A drug delivery system is a formulation or a device that enables the introduction of a drug into the body improving its efficacy and safety by controlling the rate, time, and site of release [227]. Drug delivery routes include topical, transdermal, oral, injection and nasal. Within these routes, transdermal drug delivery has several advantages over oral administration as it does not need to cross through the gastrointestinal and liver track, avoiding metabolism. Besides it represents a noninvasive route. The main disadvantage of dermal drug delivery is the barrier function of stratum corneum, the external skin layer, and its limited permeability [228]. Thus, in a passive transport system, hydrophilic ionized salts can hardly cross the epithelial barrier. Increasing drug lipophilicity enhances its skin permeability by increasing partitioning into the lipophilic environment of the skin's barrier layer [229]. An alternative method to increase permeability is the use of chemical permeation enhancers such as alcohols derivatives, sulfoxides, fatty acids, terpenes, azone, and so on [230]. However, their toxicity, irritating properties, and their incomprehensible mechanism of action make them unsuitable for clinical applications, and only few of them are used clinically.

Many APIs, as mentioned earlier, are difficult to dissolve within the body and/or FDA-approved solvents, limiting their delivery and bioavailability. Considering this, the ability of ILs to dissolve drugs provides an alternative tool for drug delivery. An increased drug solubility in the corresponding IL can allow a higher concentration of the drug, leading, for example, to an enhanced skin permeability, and can provide shelf stable solutions for dispensing. In this regard ILs have been used as formulation components to improve drug delivery as solubility enhancers, in microemulsions replacing the water IL-in-oil (IL/O), the oil (IL/W or W/IL) or replacing the surfactant, as gelators and in polymeric systems as biomembranes and patches (Fig. 12.26). However, it must be kept in mind that for such applications ILs need to be used at nontoxic concentrations. Several reviews have appeared in this area [22,24,231], and accordingly our discussion will only focus on the recent advances made for topical delivery systems.

12.4.2.1 Drug delivery strategies based on active pharmaceutical ingredient-ionic liquids

As ILs are often more lipophilic than dissociated salts, different API-ILs have been studied for dermal or transdermal delivery (Fig. 12.27) [59,93,232]. This strategy was first investigated by Megwa et al. [233], and, later, different studies have been carried out to enhance the skin permeation of protic alkylammonium salicylates [57]. The use of API-API combinations and that of more biocompatible ions has also been considered. Thus, etodolac-lidocaine **114**, a nonsteroidal antiinflammatory drug (NSAID) patch, was developed as a novel transdermal delivery system. In vitro studies showed that this IL had higher skin permeability than free etodolac [234]. The patch reached Phase I clinical assays [235]. In vitro transdermal studies were also carried out using different donepezil derived ILs based on natural oils (**115**) revealing a facilitated transdermal delivery compared to donepezil [39]. Lidocaine ibuprofenate **116** and lidocaine docusate **2**, enhanced local anesthesia relative to the commercial cream product EMLA and to conventional lidocaine hydrochloride. This was believed to be due to an improved lidocaine absorption into the skin [58]. A significant increase in the transdermal delivery of the ionic liquefied NSAIDs salicylate **44** and ibuprofenate **117** has been reported. Skin penetration of the imidazolium salicylate **44** was approximately nine times faster than that of sodium salicylate and a 10-fold increase in the

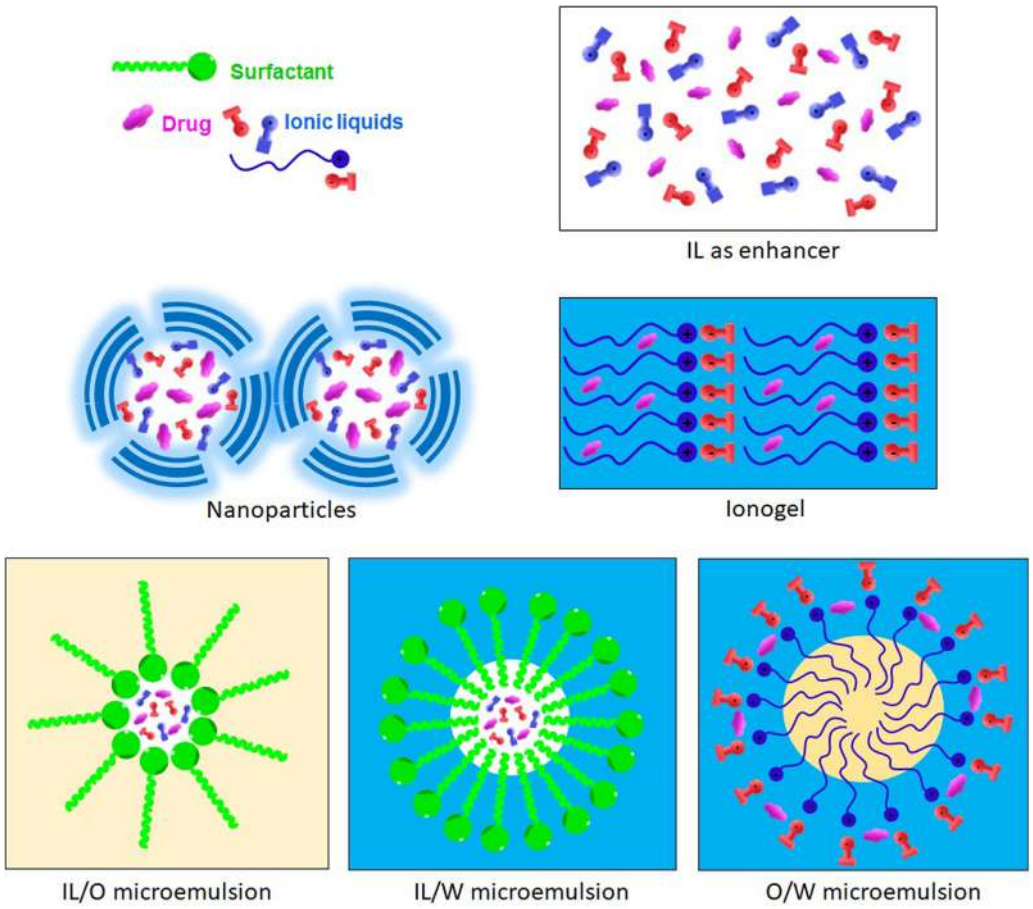


FIGURE 12.26

Drug delivery strategies using ILs.

cumulative amount of drug at 96 h was observed for the proline ester-ibuprofen IL **117** relative to the control [136,236].

Nanostructured cellulose materials with antioxidant and antiinflammatory properties for topical applications have been prepared with choline-gallic/caffeic/ellagic ILs **118, 119** and **120**. In vitro skin permeation studies showed zero order kinetics with a slow and sustained drug release [237]. Bacterial nanocellulose membranes loaded with choline-vitamin B ILs **121**, **122** and **123** demonstrated to be noncytotoxic to skin epithelial cells and suitable materials as antioxidants for skin care applications [238]. The reported data demonstrated improved membrane transport. In the case of IL **124**, presenting a rather polar tetrabutylphosphonium cation, assays in Caco-2 cell monolayers revealed that membrane transport was not improved as compared to the free API [239].

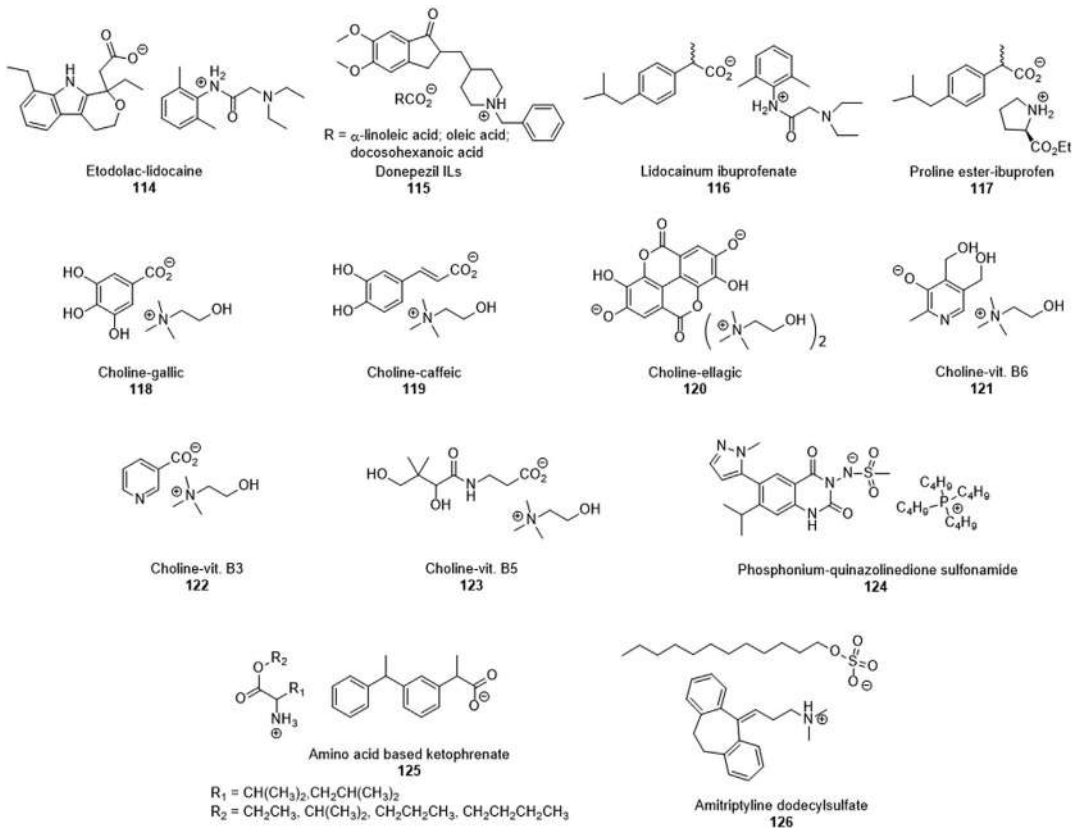


FIGURE 12.27

Some structures of API-ILs applied in drug delivery systems.

One strategy to understand pharmacokinetics and pharmacodynamics of drugs, is the study of their interactions with serum albumin proteins, which is responsible of the biological transport of hormones, steroids, fatty acids, drugs etc. In this regard, Guncheva et al. synthetized ILs **125** based in ketoprofenate and protonated amino acid esters that showed low cytotoxicity and a strong affinity (estimated binding constants 105 L/mol) to serum albumin [240].

In a different approach, IL **126** was designed as an oral drug delivery system, working as both the carrier and the active drug. This IL was able to form vesicles with higher permeability than pure amitriptyline hydrochloride, indicating a higher bioavailability [241].

12.4.2.2 Drug delivery strategies using ionic liquids not based on known active pharmaceutical ingredients

The selection of an appropriate hydrophobic−lipophobic balance in the IL, allows its application in drug delivery systems or as solubilization/enhancer agents to improve the aqueous solubility of the

corresponding API. Solubilization of drugs can be driven by different mechanisms as cosolvency, hydrotropy or micellization. For micellization a surfactant molecule is required. The resulting microemulsions are solution-like systems composed of phases of different polarities (i.e. oil and water) stabilized by surfactants and represent excellent vehicles for the solubilization and transport of water-insoluble and/or oil insoluble active compounds.

12.4.2.2.1 Ionic liquids as chemical enhancers

In their initial report in 2008, Jaitely and coworkers introduced the use of butyl, hexyl and octyl-3-methylimidazolium PF_6 as potential reservoirs for hydrophilic and hydrophobic drugs [242]. Table 12.2 shows the enhanced solubility of different drugs, with low intrinsic solubility in water, in the ammonium, pyridinium and imidazolium ILs presented in Fig. 12.28. Choline amino acid-based ILs **127** have been investigated as solubility and/or permeation enhancers for caffeine, salicylic acid, feluric acid and rutin [243,244]. ILs **127**, were more suitable than the related imidazolium ILs **128** for topical formulations, since they had higher impact in drug solubility and a lower human cytotoxicity [244]. Amphotericin B, an antifungal agent, presented good solubility in the IL **129** containing a strongly basic, hydrogen bond acceptor anion [OAc], but was not soluble in any of the related ILs displaying fatty acid carboxylates as anions [245]. The resulting solution could be poured into water (pH = 7) at high concentrations (0.10 mg/mL) and the same occurred with itraconazole (0.25 mg/mL) [245]. The low solubility of danazol in aqueous media and in lipid formulations limits its use for oral administration, while the high solubility of danazol in ILs **130** suggested their potential as enhancers for drug formulations [246]. Although paracetamol presented

Table 12.2 Solubility of different APIs in selected ILs and in water.

Drug	Water solubility mg/mL 25°C	Ionic liquids	Ionic liquids solubility mg/mL	References
Ferulic acid	0.64	127	4	[243]
Rutin	0.20	127	>7	
Amphotericin B	2.0×10^{-4}	$[C_2mim][OAc]$ 129 $[C_4NH_3][OAc]$ 129 $[C_4NH_3][Oleate]$	85 30 <5	[245]
Itraconazole	1.0×10^{-6}	$[C_2mim][OAc]$ 129 $[C_4NH_3][OAc]$ 129 $[C_4NH_3][Oleate]$	<5 <5 <5	
Danazol 337 g/mol	1×10^{-3}	130 $R = C_6H_{13}$ $n = 5$ $X = N(CN)_2$ $R = C_6H_{13}$ $n = 5$ $X = NTf_2$	>90[a] 25[a]	[246]
Ibuprofen	0.124	$[C_4mim][PF_6]$	12.18	[247]
Paracetamol	19.16	$[C_4mim][PF_6]$ $[C_6mim][PF_6]$	6.95 13.21	
Paclitaxel	<0.004	131 [Chol][Gly] [Chol][Ala] [Chol][Pro]	22.34 18.52 16.16	[248]

[a] *mg/g.*

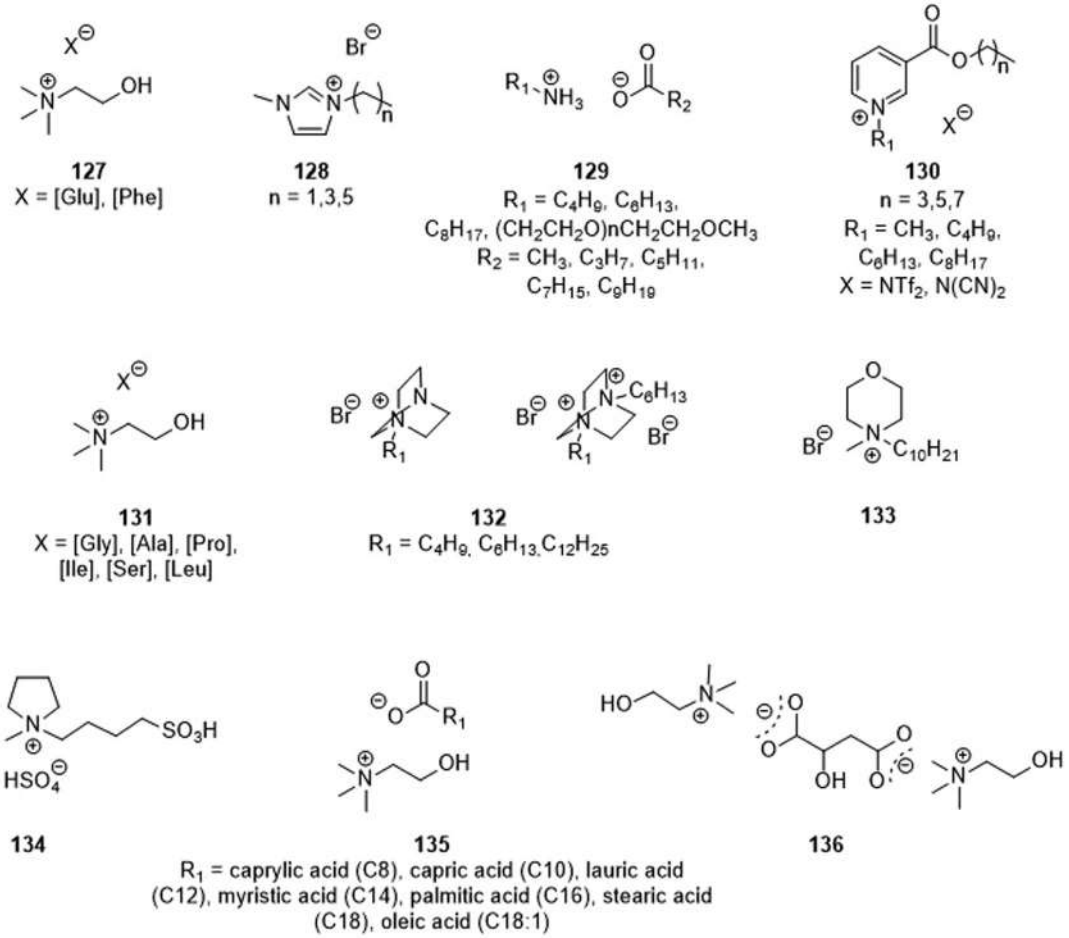


FIGURE 12.28

Structure of different ILs used as drug enhancers.

lower solubilities in a series of imidazolium containing PF_6 such as [C_4mim][PF6] or [C_6mim][PF6] than in water, the solubility of ibuprofen in those ILs was one order of magnitude higher [247]. It has been reported that choline amino acid-based ILs **131** could be an alternative vehicle to conventional organic solvents for paclitaxel formulations, as its solubility in those ILs is four orders of magnitude higher than in water [248].

A series of ILs derived from cyclic ammonium cations (**132**−**134**) were tested as permeation enhancers for transdermal diltiazem delivery. In vitro experiments showed they presented a good balance between enhancer activity and cytotoxicity [21].

Drug distribution and membrane interaction studies in ILs are essential for the development of new formulations containing ILs as excipients. In this regard, binding studies between nimesulide,

a NSAID, and serum albumin protein (HSA) in [C_2mim] ILs, evidenced that those ILs did not negatively affect the binding and were expected not to diminish the in vivo efficiency of the drug [249]. Biocompatible ILs based on choline have been reported to improve dermal delivery of hydrophilic molecules. ILs formed with fatty acid carboxylates (**135**) were used as solvents for transdermal delivery of a water-soluble antigen peptide. The oleate derivative presented the best behavior in terms of cytotoxicity, skin permeation of the antigen peptide, and affinity to the oil-based skin permeation enhancer [250]. The choline-malic acid-based IL **136** was used as a potent enhancer or vehicle for dermal delivery of hydrophilic macromolecules [251].

ILs have also been used as enhancers in chitosan and polydopamide nanocarriers [252,253]. The in vivo antitumor efficacy of [C_4mim][PF_6]-polydipamide nanoparticles loaded with doxorrubicin on mice have been studied after intravenous drug administration, results demonstrated improved effects in the combined chemotherapy and microwave thermal therapy of cancer [253].

12.4.2.2.2 Surface-active ionic liquids as microemulsion carriers

Surface-active ILs (SAILs) have been used to develop microemulsions [254], and investigated to design delivery systems. In this regard, the binding abilities of amphiphilic and gemini SAILs to different drugs have been studied [255]. The hydrophobic/hydrophilic balance of the SAIL plays a crucial role in the micellization process, the interfacial behavior, and the drug binding ability [256]. Binding studies of [C_{12}mim][Cl] and [C_{14}mim][Cl] with lidocaine hydrochloride showed that drug molecules got adsorbed on the surface of the aggregates helping in drug delivery [257]. Increasing the concentration of procaine hydrochloride decreased the critical aggregation concentration values of the surfactant [C_{14}mim][Cl]. This was attributed to the presence of cation$-\pi$ interactions between the SAIL and the drug molecules [258]. Strong synergistic interactions between sodium diclofenac and SAILs [C_{12}mim][Br]/[C_{14}mim][Br] have been observed, leading to a dramatic reduction in surface tension and formation of various types of aggregates [259]. Studies on the effect of composition and dilution on the micellar transitions in [C_{12}mim[Cl]-ibuprofen] aqueous mixtures revealed enhanced micellization and adsorption tendencies and varied aggregate assemblies in aqueous medium determined by the amphiphile mixing ratio and the total mixture concentration [260]. Furthermore, the binding of dopamine hydrochloride and acetylcholine chloride with surface-active ionic liquids such as [C_{14}mim][Br] confirmed that SAILs acted as better drug carriers compared to conventional cationic surfactants [261]. The synergistic aggregation of ibuprofen and SAIL carriers was modeled via molecular dynamics simulations [262].

12.4.2.2.3 Ionic liquids as formulation components in ionic liquid-in-oil, water/ionic liquids, ionic liquids/water microemulsions

Development of IL/O microemulsions for the dermal delivery of poorly permeating drugs has been investigated [263]. The first reported IL-oil microemulsion showed its effectiveness for the transdermal delivery of the sparingly water-soluble acyclovir (ACV) [264]. The IL/O microemulsions were successfully formulated using [C_1mim][$(MeO)_2PO_2$] in the presence of Span-20 as a main surfactant and Tween-80 as cosurfactant. The ILs [C_2mim][OAc], [N2,2,2,2][OAc] and diethylamonium acetate were used in IL/O microemulsions for ACV and methotrexate release studies, showing that the particle size and the drug carrying capacity varied with the IL nature [265].

Ex vivo permeation studies for the poorly permeating drug 5-fluorouracil (5-FU), showed a significant enhancement in flux as well as in the cumulative amount of drug permeated after 24 h

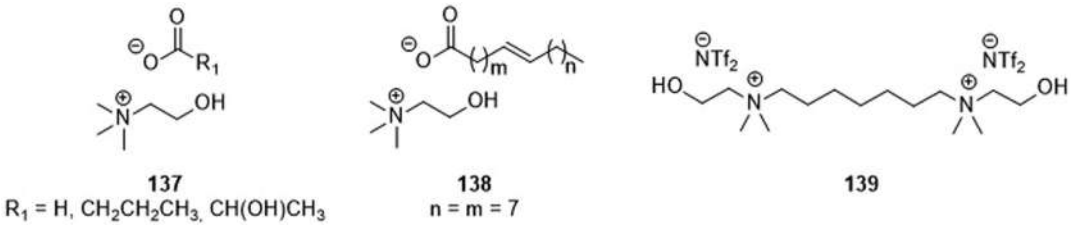


FIGURE 12.29

Structures of some ILs used in microemulsions.

using ILs. Formulations using [C_4mim][Br] were successful in treating induced mice skin tumors, whereas the aqueous solution, the conventional ointment, and the commercial cream were ineffective in regaining the normal physiology of skin [266].

Biocompatible choline ILs **137** have been designed for the transdermal delivery of ACV for IL/O microemulsions systems using SAIL **138** as the main surfactant and Span-20 as cosurfactant (Fig. 12.29), observing an increase of one order of magnitude of the drug delivered across the skin comparing with the IL or W/O carriers [267].

IL/W microemulsions have been investigated for etodolac topical delivery. Using [C_4mim][PF_6] as the IL, Tween-80 as surfactant and ethanol as cosurfactant, better permeation, antiarthritic and antiinflammatory activities were observed in comparison to other conventional formulations [268]. An IL microemulsion was also developed for the transdermal delivery of dencichine. Two ILs, [HOC_2mim]Cl and [C_4mim][$C_{12}SO_3$], showed strong enhancements of the skin permeation of dencichine, and were incorporated into both aqueous and surfactant phases. In vitro skin permeation assays suggested that the topical delivery of the drug was strongly enhanced by the ILs formulation, with an approximately 10-fold increase over that of the drug aqueous solution [269]. IL/W triphase nanoemulsions for intravenous administration of amphotericin B were developed using cholinium hexanoate and the dicholinium IL **139**, which presented negligible zebrafish in vivo toxicity [270].

12.4.2.2.4 Ionic liquids as ionogelators

Gels are materials formed by an elastic network of entangled fibers entrapping the solvent. These materials are attractive as drug delivery systems [271]. In this regard, ILs have been investigated as potential low molecular weight gelators for the encapsulation of active drugs [272]. Gels formed from imidazolium amphiphilic hydrogelators **112** ($n = 18$, X = Br) containing active drugs, could effectively release the drug in vitro and in vivo assays and demonstrated antiinflammatory capability in the treatment of rosacea, a chronic, inflammatory skin disease [273].

12.5 Biological mechanism of interactions

Most studies in this area refer to the cytotoxic properties found for some ILs. The interaction of ILs with living organisms involves different processes (Fig. 12.30): [274] (i) ILs can interact with the membrane, disturbing its phospholipid arrangement, affecting the viscoelasticity of the membrane

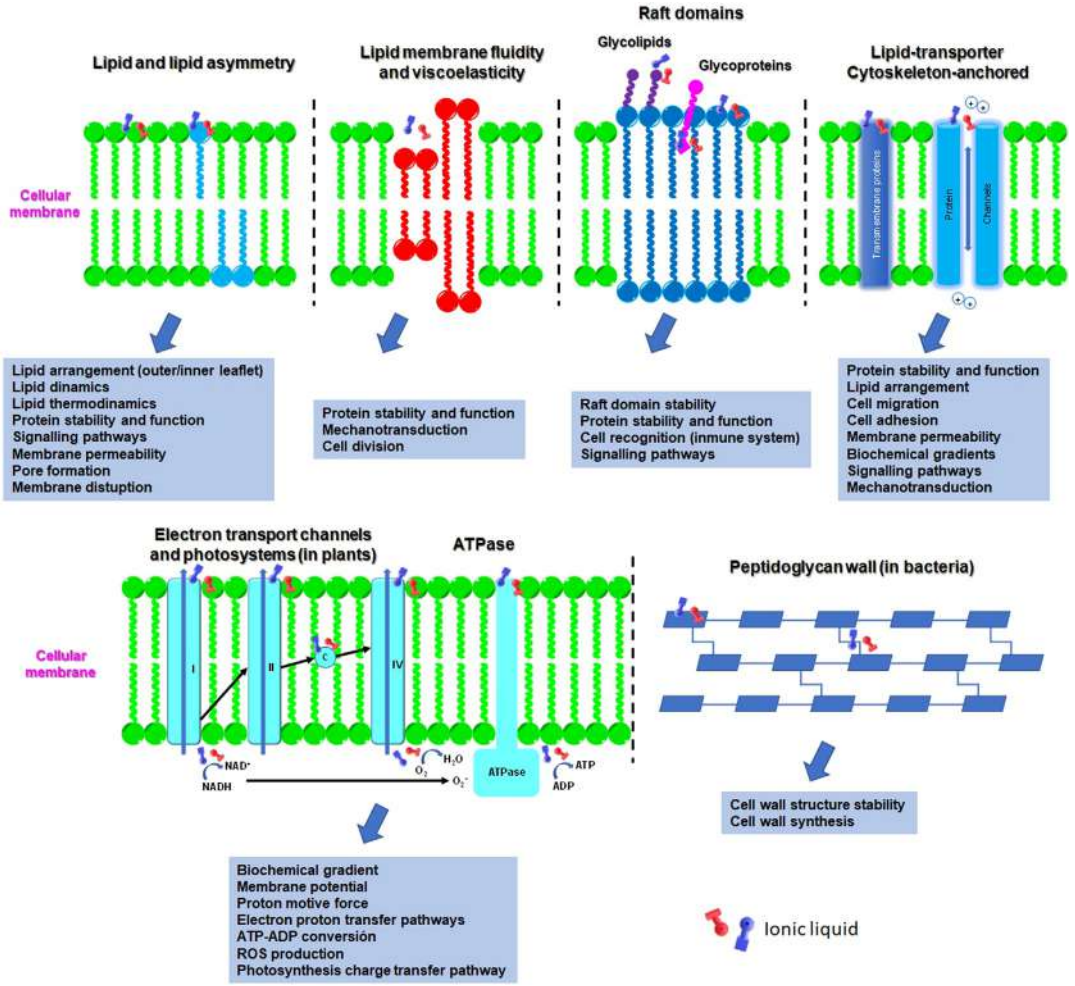


FIGURE 12.30

Potential interactions between ILs and biomembrane proposed in Ref. [274].

and fluidity, altering biological functions (mechanotransduction, division...), which could lead to cell death by apoptosis and necrosis;[275] (ii) ILs can alter membrane permeability, generating channels and leading to a change in the biomedical gradient between the extracellular media and the cytoplasm or even disrupting the entire membrane;[196,276] (iii) ILs can directly interact with membrane proteins and enzymes disrupting their biochemical function (recognition, signaling...) and diffuse into the cytoplasm [277], or can reach the intracellular region through transmembrane lipid-transporter proteins;[278] (iv) ILs can diffuse into the mitochondria and disrupt the generation of ATP;[279] (v) ILs can interfere with protein/enzyme synthesis by binding to RNA; and (vi) ILs can enter the cell nucleus reaching and interacting with the DNA [280].

To understand the molecular mechanism of membrane damage, the interaction of ILs with model membrane systems has been studied [281,282]. It has been observed how the incorporation of the ILs into the lipid membrane leads to changes in the lipid self-assembled structure and in the in-plane lateral motion, followed by cell death [283]. In general, the incorporation of the IL cations into the phospholipid bilayer becomes stronger with an increasing size of the apolar moiety [282]. ILs containing long alkyl tails can easily align with lipids and hence, accumulate within cell membrane. In this regard, membrane interactions studies with 4,5-dialkylimidazolium salts having a 1,3-dimethyl head group revealed that the long chains in the 4,5-dipentadecylimidazolium salt can self-assemble forming vesicles and fusing easily with lipidic membranes. On the contrary, the 4,5-diundecylimidazolium salt with slightly shorter chains led to imperfect hydrophobic interactions, stimulating disintegration of bilayer and plasma membranes, whereas the 4,5-diheptylimidazolium salt with significantly shorter alkyl tails passed bilayer membranes without disruption [281]. Molecular dynamic simulations studies have also shown the favorable insertion of the ILs into lipid bilayers inducing membrane disruption [284].

Inhibition of the intracellular membrane enzyme acetylcholinesterase in living cells has been observed, suggesting the enzyme as a target for ILs biological activity [285]. In silico pharmacokinetic properties of targeted ILs have been investigated, showing membrane protein p-glycoprotein inhibition [286]. Furthermore, inhibition of the activity of the acetylcholinesterase, butyrylcholinesterase and catalase enzymes in different cell lines has been observed and has been ascribed to the RNA binding affinity of ILs [287]. DNA damage, mitochondrial permeability transition, an increase in intracellular Ca^{2+}, overproduction of reactive oxygen species, gradual exhaustion of cellular adenosine triphosphate, followed by induction of apoptosis of cells, have been observed in different cell lines in the presence of imidazolium ILs [288].

Regarding the different antimicrobial activities observed against Gram positive and Gram negative strains, it has been documented that Gram positive organisms preferred a more lipophilic molecule than the Gram negative ones [177]. This has been attributed to the difference in the cell outer wall between bacterial types and strains. While Gram positive bacteria have a peptidoglycan cell wall, the outer membrane of Gram negative bacteria contains lipopolysaccharides which are cross-bridged by divalent cations, making the membrane more impermeable to lipophilic molecules. Thus, the observed trend in the antibacterial activity result could be explained by the relative lipophilicity of the compounds [289].

In addition, studies examining the toxic effects of ILs on plants reported that the oxidative stress damage is the main mechanism by which ILs exert toxicity [290]. The excessive accumulation of reactive oxygen species can induce lipid peroxidation and oxidation of DNA or proteins, which directly affects their function and cause cell damage [291].

12.6 Conclusions

ILs have received wide attention in multidisciplinary studies and offer a wide array of potential pharmaceutical and medical applications. Their unique properties and high tuneability can solve APIs solubility and permeability problems, polymorphism issues, and bioavailability to reach their final formulation stage for commercialization as new active farmaceutical ingredients. ILs have great potential as reaction media, as solubility promoters, or surfactants for the design of delivery

systems or drug formulations. In this context, their cytotoxicity can represent a problem, especially for hydrophobic ILs with long alkyl chains. On the other hand, the high biological activity makes them useful in the search of potential therapeutic agents, considering the unlimited number of possible combinations of anions and cations allow the preparation of wide libraries of active compounds. However, further investigations on ILs need to be performed to fully exploit such a potential and overcome the pharmaceutical industrial challenges. Furthermore, the biological mechanism of interaction between the IL and biological membranes requires more detailed studies, in which computational tools may play a crucial role.

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CHAPTER

Biocatalysis in subcritical and supercritical fluids

13

Željko Knez[1,2], Maja Leitgeb[1,2] and Mateja Primožič[1]

[1]*University of Maribor, Faculty of Chemistry and Chemical Engineering, Maribor, Slovenia*

[2]*University of Maribor, Faculty of Medicine, Maribor, Slovenia*

13.1 Introduction

Nowadays, the tendency of the chemical, pharmaceutical, and related industries is to move to greener, more sustainable production processes, using raw materials, producing less or no waste, and avoid the use of toxic and hazardous materials [1,2]. Considering the principles of green chemistry and sustainable development, biocatalysis can be classified as green and sustainable technology [3]. Biocatalysis refers to the use of biological systems or their parts to perform (catalyze) chemical reactions. In biocatalytic processes, natural catalysts, such as enzymes, are used for chemical transformations on organic compounds. For the catalytical purpose, both enzymes that have been isolated and enzymes inside living cells are employed.

Enzymes are generally globular proteins and as their structure may be unfold (denaturation and loss of their activity) under certain condition, a suitable medium for biocatalysis performance must be selected.

Enzymes usually act in their natural environment under water conditions. Because of this, aqueous buffers are often the choice for reaction media when enzymes are used in biochemical synthesis. Problems arise in the transfer of enzymes, as biocatalysts, into industrial processes. In many cases, many industrially important compounds are poorly soluble in an aqueous medium, so the aqueous reaction system will often not be able to provide sufficient substrate loads and consequently a successful reaction [4]. However, the product needs to be separated from the water following the reaction, which is expensive (from an economic point of view) due to its high boiling point [3].

Biocatalysis is being widely applied in the production of pharmaceuticals and some fine chemicals [3]. Further, it became more popular due to industrial interest of using green solvent systems [e.g., water, supercritical fluids (SCFs), fluorinated solvents, ionic liquids and solvent-free systems]. A suitable solution is the use of a nonaqueous solvent system to perform biocatalysis in industrial systems. Such systems are already in place for the industrial use of lipases [5–7], while for other classes of enzymes this practice is not yet so well established.

For performance of chemical reaction in industrial mmanufacture stable, selective and high productive catalysts are required with the possibility of operation under the desired process conditions [8]. Enzymes whose stability and selectivity are improved by engineering are often used for this purpose.

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The selection of the optimal solvent system is also crucial for the stability and selectivity of the enzyme and reaction products [9]. A suitable solvent can be selected for a given reaction, considering several influencing parameters (Table 13.1).

Sub (sub SCFs) and SCFs are increasingly attractive as a medium for biocatalysis. Owing to their properties such as low viscosity (similar to gas viscosity) and high density (similar to liquid density), they represent optimal solvents for biochemical reactions [10]. Their ability to adjust their properties, unlike other conventional solvents, allows reactions to be carried out with environmental, health, safety procedures, and chemical benefits (Fig. 13.1).

Table 13.1 Solvent parameters influencing the productivity of enzyme-catalyzed reaction.

Solvent parameters
Compatibility with substrates and products
Compatibility with biocatalyst
Density (e.g., low solvent density is in favor of reducing mass transfer)
Surface tension
Toxicity (e.g., low solvent toxicity enables easier waste disposal)
Flammability
Availability in a large quantity
Low cost

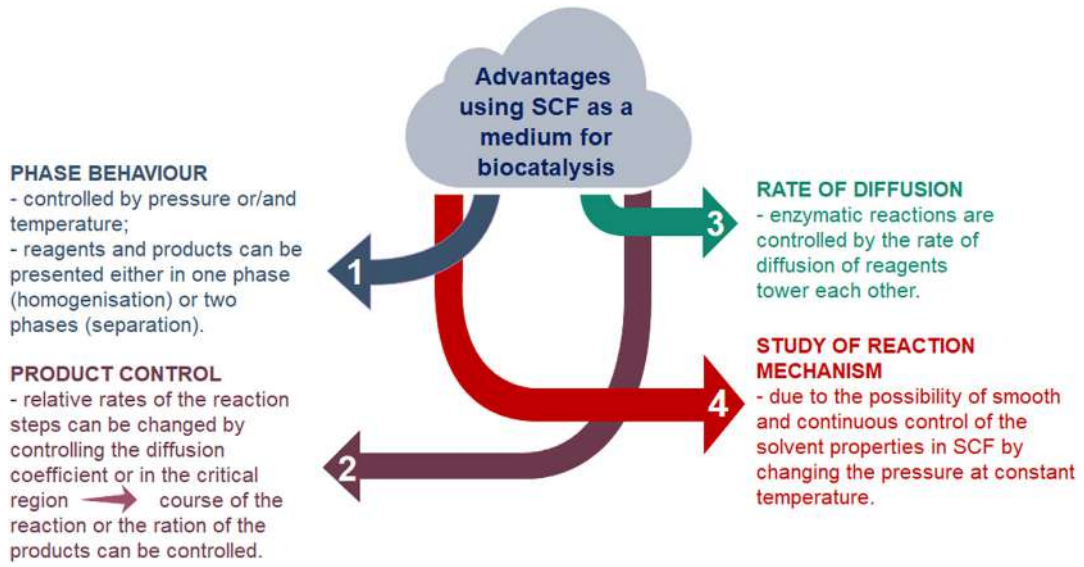

FIGURE 13.1

Advantages using SCFs for biochemical reaction (summarized from [11]).

Conducting the reaction in supercritical conditions by changing the pressure and/or temperature gives the possibility to change the properties of the solvents. The solubility of the reactants and products may increase, and the interfacial transport is limited in terms of increasing the reaction rate. As a result, selectivity can be improved, and the reaction and separation can be integrated in one step.

The advantages of using sub and SCFs as solvents for biochemical reactions are manifested in the simple further processing of the reaction systems or even the possibility of separating the interfering components that inhibit the reaction.

The combination of enzymes and SCFs is preferred because of the following: (a) the reaction equilibrium can be shifted from hydrolysis to synthesis of products; (b) the thermostability of biomolecules in SCFs is improved in comparison to that in H_2O; (c) the increase in solubility of hydrophobic materials can be achieved; (d) the solvent recycling can be easily attained; (e) the reaction and bioseparation can be integrated into a single step, since the small changes in temperature/pressure can lead in marked changes of SCFs physical properties.

Different sub and SCFs (e.g., carbon dioxide, ethane, propane, trifluoromethane, n-butane, freons) [12,13] could be used for biochemical reactions, but due to the low critical point of carbon dioxide (CO_2) 7.38 MPa and 31.2°C, it is consistent with the use of enzymes and/or labile solutes. Additional advanced properties of supercritical carbon dioxide (SC CO_2) for biochemical reaction are presented in Fig. 13.2. Further, its "naturalness" is greatly appreciated by the food, medicine and health-care-related industries [14].

Usually, the stability and activity of enzymes in SC CO_2 are preserved or even hyperactivation of the enzyme in SC CO_2 may occur [15]. Although parameters such as low critical temperature

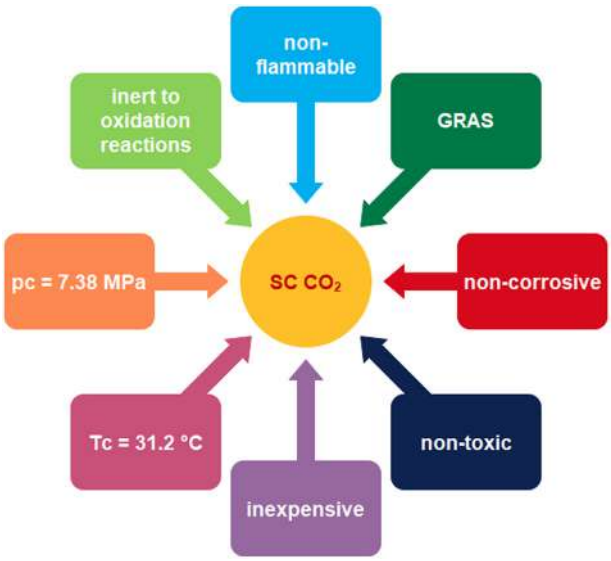

FIGURE 13.2

Advantages of SC CO_2 for biochemical reaction (summarized from [10]).

and pressure, non-toxicity, etc. are potentially beneficial for the use of SC CO_2 as a medium for enzymatic reactions; however, the presence of SC CO_2 may lead to some deficiencies, such as a decrease in pH during hydration layer of the enzyme due to carbonic acid formation and modification of the enzyme by the formation of amine complexes. In some cases, this can lead to enzyme deactivation, whereas in some other instances, carbamate formation is believed to lead to changes in enzyme catalytic features (e.g., enhanced stereoselectivity).

When the enzyme is exposed to ultrahigh pressure (>400 MPa), the irreversible structural changes in the enzyme can be caused, while within the pressure range of 10−40 MPa, only reversible conformational changes of enzyme may occur. These reversible changes usually do not affect the overall catalytic performance of the enzyme. The high temperature is always damaging for the enzyme structure, especially when applying over a long period [16]. Additional, within the supercritical region of most commonly used SCFs for enzymatic application (ranges of pressure from 1 to 40 MPa and temperature from 3°C to 60°C), an increase in pressure and/or a decrease in temperature lead to a decrease in the enzyme turnover. This occurs since the migrations of diffusion coefficients of the substrates to the active sites of enzymes are affected [16].

13.2 High-pressure reactors for biocatalysis

Various types of reactors for enzymatic reaction in SCFs can be used. Most common types are presented in Figs. 13.3−13.6. For the preliminary research on stability and activity of selected enzyme for biocatalysis, high-pressure batch stirred tank reactor is usually used (Fig. 13.3).

High-pressure batch stirred tank reactor are generally used for determination of the enzyme stability and activity as well as for screening enzyme-catalyzed reactions in SCFs. Substrate(s) are pumped into high pressure reactor. Additionally, the desired concentration of enzyme (native or immobilized) is added into reactor vessel. The high-pressure reactor with enzyme and substrate(s) is equipped with heating jacket to maintain the desired operating temperature. The initial concentration of substrate(s) should not exceed its solubility limit in selected SCFs [10]. Then, SCFs is pumped into high pressure reactor up to requested pressure. After completion of the reaction in SCFs, the slow decompression of SCFs via separator is performed. SCFs can then be condensed and recycled.

High-pressure continuous reactors are also often used to perform enzyme-catalyzed reactions in SCFs. Manly used types of high-pressure continuous reactor are high-pressure continuous packed-bed reactor (Fig. 13.4) (filled with biocatalyst) or high-pressure reactor containing membrane [high-pressure continuous flat-shape membrane reactor (Fig. 13.5) or high-pressure tubular membrane reactor (Fig. 13.6)].

High-pressure continuous packed-bed reactor is firstly field with immobilized enzyme. The column (reactor) with biocatalyst is heated using heating jacket to operational temperature. Substrates are pumped through the packed-bed reactor with high pressure liquid pump and SCFs is also pump in the system. The flow of substrates and SCFs must be adequate to ensure a sufficient retention time between the enzyme and the substrate. Depressurization of SCFs is performed in separator, where also products and unreacted substrates are collected. SCFs is condensed and recycled.

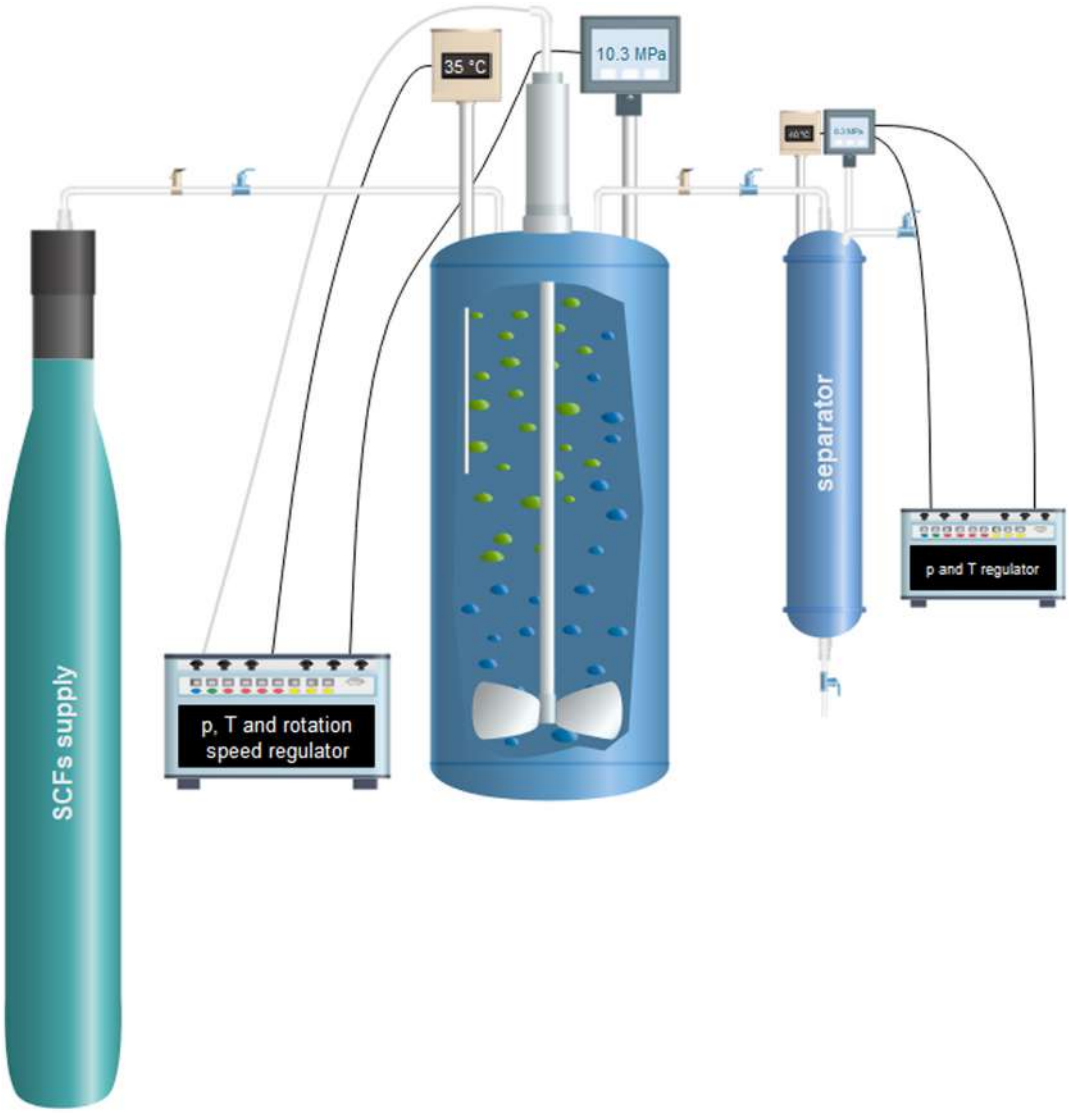

FIGURE 13.3

High-pressure batch stirred tank reactor for basic enzyme stability and activity studies in SCFs and for enzyme-catalyzed reaction performance in SCFs.

High-pressure continuous flat-shape membrane reactor contains round membrane from temperature- and pressure-resistant materials for example, polysulfone, polytetrafluoroethylene etc. The membrane is fixed between two sintered plates and fitted in the reactor. Selected amount of the enzyme is placed in the reactor. Then substrate(s) and SCFs are pumped in the reactor using high

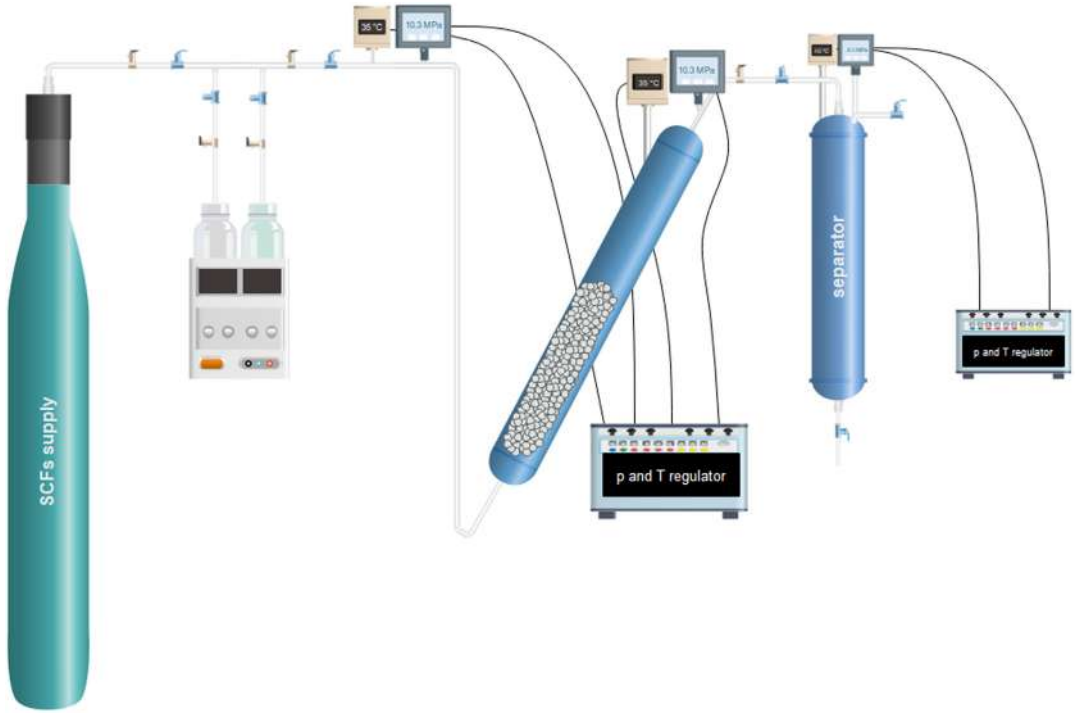

FIGURE 13.4

High-pressure continuous packed-bed reactor for enzyme-catalyzed reaction performance in SCFs.

pressure pumps. The product(s) and optional also unreacted substrate(s) are collected in separator, while SCFs is decompressed and separated from other reaction mixtures. SCFs can be recycled and reused. In this type of reactor, catalyst, which is retained by the membrane, remains in the reactor. High pressure continuous flat-shape membrane reactor is suitable for the synthesis of products having a lower molar mass than the substrates since the membrane can then serve also as a separation unit for separating unreacted substrates from the products. The products passed through the membrane and the unreacted substrate remained on the right side of the membrane where can reach the active site of the enzyme.

The high-pressure continuous tubular membrane reactor is equipped with tubular membrane(s), which can serve as carrier for enzyme immobilization. The membrane(s) are fixed in the reactor. The reactor is heated with hitting jacket to operational temperature, and substrate(s) and SCFs are pumped into the reactor using high pressure pumps. Membrane(s) are usually from ceramic materials. The problem in use of such membranes is not caused by high pressure in general but by the pressure difference, which should not exceed 1.5 MPa due to the possibility of membrane rupture. The product(s) and optional also unreacted substrate(s) are collected in separator, while SCFs is decompressed and separated from other reaction mixtures. SCFs can be recycled and reused. Additionally, membrane(s) can serve also as a separation unit for separation of product(s) from

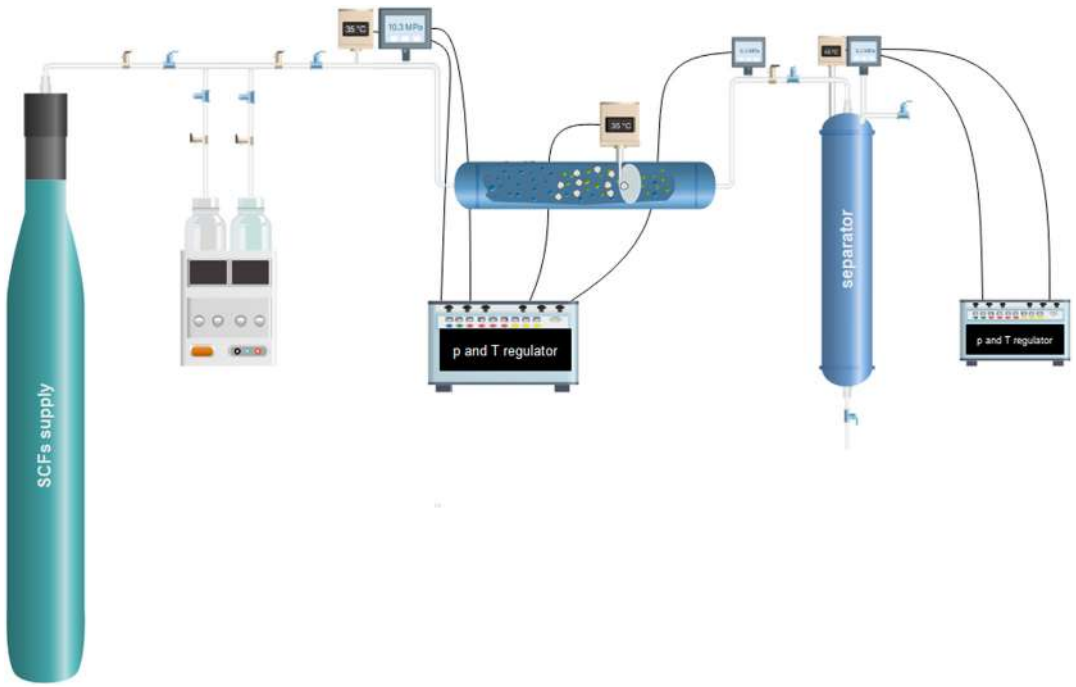

FIGURE 13.5

High-pressure continuous flat-shape membrane reactor for enzyme-catalyzed reaction performance in SCFs.

unreacted substrate(s) based on differences in molecular weights of product(s) and substrate(s) and correctly selected pore sizes of membrane(s). When the membrane is used as a carrier for enzyme immobilization, covalent immobilization of enzyme on the membrane is favored. Different crosslinkers for covalent immobilization of enzyme on membrane can be used. The most popular crosslinker is glutaraldehyde (GA), as it is soluble in aqueous solvents and may form subcovalent inter- and intralinkages [17–19].

The advantages of continuous processes especially in industrial applications are cost efficiency and relatively small implementation size, which lead to the reduction of cost and safety problems when the high-pressure equipment is used for operation at supercritical conditions.

13.3 Biochemical reactions in supercritical fluids

Many enzyme-catalyzed oxidations [20,21], hydrolyses [22–25], transesterifications [26–30], esterifications [27,31–35], and enantioselective syntheses [36–39] have been performed in SCFs in the last fifth years.

The most studied biochemical reactions in SCFs are catalyzed by lipases [3,40–42], although many other enzymes [e.g., polyphenol oxidase (PPO) [43,44], chymotrypsin [45], subtilisin [46,47],

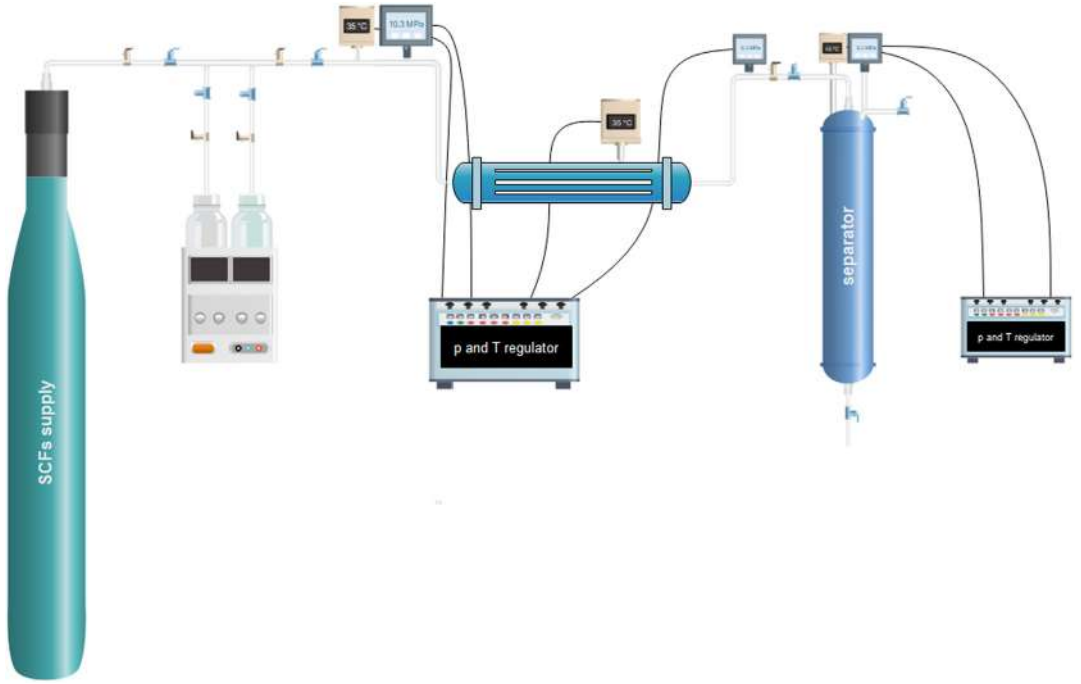

FIGURE 13.6

High-pressure continuous tubular membrane reactor for enzyme-catalyzed reaction performance in SCFs.

dehydrogenases [48–50], glucoamylase [51], and cellulase [15,52]] have been successfully applied in SCFs.

In the past, reaction of oxidation, hydrolysis, transesterification, esterification, and enantioselective synthesis catalyzed by different enzymes have been very commonly performed in SCFs [10,12,32,49,53–55].

Furthermore, the trend of using SCFs as a reaction medium for enzymatically catalyzed reactions was directed toward the polymerization process, lignocellulosic biomass (LCB) conversion and biodiesel synthesis. also, many enzyme-catalyzed reactions have been performed in biphasic ILs/SCFs media. The most commonly performed enzyme-catalyzed reactions in SCFs are shown in Fig. 13.7.

Enzyme-catalyzed polymerization in SCFs is becoming more and more attractive, due to the usage of sustainable, clean process. The combination of enzyme and SCFs for the performance of polymerization process leads to the production of residue-free products which are suitable for pharmaceutical and food applications. Polymerization using enzymes provides mild polymerization conditions, as well as high enantio- and regio-selectivity, and the use of a biocatalyst and its recyclability has prevailed, leading to the replacement of organometallic catalysts with enzymatic ones [56]. Among all SCFs, SC CO_2 and 1,1,1,2-tetrafluoroethane (R-134a), are alternative solvents for the enzymatic preparation of poly (ε-caprolactone) with molecular weight up to 50,000–80,000 Da and polylactide with molecular weight up to 12,900 Da [56].

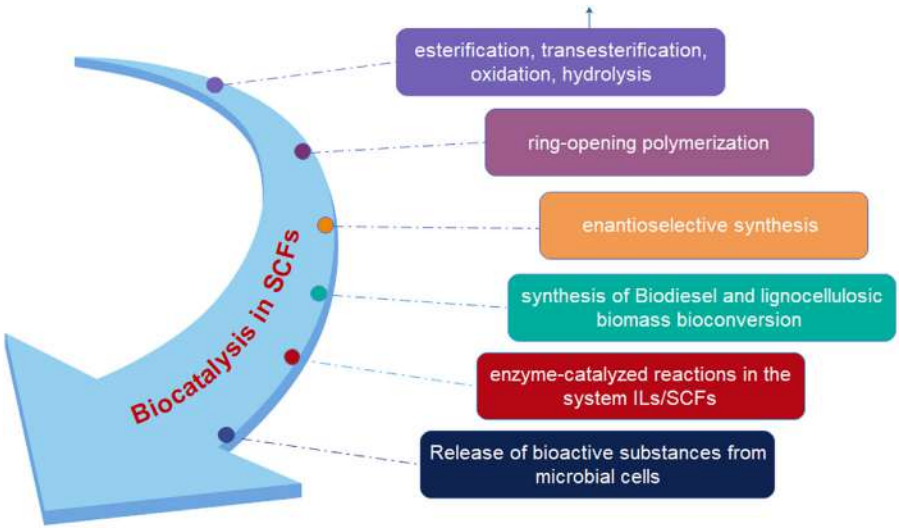

FIGURE 13.7

Enzymatic reactions in SCFs.

Additional, enzyme-catalyzed production of Biodiesel in SCFs it is becoming an increasingly interesting alternative for cleaner production of energy sources. Reducing fossil fuel stocks and raising awareness of the impact of energy production on society and the environment have spurred research into cleaner energy sources. Biodiesel and bioethanol show the potential to become primary fuels for fossil-based diesel replacement. Biodiesel produced from oil-rich raw materials is known as a green substitute for conventional petroleum diesel. The use of alternative solvents (SCF system, ILs, SCFs/ILs, etc.) for enzyme-catalyzed biodiesel production [29,57–60] demonstrates the possible alternative synthesis technique. Using SCFs for biodiesel production leads to an energy saving of 71.6% [59]. Dimethyl carbonate and methyl acetate produce high fatty acid methyl esters yields and valuable by-products when used as an acyl acceptor to produce biodiesel. Dimethyl carbonate is often used with lipase-based biocatalysts, whereas methyl acetate is preferred for use under supercritical reaction conditions [61]. Further, enzymatic hydrolysis of LCB in SC CO_2 is also a promising approach that can accommodate mild reaction conditions as it is a process reducing environmental impact and energy consumption and can significantly enhance biorefining of LCB at commercial scale [62].

A further possibility of carrying out the enzyme-catalyzed reaction in the presence of SCFs is a combination of SCFs with ILs, in a biphasic ILs/SCFs system. Such a system, where SCF is a representative of a nonaqueous reaction medium, represents alternatives to organic solvents for the production of pure products directly using pure chemical processes.

As a first approach to attain integral green bioprocesses in nonaqueous media, two-phase systems based on ILs and SC CO_2 have been suggested, where ILs provide an adequate microenvironment for the high catalytic efficiency of enzymes, while SC CO_2 can act as extracting, dissolving, or transporting phase, making easier recovery of the products [63]. Due to the miscibility switch

phenomenon of SC CO_2, two immiscible phases can form one homogeneous fluid phase, where the reaction can be performed. To facilitate product recovery after the reaction was completed, the homogeneous fluid phase can be split into two or three phases upon pressure decrease [64]. For the performance of enzyme-catalyzed reactions in ILs/SC CO_2 biphasic system, enzyme behavior in both phases as well as in biphasic system has to be studied. These behavior parameters are very important especially for the implementation of a continuous process for multi-step synthesis (i.e. dynamic kinetic resolution of sec-alcohols, synthesis of biodiesel, etc.) [65,66].

Recent implementation of enzymes as biocatalyst for enzyme-catalyzed reaction in SCFs in the last five years are summarized in Table 13.2.

When the enzyme-catalyzed reactions are performed in different reaction media, recovered enzymes can often lost their catalytic activity followed by possible denaturation of the biocatalyst. The activity and stability of enzyme can be improved by using SCFs as potential media for enzyme-catalyzed reactions [15,85–88]. For example, it is well known that improvement in the *Candida antarctica* lipase B (CALB) activity after incubation in SC CO_2 may occur. Nyari et al. [85] reported about the 315% residual activity of CALB after activation in SC CO_2. Pretreatment of immobilized cellulase in the form of cross-linked enzyme aggregates (CLEAs) in SC CO_2 at 10 MPa, 50°C for 3 h resulted in hyperactivation (residual activity was 143%) [15]. Furthermore, high-pressure treatments increase the activity of chicken egg lysozyme, as SC CO_2-treated lysozyme had a smaller reduction in storage activity compared to other gases, while maintaining residual activity above 100% [89].

This activity and stability improvement may occur due to conformational changes [87]. However, the enzyme activity changes are significantly dependent on the enzyme species and on the experimental conditions (e.g., temperature and pressure) in the reaction system. Therefore, stability study of individual selected enzyme in SCFs should be done before performing enzyme-catalyzed reaction in chosen SCFs.

Denaturation mechanism of enzyme in SC CO_2 is conditioned by the structural properties of the different residues and lysine is not the only responsible of the denaturation mechanism. Denaturation process occurs by escaping of many surfaces charged and polar residues from the solvent and forming non-native H bonds (destruction of essential native H bonds of the enzyme) with the other residues of the enzyme in a cooperative process. This changes the interaction pattern of the enzyme residues in SC CO_2. The creation of more interactions among the polar and charged residues at the enzyme surface and increasing the surface hydrophobicity could be effective ways to stabilize the enzymes in SC CO_2 [90].

An overview of process parameters influencing enzyme activity and stability, and limitation of biocatalysis performance in SCFs are discussed by Knez and co-authors [53,91].

13.3.1 Enzyme inactivation

Microbial and enzyme inactivation by SC CO_2 is a promising non-thermal method for the pasteurization of liquid food. The inactivation of an enzyme occurs, when protein migrates from the pure aqueous phase to the CO_2/water interface and where it is deactivated. Hydrophobic cores are released to the CO_2 phase while the hydrophilic surface residues are escaped to the aqueous phase. Consequently, the protein is denaturized to a flat and extended conformation [92].

Table 13.2 Performance of enzyme-catalyzed reaction in SCFs in the last five years, literature review.

Enzyme	Reaction	SCFs	Reaction parameter	Yield/conversion	Type of reactor	References
Lipase B from *Candida antarctica* (Novozym 435)	Transesterification	SC CO_2	10 MPa 60°C 20 h	67.26%	Batch	[29]
Mixture of lipase B from *C. antarctica* (Novozym 435) and lipase from *Thermomyces lanuginosus* (Lipozyme TL IM)	Esterification	SC CO_2	25 MPa 50°C 24 h	96%	Batch	[67]
Lipase B from *C. antarctica* (Lipozyme 435)	Esterification	SC CO_2	15 MPa 60°C 1 h	88.4%	Batch	[31]
Lipase B from *C. antarctica* (Novozym 435) coated with $[C_{18}tma][NTf_2]$	Transesterification	SC CO_2	18 MPa 60°C flow (SC CO_2) 1.5 mL/min 14 h	100%	Continuous	[68]
Lipase B from *C. antarctica* (Novozym 435)	Interesterification	SC CO_2	27.6 MPa 70°C 4 h	65%	Batch	[69]
C. antarctica B lipase (CAL-B) immobilized on functionalized multiwalled carbon nanotubes	Esterification	SC CO_2	20 MPa 55°C flow (SC CO_2) 3.94 mL/min 6 s	10%	Packed-bed	[70]
Lipase B from *C. antarctica* (Novozym 435)	Aza-Michael addition	SC CO_2	10 MPa 40°C 1 h	93%	Batch	[71]
Lipase B from *C. antarctica* (Novozym 435) or lipase from *Rhizmucor miehei* (Lipozyme RM)	Transesterification	SC CO_2	10 MPa 35°C 6 h	84.4%	Batch	[72]
Lipase B from *C. antarctica* (Novozym 435)	Ring opening polymerization	SC CO_2	21.7 MPa 60°C 7 h	96%	Batch	[73]
Lipases B from *C. antarctica* (Novozym 435 or Lipozyme 435)	Esterification	SC CO_2	10 MPa 40°C 1 h	54.99% (Novozym 435) 64.79% (Lypozyme 435)	Batch	[74]

(*Continued*)

Table 13.2 Performance of enzyme-catalyzed reaction in SCFs in the last five years, literature review. *Continued*

Enzyme	Reaction	SCFs	Reaction parameter	Yield/conversion	Type of reactor	References
Lipases B from *C. antarctica* (Novozym 435 or Lipozyme 435)	Acidolysis	SC CO_2	10 MPa 50°C 5 h	63.2% (Novozym 435) 61.2% (Lypozyme 435)	Batch	[75]
Lipase B from *C. antarctica* (Novozym 435)	Enantioselective transesterification	SC CO_2	20 MPa 50°C 3−5 h	Equilibrium conversion 90%	Batch	[76]
Lipase B from *C. antarctica* (Novozym 435)	Epoxidation	SC CO_2	10 MPa 40°C 0.5−1 h	83%−98%	Batch	[77]
C. antarctica lipase B variant (CalB 1422)immobilized on a methacrylic resin	Transesterification	SC CO_2	15 MPa 40°C 6 h	87%	Batch	[78]
Lipase B from *C. antarctica* (Novozym 435)	Esterification	SC CO_2	15 MPa 40°C 3 h (batch) 1 h (packed-bed)	79.8% (batch) 100% (packed-bed)	Batch and packed-bed	[79]
Lipase from *R. miehei* (*Lipozyme RM IM)*	Interesterification	SC CO_2	8 MPa 70°C 2 h	Presence of on trans-fatty acids	Batch	[80]
Lipase from *R. miehei* (*Lipozyme RM IM)*	Ethanolysis	SC CO_2	30 MPa 50°C 24 h	86%	Batch	[81]
Lipase B from *C. antarctica* (Novozym 435)	Ring opening polymerization	SC CO_2	20 MPa 70°C 2 h	64%	Batch	[82]
Lipase (Steapsin) ex. *Microorganism* covalently immobilized on immobead-350 support matrix	Transesterification	SC CO_2	11 MPa 50°C 14 h	94%	Batch	[83]
Lipase B from *C. antarctica* (Novozym 435)	Esterification	SC CO_2/ [Bmim][BF_4]	12 MPa 50°C flow (SC CO_2) 1.5 L/min 2 h	95.5%	Packed-bed	[84]

Numerous studies have been performed on enzymatic browning in fruits and vegetables in the past. In this sense very important is naturally present enzyme PPO. With the oxidation of o-diphenols which is catalyzed by this enzyme *o*-quinones are produced and involved in food browning. Such deteriorated food products cause large economic losses. Enzyme activity doesn't affect only fruits and vegetables but also other food products. Enzymes that are naturally present in flour are responsible for its quality. The main cause of undesirable darkening in flour and other vegetable products is PPO activity [93–95]. On the basis that the research in the past showed successful enzyme inactivation with SC CO_2 this was used for inactivation of PPO and prolongation of the flour shelf life with the browning process prevention. The technology is non-thermal and assures that the flour quality remains same or is even improved after the treatment. A 35% decrease in PPO activity was achieved after SC CO_2 exposure at 30 MPa, 35°C for 24 h [96].

Murtaza et al. [93] reported that high quality apple juices with low enzyme activity of PPO was obtained using high-pressure technology. At 25°C and 10 MPa, the PPO activity decreased only for 10%, drastically deactivation to 64.88% was detected at critical state of SC CO_2 (31.1°C and 7.38 MPa) but the complete inactivation of PPO occurred at 55°C and 25 MPa.

13.4 Other applications of supercritical fluids in biocatalysis

13.4.1 Release of bioactive substances from microbial and plant cells

It is well known that microbial cells are rich in bioactive substances of which more and more important for industrial use are enzymes. One possibility to obtain intracellular enzymes from microbial cells is the use of SC CO_2. Its characteristics go hand in hand with the biotechnological production conditions requirements, especially important in the food and pharmaceutical industries. Mild temperatures, lack of toxicity, good selectivity, ultra-pure products, non-carcinogenesis and thermodynamical stability are easy to achieve with this solvent. In such system products do not include the excess solvent. SC CO_2 can be used as a medium for cell disruption as well as a medium for biochemical reaction and bioseparation. From the an economical point of view, the integration of all three processes into a single step is prudent [97].

Baker's yeast or *Saccharomyces cerevisiae*, an industrial important microorganism contains many different enzymes including alcohol dehydrogenase, the most important one. To obtain proteins and enzymes from this microorganism SC CO_2 was used [98]. The mentioned fluid causes damage or disturbance of cellular compounds (cell membrane, enzymes and proteins). This causes microbial inactivation followed by extraction of intracellular substances from cells. Since such system contains suspension culture, using pressurized CO_2 changes the acidity. This decreases and weakens the microbial resistance to inactivation, by inhibiting microbial growth. Furthermore, the intracellular enzymes thus obtained can be further used as biocatalysts in cascade reactions performed in SCFs.

Due to the growing demand of the industrial market for enzymes, microbial cells, which can be cultured in large quantities, can present the primary source of enzymes. Extremozymes from halophilic fungi are interesting for industrial and biotechnological applications, due to their harsh conditions action. Extremozymes with high catalytic activities after exposure of fungi cell suspension of *T. salinum*, *Wallemia ichthyophaga, Hortaea werneckii* and *Pentagramma triangularis* to SC CO_2 were acquired [97].

Biological active compounds in many plants are mostly present as glycosides. Flavonoids such as isorhamnetin conjugates can be found in plants also in glycoside-bound form linked to cell walls [99]. To overcome the drawbacks of chemical methods for cell wall degradation including thermal degradation, new modern and advanced technologies could be used. Degradation and disruption of the cell-wall matrix via SC CO_2 assisted enzymatic hydrolysis was used for isolation of isorhamnetin glycosides with anti-inflammatory activity from *O. ficus-indica* (L.) Mill. For the polysaccharide degradation enzymes Rapidase Maxi Fruit (RMF) from *Aspergillus niger* and Viscozyme from *Aspergillus* sp. were used. RMF enzyme was more selective for the extraction of isorhamnetin-3-*O*-glucosyl-rhmanoside (IG5) in SC CO_2 at 70°C and 8 MPa for 40 min or in combination with isorhamnetin-3-*O*-glucosyl-rhamnosyl-pentoside (IG2), while Viscozyme selectively extracted triglycosides isorhamnetin-3-*O*-glucosyl-rhamnosyl-rhamnoside (IG1) and IG2 in SC CO_2 at 70°C and 8 MPa for 40 min [100]. Additionally, Krakowska-Sieprawska et al. [101,102] reported that the combination of enzymatic hydrolysis using Kemzyme (mix of xylanase, beta-glucanase, cellulase, amylase, and protease) with SC CO_2 treatment of *Medicago sativa* L. leaves led to the production of polyphenolic compounds with high concentration.

13.4.2 Scaffolds

Much research has been recently done in the field of tissue engineering applications. Scaffolds with high surface area to volume ratios are required for application in medicine. This enables effective cell seeding onto the biomaterial support and rapid proliferation.

Using SCF technology enables to incorporate bioactive molecules into biodegradable porous scaffolds. The viscosity of the mixture is decreased when CO_2 is dissolved in the polymer because of the fact that the polymer is plasticized and weak interactions with the functional groups of the polymer appear. This enables incorporation of insoluble particles into the polymer. At the same time the size and distribution of pores may be controlled only with proper selection of process conditions, such as temperature, depressurization rate and solubilization pressure [103,104]. The very important fact for before-mentioned applications is that after processing no solvent residues remain in the material [105,106]. An important aim for biomedical applications is controlled protein delivery from these materials. A two-stage protein release profile was observed when BSA was loaded into the porous poly(ε-caprolactone)-chitosan PCL-CS scaffolds prepared by using SCF technique. The release profile showed that in the first few days larger concentrations of the protein were released but still the rest of the protein was released in 16−20 days [107]. PCL, a synthetic polymer-based biodegradable scaffolds were prepared using foaming technique with SC CO_2. This material may be used for soft- and hard-tissue engineering. Chitosan (CS), an antimicrobial and biocompatible agent and calcium phosphate ceramic hydroxyapatite were incorporated to enhance PCL's poor biological interactions and increase cellular adhesion, osteoinductivity and calcium deposition in the porous composite. Such material may be used as a protein release carrier. To achieve this, transglutaminase (TGM), a thermo-sensitive enzyme was incorporated. This therapeutic enzyme acts on the cell surface. Partnering with fibronectin promotes integrin-mediated adhesion in a transaminase-independent manner leading to extracellular matrix production and stabilization [108]. In the presence of GA as a cross-linker and standard protein bovine serum albumin, TGM activity was preserved. The important fact was also that protein release patterns were extended from rapid profile lasting one week to a prolonged release lasting at least one month

[109]. The composition of material and operation parameters can influence the structure of synthesized scaffold using SCFs technology (Fig. 13.8). The structure and porosity of the synthesized scaffold is very important when the bioactive components should be incorporated into it.

New processes are in a development for the production of biopolymers through enzyme ring opening (e-ROP) polymerization reaction. The e-ROP of ε-CL using Novozym-435 as a biocatalyst in continuous mode and in SC CO_2 with dichloromethane (DCM) as a cosolvent was studied. Regarding the average molecular weight for the experiments performed with mass ratio of SC CO_2 and 50 wt.% of ε-CL + 50 wt.% of DCM mixture 2:1 the reaction yield was 94.1%. CAL-B enzyme proved to be robust for ε-CL polymerization even after several reuses. [110].

Yadav and Varghese [88] used SC CO_2 as a medium for foaming and for the immobilization of lipase from *C. antarctica* B in mesocellular foam silica [CALB@MCF(SC CO_2)]. The prepared

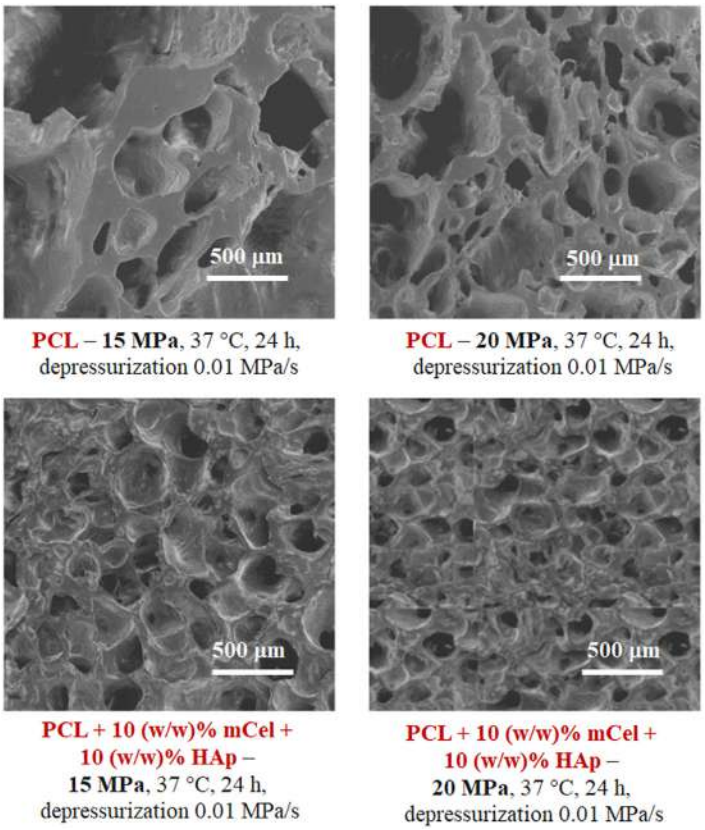

FIGURE 13.8

Influence of operation parameter (pressure 15 and 20 MPa) and composition of material (*HAp*, calcium phosphate ceramic hydroxyapatite; *mCel*, microcrystalline cellulose, *PCL*, poly(ε-caprolactone)) on the scaffold structure (SEM analysis).

immobilized enzyme was used for flavor ester (hexyl laurate) synthesis. In comparison to conventional immobilization, supercritical assisted immobilization significantly reduced enzyme leaching during repeated reactions. Additionally, increase in activity and stability of CALB@MCF(SC CO_2) was detected.

Recent, the fabrication of catalytically active nanomaterials as artificial enzymes (nanozymes) has become a new growing field of bionic chemistry research aimed at designing functional nanomaterials with various mimicked properties of natural enzymes. For the synthesis of monodisperse, and uniform-sized manganese (III) oxide (Mn_2O_3)-based hollow containers SCF-assisted procedure was used. Obtained nanozymes showed high efficiency for glucose sensing [111].

13.4.3 Bleaching using enzymes and supercritical fluids

Nowadays, different cellulose fibers for example, flax fibers are widely used for clothing and for decorative textiles production. Due to low price, easy availability and antibacterial activity, flax is very interesting from an economic point of view for the textile industry [112]. The flax fiber mainly contains cellulose, hemicellulose, lignin, pectin and wax. Their chemical composition, hemicellulose, lignin, pectin content, influence the physical properties (ductility, softness as well as crimpnes) of flax fiber [113,114]. Combination of enzyme treatment and SC CO_2 for scouring and bleaching of flax roves present a novel and eco-friendly approach for improvement of fiber quality. Different enzymes such as lipase, xylanase, cellulase etc. are used for textile bleaching. Significant improvements in the whiteness and residual gum content of flax roves were achieved after treatment in SC CO_2 with combination of xylanase and cellulase. The high-pressure enzymatic process for the scouring and bleaching of flax roves was optimized; enzyme loading 3%, an enzyme ratio of 2:1 (xylanase: cellulase), 50°C, 20 MPa, a CO_2 flow rate of 30 g/min for 1.5 h [113]. Bamboo fiber was treated using a high-pressure enzyme hydrolysis process at 70°C, 90 MPa for 6 h using enzyme "Marugoto A" (cellulase from soybeans) in supercritical water was performed. Results show that high pressure enzyme hydrolysis is an effective process for cellulose bamboo fiber production with elevated crystallinity and higher thermal stability compared to the pulping and conventional bleaching processes [115]. Additionally, environmentally-friendly strategy for integrated bio-treatment of gray cotton for removal of impurities using enzyme-catalyzed reaction in SC CO_2 was developed [116].

13.5 Conclusion: the state of the art

Biocatalysis engineering concerns the development of complete enzymatic systems, which entails engineering its different components: engineering of substrate, medium, protein (enzyme), biocatalyst (formulation), biocatalytic cascade, and reactor [117]. Therefore, the application of SCFs as a "green" medium for biocatalysis has to be engineered to achieve optimal process results. As the previous research indicated that the activity of enzymes in nonaqueous media is dependent on solvent properties [12,53,118], SCFs were recognized as attractive media in which the biocatalytic reactions can be performed and even controlled. Different parameters such as pressure, temperature, pH etc. are key parameters affecting enzyme activity and stability in SCFs. Optimization of these

parameters for enzyme-catalyzed reaction performance in SCFs may even lead to hyperactivation of the enzyme. Biocatalysis in sub and SCF has not yet been performed on an industrial scale, due to possible instability and inactivation of the enzyme at high pressures or more costly process due to higher equipment costs.

The stability and activity of enzymes depend also on their species; therefore, new enzymes, stable in harsh conditions for example, extremozymes (enzymes from extremophiles), can be selected as a catalyst for biocatalysis in SCFs. Extremophiles are organisms living in deep-sea, hot springs, high sulfursprings, high salts environments, high pressure, and low-temperature environments, etc ... and are therefore adapted to the extreme conditions, which is also reflected in the resistance and adaptation of their enzymes to extreme conditions. The isolation of extremozymes from extremophilic microorganisms and reaction can take place in a single step in the case of the use of extremophilic enzymes for biocatalysis in SCFs. This represents an advantage from both an economic and a sustainable point of view.

Further, SC CO_2 is also the solvent that can replace toxic organic solvents in enzyme-catalyzed polymerization. Owing to the favorable transport properties of SC CO_2, mass transfer in enzymatic reactions can be accelerated, as well as SC CO_2 can improve enzyme activity and enzyme stability, and it can be easily separated from the final product by simple depressurization of the system, followed by completely removing of solvent traces [119]. The potential disadvantage of supercritical polymerization is reflected in its practicality of scaling up [120].

Nowadays, efficient methodologies for enantiomerically pure component production are of great importance; therefore, enzymatic-assisted resolution is an attractive option. The most commonly used resolution method today is based on the enzymatic conversion of one enantiomer of the racemate, while the other remains unchanged since its conversion rate is much slower [10]. The tendency to obtain individual enantiomers instead of racemic mixtures is increasing in the pharmaceutical and agrochemical industries, due to the desired activity of one of the enantiomers, as usually, the other enantiomer has no activity or even expresses unwanted side effects. The implementation of the enzyme-catalyzed resolution of enantiomers in SCFs (most commonly in SC CO_2) allows the separation of the product without the presence of an organic solvent. A combination of ILs and SC CO_2 for the enzyme-catalyzed racemic resolution can have several advantages such as high enzyme stability and sometimes even enhanced activity and stereoselectivity (impact of ILs) as well as extraction of the products in a completely solvent-free form (impact of SC CO_2) [121].

The preparation of scaffolds with desirable properties for tissue engineering applications is of great importance today, especially in medical applications. The development of advanced technologies for novel biodegradable scaffolds production with bioactive delivery capability is delivery is a priority domain in the field of tissue engineering applications. The use of SC CO_2 to create porous polymer materials has recently proven to have great potential compared to conventional synthetic pathways. Applications of SC CO_2 in tissue engineering include the use of dense gas as a polymer swelling agent to help impregnate the scaffold with desirable additives such as drugs, enzymes, and other bioactive compounds, and the use of CO_2 as a compressed liquid to obtain various polymer morphologies [122]. The use of SCFs in this field is in the research phase but represents an advanced and promising technology with the possibility of producing a wide variety of scaffolds materials with unique properties.

Research opportunities in the field of biocatalysis under unconventional conditions are without limitations. Additional studies of the stability and activity of more specific enzymes (superoxide

dismutase, catalase etc.) in SCFs for possible application in different pharmaceutical synthesis should be performed. The field of application of whole cells for the biocatalysis in SCFs is also unexplored, as it is possible to perform the release of enzyme from for example, microbial cells and biocatalysis in one step. The most pronounced benefit of using SCF in biocatalysis is the lack of solvents in the products and the possibility of solvent recycling, while reducing the negative impact on the environment. In addition, the possibility of using different types of high-pressure reactors allows different types of reactions to be performed, where the reaction and separation can be carried out in one step.

Often, when using SCFs for biocatalysis, there are concerns about the economy of the process. By optimizing the process, reusing the catalyst, and considering the sustainability of the process, such a process can also represent an "economical" process from a cost as well as an environmental point of view.

Because of intensive development of equipment producers, there is no limitation for scale up to industrial scale regarding the process equipment.

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CHAPTER

Biocatalytic processes in ionic liquids and supercritical carbon dioxide biphasic systems

14

Rocio Villa[1], Antonio Donaire[2], Susana Nieto[1], Eduardo García-Verdugo[3] and Pedro Lozano[1]

[1]*Department of Biochemistry and Molecular Biology B and Immunology, Faculty of Chemistry, University of Murcia, Murcia, Spain* [2]*Department of Inorganic Chemistry, Faculty of Chemistry, University of Murcia, Murcia, Spain*

[3]*Inorganic and Organic Chemistry Department, E.S.T.C.E., Universitat Jaume I, Castelló, Spain*

14.1 Green chemistry: biocatalysis in organic versus neoteric solvents

Chemistry is one of the sciences that has contributed the most to the well-being and progress of mankind. Water chlorination [1,2], nitrogen fixation (agriculture) [3], the development of fuels [4], pharmaceutical compounds, cleaning products, and products related to communication and computer technologies are among the daily events that make us live longer and better than ever before in history [5]. Paradoxically, a nonnegligible percentage of the society has a negative concept of Chemistry [6,7]. It is probably a matter of unproven prejudices, but the chemical industry's action partially contributes to the deterioration of the environment.

Indeed, most of the chemical syntheses developed in the last century and still being carried out are produced in organic solvents [8]. The annual production of organic solvents is estimated to be in the order of 20 million MT/year [9]. Organic solvents are usually volatile (so they are known as volatile organic solvents, VOSs), toxic, flammable and hazardous to human health and the environment. Moreover, these chemical processes are often carried out under drastic conditions (high temperatures or pressures) and in many cases produce nonrecyclable waste and by-products which are released to the atmosphere and are often highly harmful to the global ecosystem. For instance, it was estimated that methanol, hexane, toluene, carbon disulfide and *n*-butanol were released in large quantities (45,731, 15,660, 8774, 3970 and 3901 MT in 2016, respectively) into the atmosphere and, as a consequence, these and many other VOSs have also been observed in high concentrations in waters and soils [8]. Thus, there is increased social pressure, transferred to the political level, which pushes chemists to develop new chemical processes in greener environments and milder conditions for achieving a sustainable chemical industry

The use of safer and environmentally benign nonaqueous solvents is only one of the goals of sustainable chemistry. The sustainability of chemical processes begins with catalysis, because the selectivity in chemical transformations is directly related with several of the principles of green chemistry, for example, prevention, atomic economy, less hazardous synthesis, reduction of derivatives, etc. The inherent formation of wastes/contaminants and undesired by-products in classical synthetic processes, which are based on the use of stoichiometric amounts of reagents, can be

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minimized by using catalytic steps. The development of efficient catalytic processes leads to significant savings in production costs for industry, as well as in environmental impacts. The use of bio-renewable raw materials, the design of flow processes, the application of alternative reaction activation methodologies (e.g., microwave and ultrasonic irradiations, etc.) for improving catalytic efficiency over conventional energy sources, are other greener aspects to be taken into account for developing clean chemical processes. All these sustainable approaches may be integrated for developing selective processes of transformation and separation, able to directly provide pure products, including the reuse of all the elements of the reaction system, for example, catalysts, solvents, etc.

In the search for safer new environmentally benign solvents or green solvents, which could be easily be recovered and recycled, and would still allow performing efficient catalysis, a series of environmental, health, and safety requirements should be considered(Fig. 14.1).

The neoteric solvents, most of them considered as green solvents [10], can modify their physicochemical characteristics depending either on their composition and on external variables (typically, pressure and temperature). Thus, key solvent properties, such as hydrophilic/hydrophobic character, can be finely tuned to suit the analogous features of substrates, products or catalysts, and to direct affect the course of a chemical reaction. A multitude of neoteric solvents has been described, such as supercritical fluids (SCFs) [11], ionic liquids (ILs) [12], deep eutectic solvents [13], fluorinated solvents [14], liquid polymers [10], or supramolecular solvents [15], among others, as alternatives for replacing the nonaqueous hazardous solvents with environmentally benign ones.

Currently, ILs and SCFs, which are nonaqueous green solvents, have received most attention worldwide. ILs are a new class of liquid solvents that have led to a new green chemical revolution, because of their unique array of physical-chemical properties, which make them suitable for numerous industrial applications. SCFs, especially supercritical carbon dioxide ($scCO_2$), are another class of useful solvents with unique array of properties that can be applied in clean reaction, extraction, and fractionation processes. Furthermore, the discovery that ILs and $scCO_2$ form biphasic systems was crucial for further developments in nonaqueous multiphase green catalytic synthetic transformations, and lead to processes where most of these requirements can be satisfied [11].

Enzymes are catalytic proteins obtained from living systems; they show a high level of activity and selectivity (stereo-, chimio- and regio-) toward catalyzed reactions using wide variety of substrates/chemicals. Furthermore, their application in chemical processes greatly improves the

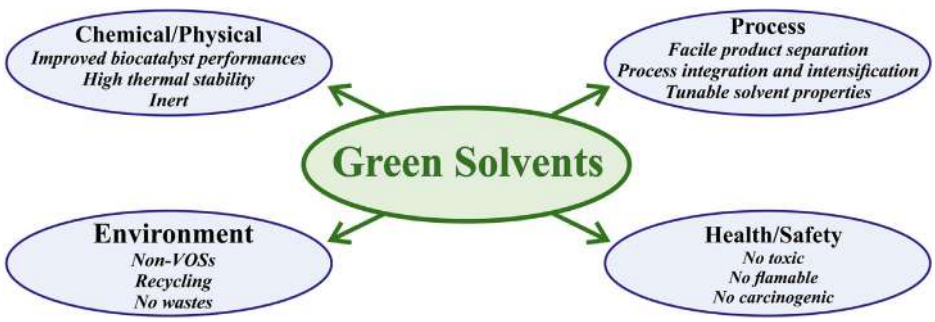

FIGURE 14.1

Requirements for neoteric solvents to be considered as green solvents.

efficiency when using nonaqueous environments (e.g., the improved solubility for hydrophobic substrates, the easy recovery of enzyme because its insolubility, the shifting of thermodynamic reaction equilibrium to favor synthetic processes over hydrolysis, the elimination of microbial contamination in reactors, etc.) [16]. It has been demonstrated that many enzymes maintain their native three-dimensional structure and are active in these neoteric media of different polarities (Fig. 14.2). This feature has opened a wide range of possibilities for their industrial exploitation both in these solvents alone and in biphasic combinations between them.

Among all the nonaqueous neoteric solvents, the use of biphasic systems based on both ILs and $scCO_2$ has enhanced the potential of enzymes because of the improvements to their catalytic properties and operational performance. By using several examples, this chapter tries to show the excellent suitability of enzymes when combined with these neoteric solvents, as an important "arsenal" of green tools to develop integral clean chemical processes of industrial interest in the near future.

14.2 Supercritical fluids and supercritical carbon dioxide

A SCF is any molecule at a temperature and pressure higher than their critical points (P_c and T_c), having densities and solvation power comparable to those of liquids, while their diffusivities and viscosities are similar to those of gases. These characteristics suit them as ideal solvents for using in extraction, fractionation and analytical processes, as well as attractive as a medium for biocatalytic transformations, especially when reactions are limited by the rate of diffusion rather than by any intrinsic kinetics. Thus, the key feature of SCFs as solvents for biocatalysis is the sensitivity of

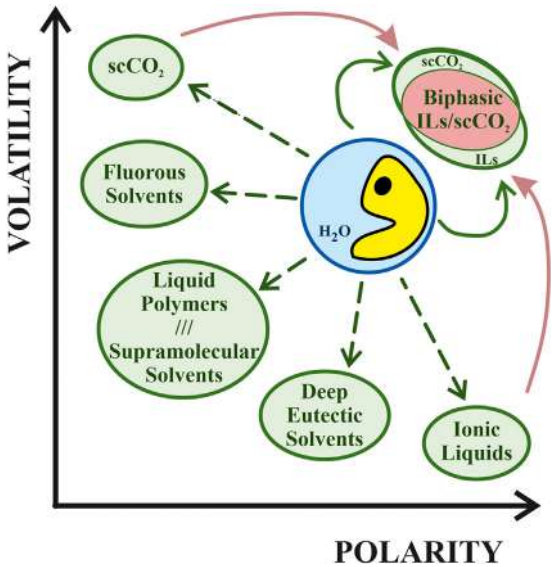

FIGURE 14.2

Solvents in which enzymes are active, classified as a function of its polarity and volatility.

its density to both pressure and temperature, mainly near the critical point, where small changes in pressure and temperature modify all density-dependent solvent properties (e.g., dielectric constant, Hildebrand solubility parameter, partition coefficients, etc.) [17].

Despite the great diversity of SCFs used as solvents for chemical synthesis (Table 14.1), the CO_2 is by far the solvent most widely used as SCF [18]. This is because $scCO_2$ has mild critical points of temperature and pressure (see Table 14.1), low cost, low reactivity, is nonflammable, has no toxicity and it is easily recyclable [19]. As a solvent, $scCO_2$ is noncorrosive and nonexplosive, lacks both surface tension and enthalpy of vaporization and has a low viscosity, which greatly facilitates the mass transfer of reactants and products and is affordable and thus, economical. All this has led to the naming of $scCO_2$ as a green solvent.

Carbon dioxide is nonpolar and therefore $scCO_2$ is a very good solvent for hydrophobic compounds. In addition, solubility properties of $scCO_2$ are finely tunable just slightly adjusting P or T. Indeed, small changes in P and T modify $scCO_2$ density, which, in turn, varies the Hildebrand solubility parameter, related to the ability to separate solvent molecules from the solute, that is the ability to dissolve different types of solutes in whatever solvent [20]. Although this solubility, in the case of pure $scCO_2$, mainly refers to hydrophobic compounds, the addition of small amounts of polar solvents (e.g., methanol, ethanol, *tert*-butanol-, acetone, etc.) greatly increases the solubility of hydrophilic compounds. Not only cosolvents but also additives or modifiers help to the solubilization of many different solutes, such as hydrocarbons, fluorocarbons, carbonyl compounds, siloxanes and polymers [21–23]. In summary, the combination of P, T and the addition of cosolvents or additives allows the fine modification of the range of substances that can be dissolved into $scCO_2$.

Owing to its ability to solubilize lipophilic compounds, $scCO_2$ has been widely used for decades in extraction processes in the food, beverage, pharmaceutical and cosmetic industries; these processes are called SCF extraction (SFE) [23,24]. Once the compound of interest has been extracted and dissolved into the supercritical phase, separation can easily be carried out by simply by depressurization, which releases the CO_2 gas, yielding a practically pure compound. The major drawback

Table 14.1 Critical parameters of most commonly used supercritical fluids.

Solvent	T_c (°C)	P_c (MPa)	Critical density (g/cm^3)
Carbon dioxide*	30.95	7.38	0.469
Water	373.95	22.06	0.322
Methane*	− 82.75	4.60	0.162
Ethane*	32.15	4.87	0.203
Propane*	96.65	4.25	0.217
Ethylene*	9.25	5.04	0.215
Sulfur hexafluoride*	45.57	3.76	0.742
Trifluoromethane*	25.95	4.82	0.107
Acetone	234.95	4.70	0.278
Methanol	239.45	8.09	0.272
Nitrous oxide	33.45	7.35	0.452
Xe	16.58	5.84	0.110

**Those used in biocatalysts are marked with a star (from ref. [18]).*

of using supercritical CO_2 at an industrial level is the high technology required to achieve stable and safe high pressures in large-scale compartments, which makes the technique expensive. The extraction of caffeine from green coffee beans is one of the paradigmatic examples where SFE by $scCO_2$ has proven to be an efficient, economical, and environmentally sustainable method [25]. In this case, the use of cosolvents such as ethyl lactate, ethanol or ethyl acetate, and in that order, substantially increase the $scCO_2$ extraction capacity [26]. The extraction of palm oil natural fats from kernels is another example of the efficient use of $scCO_2$. The yield and purity of lipids obtained from these processes are comparable or better than those obtained in VOSs, and do not give rise to their undesirable environmental effects [27]. Another example, in this case developed recently, is the extraction of biodiesel. An entire refining industry is developing based on this technology [28,29]. Supercritical CO_2 has also been used as a solvent in a multitude of synthesis reactions such as hydrogenations, hydroformylations or selective oxidations [20]. These applications of $scCO_2$ are beyond the scope of this chapter.

14.3 Ionic liquids

ILs are salts, and therefore entirely composed of ions, which are liquid below 100°C or even close to room temperature [30]. By using the solid sodium chloride as representative example, molten sodium chloride (above 800°C) is an ionic liquid, while an aqueous solution of this salt is an ionic solution. Typical room temperature ILs are liquid salts based on organic cations (e.g., 1,3-dialkylimidazolium, tetraalkylammonium, N-alkylpyridinium and tetraalkylphosphonium, etc.), paired with a variety of anions, having or not strongly delocalized negative charge (e.g., halides, BF_4^-, PF_6^-, methyl sulfate, trifluoromethanesulfonate, bis(trifluorometylsulfonyl)imine or bistriflimide, etc) (Fig. 14.3). The combination of these cations and anions become in colorless, low viscosity and easily manipulable liquid materials with very interesting solvent properties [31].

The plethora of unique characteristics of ILs are headed by its negligible vapor pressure (they do not therefore evaporate), as well as excellent thermal stability (above 300°C in many cases), a high ability to dissolve a wide range of organic and inorganic compounds, including gases (e.g., H_2, CO_2, CO, etc.), a nonflammable nature, a high conductivity and a large electrochemical window [32]. Moreover, their polarities, hydrophilicities/hydrophobicities and solvent miscibility can be tuned by selecting the appropriate characteristics of the anion and the cation (e.g., length of the alkyl chain). Due to the large number of cations and anions capable of forming ILs, the number of theoretical combinations that can exist of these is gigantic (estimated to be on the order of 10^{12} for binary combinations [33]). Thus, ILs can be designed to be miscible or immiscible with water or some organic solvents (e.g., hexane, benzene, ether, i-PrOH, etc.), making them more useful for recovering products from the reaction mixture liquid-liquid extraction with molecular solvents. Spectroscopic measurements of solvatochromic and fluorescence probe molecules in ILs suggest that these neoteric solvents are polar. For ILs based on 1-alkyl-3-methylimidazolium as the cation, the polarity slightly decreases through the series, $[NO_3^-] > [BF_4^-] > [NTf_2^-] > [PF_6^-]$, although all ILs are in the same polarity region as 2-aminoethanol and lower than alcohols such as methanol, ethanol and butanol. Furthermore, even for those ILs nonmiscible with water, they are hygroscopic compounds (e.g., 1%−2% water content), which represent an interesting characteristic with regards

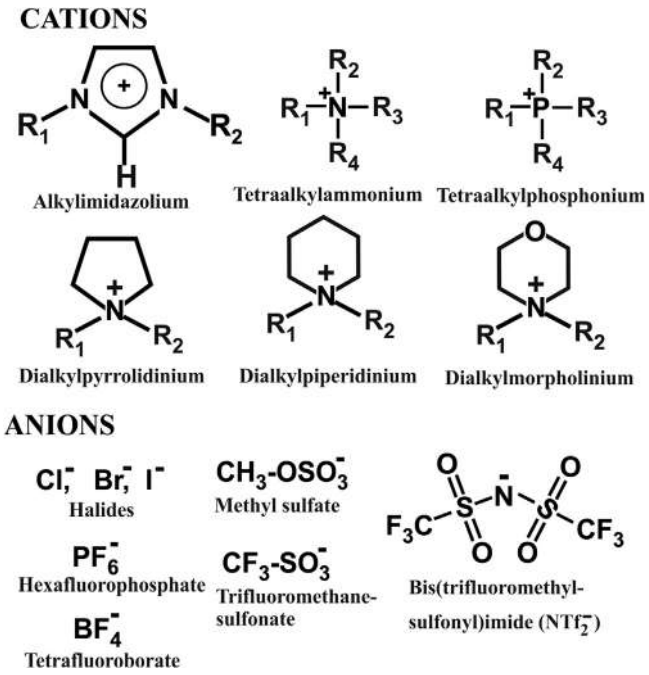

FIGURE 14.3

Structures of typical cations and anions of ILs used for catalytic processes.

their applications in biocatalytic processes, because of the importance of enzyme-water interactions for the maintenance of the active conformation of the enzyme [34]. All of these properties, including the fact that they are reusable and recyclable, allow ILs to be considered ideal solvents for Green Chemistry.

In this sense, the applicability of ILs in catalysis and biocatalysis is immense. For example, organocatalysis has been extensively developed where the IL anion or cation itself acts as a catalyst [35,36]. On the other hand, there are also countless examples of biocatalysis reactions in, for example, carbohydrate catalysis or biomass processing [37–39]. In this chapter, the capabilities of ILs to act as solvents in biocatalysis mainly by forming biphasic systems with $scCO_2$ will be highlighted.

14.4 Biocatalysts in nonaqueous environments

Enzymes are the molecules that evolution has designed for the acceleration of the vast number of chemical reactions that occur in living systems. They are extraordinarily efficient and selective stable machines, not only in respect to single substrates but also from the chemo-, regio- and stereoselective point of view [16]. Being water the universal solvent par excellence and for life, in particular, most enzymes work in aqueous solution. Moreover, enzymes operate in moderate

temperature and pH ranges, which allow reactions to be carried out without high energetic costs. However, these conditions also limit the use of enzymes in fully aqueous solutions and their applicability on an industrial scale.

Enzymatic catalysis in aqueous media has been used, among many other processes, in the hydrolysis of cellulose, in fruit juice clarification, in the degradation of polyethylene in the treatment of wastewater, etc., and numerous syntheses in bulk and fine chemicals [16]. However, some problems make the application of biocatalysts in aqueous media relatively restrictive: (a) enzymes are soluble in aqueous media, so that, except when they are immobilized and even, in that case, their recovery is not easy; (b) there are many reagents and products that are not miscible in water and whose synthesis reactions cannot be carried out in aqueous media; (c) the conditions under which enzymes work in water are mild and yields can be low, so that, with few exceptions, these processes can be economically improved when carried out in other solvents; (d) the separation of reagents and products is usually energetically costly; (e) water is the natural medium for microorganisms, consequently contamination of these media is frequent.

Enzymes, however, are not only stable in aqueous media, but also in highly nonapolar media (organic solvents) and other nonconventional solvents, with different features (see Fig. 14.2), which have made them possible to extend their applicability tremendously (see below). Also, combinations of water with organic solvents (aqueous biphasic systems), which have the advantages of the existence of two phases, have proven to be a very economically attractive solution to the above-mentioned problems [40]. However, by using VOSs, these processes damage the environment and do not fulfill the minimal requirements to be considered green.

At the beginning of the last century, it had already been described that certain enzymes were active in water-immiscible organic solvents [41,42]. However, it was Zaks and Klibanov in 1984 who first applied biocatalysis in such solvents [43]. Since then, it has been shown that many enzymes are stable in organic solvents, maintaining their tertiary structure and being active for long periods and catalytic cycles. Indeed, a multitude of spectroscopic (EPR, IR, fluorescence, CD and ^{1}H NMR) [44] and theoretical (molecular dynamics) studies [45] have shown that in nonpolar solvents with a minimal amount of water, the enzymes are surrounded by a solvation sphere due to which they maintain their native three-dimensional structure [46,47]. The nonpolarity of the solvent is a determining factor in enzyme stability. Lyophilized proteins contain strongly trapped water molecules that make them maintain their active conformation at local and global levels. When the proteins are dissolved in a highly hydrophobic solvent, these water molecules are captured by the protein and cannot escape due to their immiscibility with the solvent. On the contrary, an organic solvent of certain polarity can withdraw water molecules from the solvation sphere of the protein and, as a consequence, different domains or substructures of it lose their native conformation, which ultimately results in their complete denaturation in that medium [48]. Thus, a minimal quantity of water molecules is necessary for the enzyme to carry out its function. However, large amounts of water also take to enzyme denaturation.

A key point in maintaining protein stability is the freeze-drying process followed by enzyme solubilization. Normally, enzymes are lyophilized and then solubilized in the corresponding solvent. In the lyophilized state, proteins maintain the coordination sphere of water molecules, more strongly trapped than those of bulk water (sublimated in the process). When enzymes are dissolved in a nonpolar organic solvent they maintain this solvation sphere and are therefore active. If this solvation sphere did not exist, enzymes would not be active in organic solvents; in fact, completely

dehydrated enzymes are not active in such organic solvents [49]. However, freeze-drying/solubilization processes denature the enzymes and deactivate them, so other much less aggressive processes have been devised. For example, desiccation of enzymes by *n*-propanol treatment leads to enzymes that are more active and produce higher enantiomeric excesses than when the enzymes have been subjected to freeze-drying treatment and then dissolved under the same conditions. In the kinetic resolution of *rac*-β-citronellol by *Rhizomucor miehei* lipase, the lyophilized enzyme gave 30% conversion with an enantiomeric excess (*ee*) of 78%, whereas with the enzyme rinsed with *n*-propanol 45% conversion (*ee* 78%) was obtained [50]. The higher activity of the enzyme rinsed with *n*-propanol is believed to be due lyophilization, which causes irreversible structural changes in the protein. These changes do not occur with *n*-propanol desiccation [51].

The use of enzymes in organic solvents presents some relevant advantages, being one of them is the application of enzyme-catalyzed chemical reactions involving hydrophobic substrates, which are not or are scarcely soluble in water. In addition, reactions catalyzed by hydrolases (lipases, proteases, glycosidases, ...), which in the natural environment mainly perform reactions in one direction (where water is abundant), can be used in the reverse direction; in other words, the reaction can be directed almost at will. One of the most attractive characteristics of these enzymes is their capacity to carry out not only the specific reaction for which they are designed with the corresponding natural substrate but also many other similar reactions with analogous functional groups and a multitude of similar substrates. Lipases (*EC 3.1.1.3*) are probably the most used enzymes among hydrolytic enzymes at the industrial level. They are used in the hydrophobic/hydrophilic interfaces and so they can esterify water-soluble substrates. Due to their promiscuity, lipases have been fully exploited in many different kinds of reactions (hydrolysis, esterification, transesterification, aminolysis, etc.) and, at a large scale, in the organic synthesis, paper manufacture, pharmaceutical, agrochemical, cosmetic or flavor industry and detergents [52]. More than 1000 MT per year are used in lipases as additives to detergents. Another good advantage of organic solvents is the fact that they are also antimicrobial, preventing contamination and degradation of the biocatalyst.

For all these reasons, most reactions with biocatalysts at the industrial level are carried out in VOSs. However, the use of VOSs is, as previosuly indicated, unhealthy and harmful to the living conditions. For instance, the main method of separation of products, substrates and biocatalysts when using VOSs is distillation, a process in which a large amount of the solvent is lost to the atmosphere, which is extremely hazardous to the environment [8]. Because of the high degree of VOSs contamination, the investigation of biocatalytic routes in neoteric solvents that have all the advantages of VOSs but are environmentally friendly, that is, neoteric solvents, and thus, they have generated enormous interest since the beginning of the century [10]. Many of these routes are also already applied at a large scale [53].

14.5 Biocatalysis in supercritical CO_2

SFE using supercritical CO_2 and catalytic processes have been widely used since the last decades of the last century on large scale. In 1985, two groups of researchers, Randolph [54] and Hammond [55] independently demonstrated that biocatalysis was also possible in scCO_2. Since then, its applicability has been developed at both laboratory and industrial levels [53].

Table 14.2 lists some advantages and disadvantages of $scCO_2$ as a solvent for biocatalysis. Like nonpolar VOSs, $scCO_2$ extensively solubilizes hydrophobic compounds, which allows its use in reactions involving hydrophobic substrates; unlike VOSs, $scCO_2$ does not present any of the environmental problems described for them. Furthermore, enzymes are not soluble in $scCO_2$, and several free or immobilized enzymes (lipases, trypsin, chymotrypsin, penicillin acylase, alcohol dehydrogenase, cholesterol oxidase, etc.) are suitable for catalyzing chemical transformations (e.g., esterification, hydrolysis, alcoholysis, asymmetric synthesis etc.) in $scCO_2$.

Supercritical CO_2 allows reactions in which water is an essential element of the reaction. Lipases are excellent biocatalysts in $scCO_2$ [52,56]. Esterification and transesterification synthetic reactions are very relevant because they give rise to many different applications. For example, in the cosmetics industry, there are many compounds that are naturally hydrophilic and are therefore rapidly absorbed by the skin so that their duration and action are minimal. The esterification or transesterification of these compounds to form long-chain esters produces lipophilic or amphipathic compounds, usually with emulsifying properties that lengthen the duration process on the skin [57].

A fundamental aspect in the stabilization of proteins is their immobilization. The immobilization of proteins on very diverse supports and by means of different physical or chemical techniques basically produces two advantages with respect to the native protein:[48,58,59] their stabilization, extending the range of action (*P, T*, pH, etc.) and their recyclability, as they can be separated from the solvent, by means of physical methods (centrifugation or distillation, among others). This last aspect is crucial since the high cost of biocatalysts is one of the main problems of their applicability at a large scale. It is even more critical when it comes to biocatalysts in $scCO_2$, a solvent that also produces enzyme deactivation (see Table 14.2). The poor stability exhibited by free or immobilized enzymes in SCFs is probably the main drawback of these solvents in industrial biocatalytic processes. The adverse effects of $scCO_2$ on enzymes

Table 14.2 Some advantages and disadvantages of biocatalysis in supercritical CO_2.

Advantages	Disadvantages
Nonpolar solvent: solubilization of many nonpolar substrates.	CO_2 is acidic: reacting with the solvation sphere of the enzyme acidifies the medium, denaturing the protein.
High mass transfer rates: substrates quickly reach the active center of the enzymes.	Enzymes are deactivated in $scCO_2$: CO_2 reacts slowly with the ε-amino groups of lysine giving carbamates.
Enzymes poorly soluble in $scCO_2$: easy enzyme recovery.	Pressurization/depressurization processes deactivate the enzyme.
Activity and selectivity are finely tuned by external P and T changes.	CO_2 is highly nonpolar and tends to destabilize enzymes: in general, $scCO_2$ is not optimal for homogeneous biocatalysis.
Reactions involving water (lipases and hydrolases as biocatalysts) can be modulated at will, as well as directing the course of the reaction (use of molecular sieves).	
Eco-friendly solvent	
Possibility of increasing the solubility of hydrophilic compounds by using cosolvents.	

stability have been attributed to local changes in the pH of the hydration layer, or by conformational changes produced during the pressurization/depressurization steps, as well as, by the ability of CO_2 to form carbamates with free amine groups on the protein surface, resulting in changes in the secondary structure. In this context, the immobilization of enzymes by sol-gel entrapment in silica-aerogels has been described a clearly stabilizing approach against enzyme deactivation by $scCO_2$, whereby enzyme molecules are included within a rigid glass framework [56].

Lipases and esterases are the most widely used enzymes for biotransformations in $scCO_2$, because of the excellent ability of this fluid to dissolve and transport hydrophobic compounds. In a representative example, the biocatalytic synthesis of polyphenol esters, such as the antioxidant arbutin (hydroquinone β-D-glucopyranoside) has successfully been carried out in $scCO_2$ by transesterification with palmitic acid ethyl ester using Immobilized Novozym 435 from *Candida antarctica* as biocatalyst (Fig. 14.4) [60]. Working in batch mode and applying hexane/propylene glycol as cosolvent a yield of 85.2% was obtained in $scCO_2$ (10 MPa) after 20 h reaction at 60°C. Increasing the CO_2 pressure (up to 24 MPa) worsened the yield. The water content of the reaction media was shown as a key parameter, being obtained the best results a 1.3%. For lower water content the enzyme activity was reduced, while the higher water content produced enzyme deactivation.

For Novozyme 435-catalyzed the synthesis of butyl butyrate in $scCO_2$, it was observed that an increase in temperature from 40°C to 60°C provides an improvement in enzyme activity at all the assayed pressures (80−150 bar). However, at a fixed temperature in the above range, an increase in pressure resulted in a decrease in the synthetic activity of the enzyme, being attributed to the increase in $scCO_2$ density [61].

In another representative example, the biocatalytic synthesis of biodiesel by using alperujo oil and methanol as substrates has also been studied in $scCO_2$ (Fig. 14.5) [62]. Two different commercial immobilized lipases, such are Novozym 435 (*C. antarctica* lipase B immobilized on the macroporous acrylic resin Lewatit VP OC1600), and Lipozyme TL IM (*Thermomyces lanuginosus* lipase immobilized on silica gel) have been assayed for biodiesel synthesis in $scCO_2$, being obtained up to 96% biodiesel yield after 24 h of reaction at 50°C, 250 bar. However, the reuse of the enzyme was not assayed because of its deactivation, being attributed to the influence of temperature, the enzyme leaching during depressurization step, as well as the interaction of methanol and $scCO_2$ with the enzyme, pointing out the weakness of this neoteric solvent for developing biocatalytic processes.

The biocatalytic synthesis of isoamyl acetate in $scCO_2$ is another example (Fig. 14.6) [63]. Here different acyl donors (acetate anhydride, ethyl acetate, acetic acid) were tested and the best was shown to be the anhydrous one. The conversions obtained in $scCO_2$ were also compared, being superior to the one

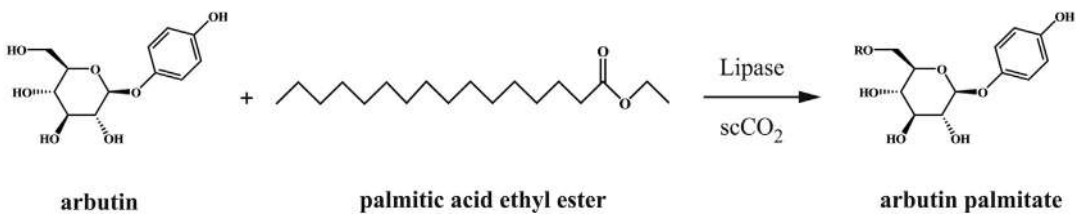

FIGURE 14.4

Schematic of the biocatalytic synthesis of arbutin palmitate.

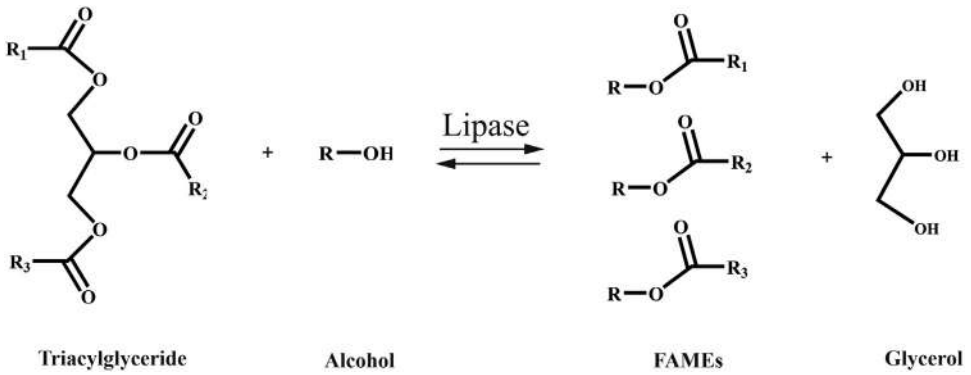

FIGURE 14.5

Schematic of the biocatalytic synthesis of biodiesel (FAMEs) by transesterification of triacylglycerides with methanol.

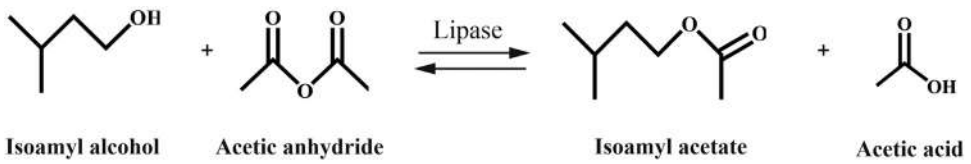

FIGURE 14.6

Schematic of the biocatalytic synthesis of isoamyl acetate from isoamyl alcohol and acetic anhydride.

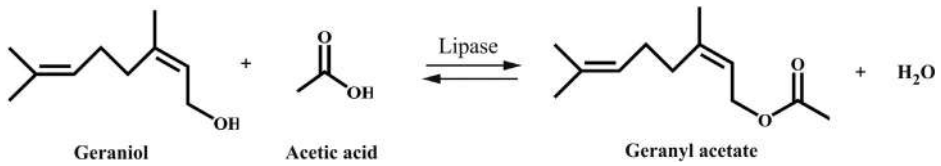

FIGURE 14.7

Schematic of the biocatalytic synthesis of geranyl acetate by direct esterification of geraniol with acetic acid.

obtained in *n*-hexane (1.3 times higher) and in a solvent-free system (1.2 times higher). Under the best conditions (60°C, 15 MPa, 1:1 molar ratio between substrates), the best conversion rate (88.4%) and specific productivity (36.9%) were obtained, although the reuse of the enzyme not considered.

In another example, the immobilized *C. antarctica B* lipase onto carbon nanotubes has been used as biocatalyst for the *O*-acylation reaction of geraniol with acetic acid, producing geranyl acetate in $scCO_2$ (Fig. 14.7) [64]. Now, it should be noted that the moderate level of activity shown by the enzyme for the first cycle (9.9% product yield), is emphasized by a drastically decay in activity during reuse from cycle 1−5 (less than 2%), being attributed to the enzyme deactivation phenomena produced by pressurization/depressurization processes.

Another key parameter in $scCO_2$ reactions is temperature. Although the increase of the temperature always favors diffusivity and enhances the enzyme activity, it should be noted that temperature also determines the number of water molecules that can be held in the solvation sphere of the protein. It has been determined that $scCO_2$ can dissolve between 0.3% and 0.5% water (v/v). At high temperatures the enzyme begins to lose its water molecules from the solvation sphere, resulting in denaturation of the enzyme with subsequent deactivation. In turn, the amount of water is a critical parameter. Moreover, the amount of water during the whole process must be more or less constant to maintain optimal enzyme activity. As indicated in Table 14.2, $scCO_2$ deactivates the enzyme, both by acidification of the water in the solvation sphere and by the reaction between amino groups of the protein with CO_2 to form carbamates. In the reuse processes, which involve depressurization and pressurization steps, analogous to the freeze-drying processes discussed above, substantial losses of enzyme activity also occur [65].

All these drawbacks lead to the fact that the use of $scCO_2$ in single-phase systems is not of excellent performance, in particular, due to the difficult reusability of the biocatalyst. Thus, the review published by Beckman and Russel in 1999 states in the final sentence: "*...the advantages of replacing conventional organic solvents with supercritical fluids have not been fully demonstrated yet*" [17].

14.6 Essentials of biocatalysis in ionic liquids

From tree pioneering works published in 2000, ILs have emerged as exceptionally interesting nonaqueous reaction media for biotransformations because of their unique solvent properties, headed by their negligible vapor pressure, and their exceptional ability of water-immiscible ILs to maintain enzymes in native folding and active functionality. In the first, Cull et al. [66] demonstrated, in a biphasic [Bmim][PF_6]-water system, that *Rhodococcus* R312 could transform 1,3-dicyanobenzene to cyanobenzamide and subsequently to 3-cyanobenzoic acid (Fig. 14.8A). In the second, Erbeldinger et al. [67] shows the suitability of thermolysin as catalyst for the synthesis of *Z*-aspartame from carbobenzoxy-L-aspartate and L-phenylalanine methyl ester hydrochloride in the same ionic liquid in the presence of 5% water (Fig. 14.8B). But it was Madeira et al. [68] who demonstrated the broad suitability of ILs for lipase-catalyzed different synthetic reactions, that is transesterification, ammonolysis and epoxidation. For the transesterification reaction of ethyl butanoate with 1-butanol (Fig. 14.8C) they obtained 81% yield after 4 h in the two ILs used ([Bmim][BF_4]) or hexafluorophosphate ([Bmim][PF_6]), a slightly higher conversion than that obtained in *tert*-butyl alcohol.

As for organic solvents, enzymes in water-immiscible ILs fold by adhering to a essential solvation sphere to maintain the 3D native structure, as well as a high level of activity. Water is key parameter for these nonconventional reaction media, and in the same way of VOSs, the hydrophillic character of water-miscible ILs (e.g., [Bmim][Cl], etc.) promotes a water stripping phenomenon on enzymes leading to the irreversible unfolding and full deactivation of biocatalysts [56].

Despite of all the excellent catalytic properties shown by enzymes in water-immiscible IL systems that are shown in several chapter of this book, the recovery of products after the biocatalytic reaction should be considered a key step when integrally clean chemical process will be developed. Since many molecular solvents (e.g., water, hexane, toluene) are immiscible with ILs, biphasic systems based on IL/water or IL/organic solvent have been assayed as the most straighforward approache for product separation. However, the use of VOSs is not recommented when integral

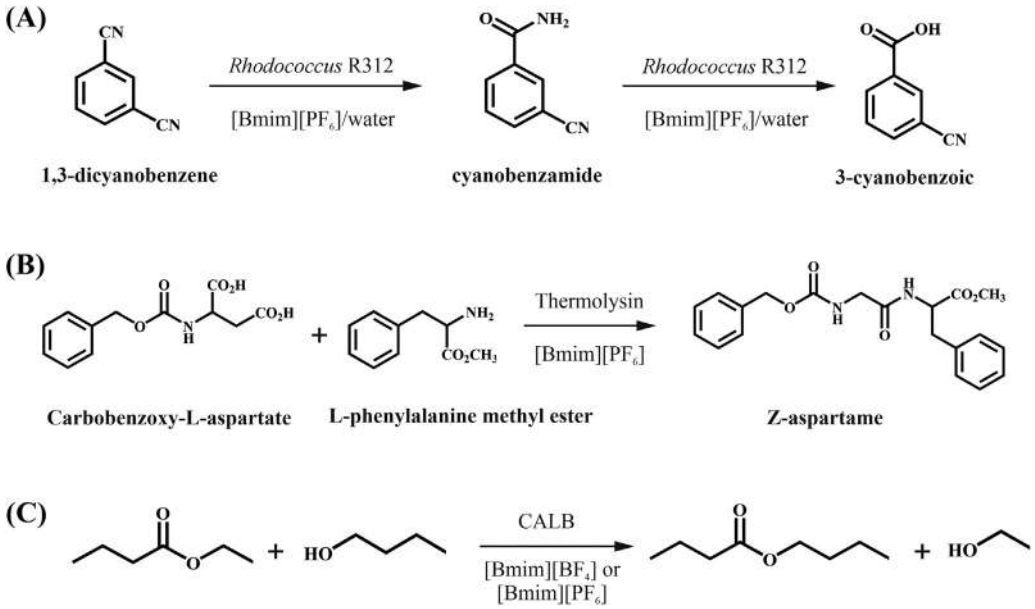

FIGURE 14.8

(A) Schematictic reactions for the biocatalytic synthesis of 3-cyanobenzoic acid from 1,3-dicyanobenzene; (B) Thermolysin-catalyzed the Z-aspartame synthesis; (C) Lipase-catalyzed the synthesis of ethyl butyrate by transesterification of ethyl butyrate with 1-butanol (see text for details).

green approaches will be developed. An original and clean strategy developed for the separation of reactants, products, catalysts with respect the IL reaction medim consists of the use of socalled sponge-like ILs (SLIL, see Chapter 8), being based in the selective separation of molecular compounds by the unique behavior of these SLILs with temperature [69].

Based on the unique solvent properties of ILs with respect molecular solvents, the use of water, or other "green" molecular solvents, that from biphasic systems when combined with ILs is a common experimental approach, to achieve the product separation, as well the recovery of the IL for further reuse [34]. As mentioned above, the $scCO_2$ has also been shown to exhibit properties suitable for biocatalysis reactions, although they deactivate the catalyst, preventing its reuse. The biphasic IL/$scCO_2$ systems in which IL provides the enzyme with stability, while $scCO_2$ solubilizes and carries away reagents and products make this conjugation an approach with many advantages in biocatalysis.

14.7 Phase behavior of ionic liquids and $scCO_2$ mixtures

Among all the unique properties of ILs as neoteric solvents, the particular behavior of ILs and $scCO_2$ mixtures should principally be analyzed. ILs can dissolve gases to a large extent, depending on the nature of the IL, pressure and temperature. Indeed, CO_2 is highly dissolved in many ILs, while ILs is

not measurably soluble in the $scCO_2$. Thus, the pioneering work of Brennecke's group in 1999 demonstrated that ILs and $scCO_2$ form biphasic systems, where $scCO_2$ is able to extract from ILs previously dissolved hydrophobic compounds [70]. In a representative example, it was shown that the solubility of CO_2 in IL [Bmim][PF_6] was as high as 0.75 mole fraction CO_2/IL at 8.3 MPa, whereas the solubility of IL in $scCO_2$ was residual under these conditions (lower 10^{-5} IL/$scCO_2$ mole fraction). In this context, the extraction of a nonvolatile organic compound, as naphtalene, dissolved in IL was carried out with $scCO_2$ (40°C, 13.8 MPa), recovering up to 94%−96% yield. The final separation of $scCO_2$ from naphthalene was immediate by decreasing pressure. The high solubility of CO_2 in ILs is attributed to hydrophobic, enthalpic-type interactions of the molecule with both anions and side chains of IL cations [71]. The very low solubility of ILs in $scCO_2$ has been attributed both to the low vapor solubility of ILs, being unable to separate its ions from each other.

Further studies showed how, as the pressure increases, the solubility of CO_2 into the IL-rich phase increases dramatically, reaching solubility data up 0.32 mole fraction at 93 bar and 40°C. The same study clearly showed how the dependence of CO_2 solubility in [Bmim][PF_6] on temperature was quite low, and there was only small decrease in solubility when the temperature was increased from 40°C to 60°C. Studies on [Bmim][PF_6]/$scCO_2$ phase behavior in high-pressure conditions (up to 970 bar) found two distinct phases in all conditions studied [70]. Although the solubility of CO_2 in this IL increases with pressure, the mixtures never become a single phase (e.g., a 5×10^{-7} mole fraction of [Bmim][PF_6] is obtained in CO_2 at 40°C and 138 bar). Furthermore, it should be noted that the water content in the IL phase affect the phase behavior with CO_2, due to the increase in CO_2 solubility when ILs are previously dried. However, the hygroscopic character of ILs is very high, resulting in a rapid re-hydration by atmospheric humidity after drying (e.g., the water content of [Bmim][PF_6] after drying is approximately 0.15% w/w, and increased up to 2% when exposed to the atmosphere). A similar phase behavior to [Bmim][PF_6] was observed for other IL-$scCO_2$ biphasic systems (e.g., [Omim][PF_6], [Omim][BF_4], [Bmim][NO_3], [Emim][$EtSO_4$], [Bpy][BF_4]), where the solubility of CO_2 in the IL-rich phase being increased proportionally with the length in the alkyl chain length of the cation for the case of fluorinated anions [72].

Furthermore, it should be noticed that the presence of other components (e.g., substrates, products, solvents etc.) in the $scCO_2$ phase, which may act as cosolvents, may enhance the solubility of ILs in this $scCO_2$ phase. Taking the [Bmim][PF_6]/$scCO_2$ binary system as representative example, the addition of ethanol or acetone dramatically increases the solubility of the IL in the supercritical phase as a result of the strong interaction of these molecular solvents with the IL. The ability of cosolvents to increase the solubility of ILs (e.g., [Bmim][PF_6], etc.) in the $scCO_2$ phase is in agreement with the dipole moment of cosolvent (i.e., acetonitrile > acetone > methanol > ethanol > hexane) [73].

Studies devoted to the extraction with $scCO_2$ of solutes dissolved into an IL phase (e.g., aliphatic or aromatic solutes containing different substituent groups, such are halogen, alcohol, amide, ester, ketone, etc) showed that all solutes can be easily recovered (i.e., up to 95% yield). For the case of compounds with a low solubility or even immiscibility with the IL phase (e.g., benzene or chlorobenzene are immiscible with [Bmim][PF_6]), a minimum amount of CO_2 was required. For the case of IL/organic solvent/CO_2 ternary systems, it should be noticed the formation of three phases in supercritical conditions (e.g., methanol/[Bmim][PF_6]/$scCO_2$, Fig. 14.9) [74,75].

For this case, it should be noted that the mixture of methanol and [Bmim][PF_6] are fully miscible in all proportions at ambient conditions, resulting in a gas-liquid biphasic system under atmospheric pressure of CO_2 (Case *a*: Room *P*). The pressurization of the methanol/IL mixture (*L* phase) with CO_2 becomes in the formation of two liquid phases (L_1 and L_2, Case *b*). The bottom

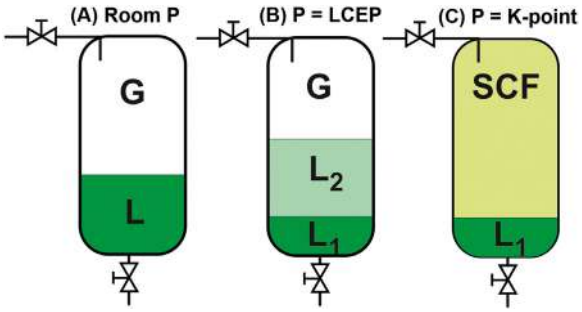

FIGURE 14.9

Schematic of the influence of pressure on the phase behavior of IL-organic solvent-CO_2 ternary systems, (A) room pressure; (B) lower critical endpoint pressure; (C) *K*-point pressure. (*L*, L_1 and L_2, liquid phases; *G*, gas phase; *SCF*, supercritical phase) [74].

densest liquid phase (L_1) is rich in the IL, while the other liquid phase (L_2) is rich in the molecular solvent, and the upper gas phase (G) is mostly CO_2 with some organic compounds. The pressure and temperature conditions in which the second liquid phase appears is called the lower critical endpoint. In these conditions, the organic compound-rich phase expands significantly with increased CO_2 pressure, while the IL-rich phase expands relatively little. The further increase in CO_2 pressure may induce another critical point, called K-point, at which the organic solvent-rich phase (L_2) merges with the gas phase (G), while the resulting scCO_2-organic solvent phase contains no detectable IL. This genuine phase behavior has practical implications for carrying out both reaction and separation processes IL/scCO_2 biphasic systems, allowing the extraction of solutes from IL phase without any cross contamination [74].

14.8 Biocatalytic processes in ionic liquid/scCO$_2$ biphasic systems

14.8.1 Pioneering works: a new strategy found

Despite the clear advantages of using ILs as nonaqueous reaction media for enzyme catalysis, a breakdown point in the greenness of any chemical process occurs if products are recovered by liquid-liquid extraction with organic solvents. The use of IL/scCO_2 biphasic systems as reaction media for enzyme catalysis has opened up new opportunities for the development of integral green processes in nonaqueous environments. In 2002, and almost simultaneously, the Lozano's [76] and Leitner's [77] groups demonstrated the feasibility of biocatalytic synthesis in a biphasic IL/scCO_2 system using two types of transesterification reactions. Lozano and coworkers devised a flow reactor in which CALB enzyme was dissolved in an IL ([Emim][NTf_2] or [Bmim][NTf_2], working phase) and the reactants were added to the scCO_2 (extractive phase, Fig. 14.10). The process was carried out in continuous mode. After the time required for the reaction, the product was dragged by the scCO_2 and it was collected practically pure by depressurization, while the CO_2 (*gas phase*) can be recycled. The first reaction they work with was the synthesis of butyl butyrate by transesterification of vinyl butyrate with *n*-butanol (Fig. 14.11).

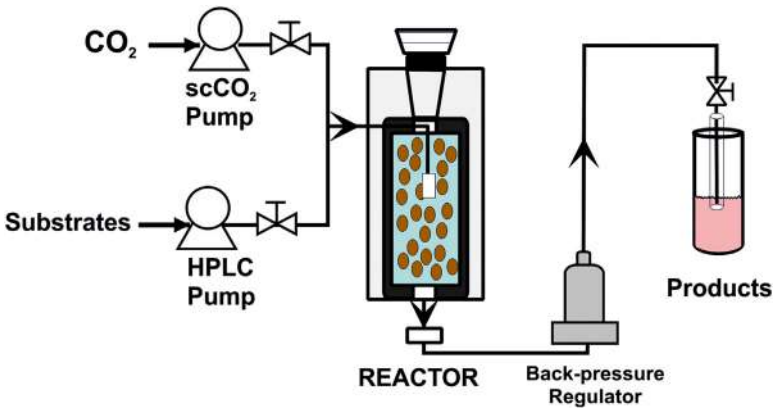

FIGURE 14.10

Experimental set-up of the continuous flow biocatalytic reactor for producing butyl butyrate in IL/$scCO_2$ biphasic systems [76].

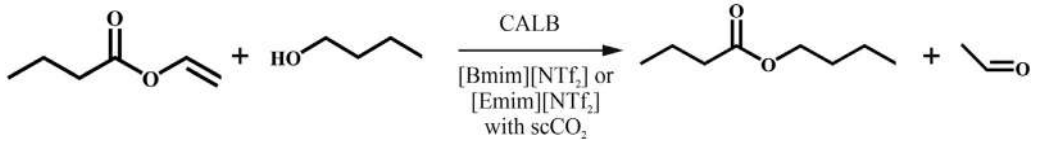

FIGURE 14.11

Lipase-catalyzed the synthesis of butyl butyrate by transesterification of vinyl butyrate with 1-butanol in ILs/$scCO_2$ biphasic systems.

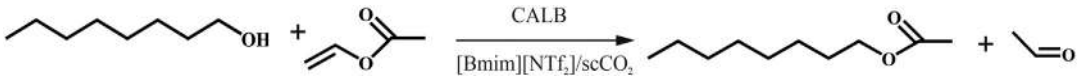

FIGURE 14.12

Lipase-catalyzed the synthesis of acetyl octanoate by transesterification of vinyl acetate with octan-1-ol in an IL/$scCO_2$ biphasic system.

The reaction occurred at the IL/$scCO_2$ interface (Fig. 14.12). By modifying P and T (i.e., $scCO_2$ density) the best yield ($>50\%$) and the highest degree of selectivity ($>99\%$) for ester synthesis was obtained at 100°C and 15.0 MPa. At this high temperature (the highest of those tested) the water content was minimal ($<4\%$), which largely prevented possible secondary esterifications. In these conditions, the enzyme showed an exceptional level of activity, enantioselectivity (ee$>$ 99.9) and stability after 11 operation cycles of 4 h continuous work, being attributed to the protective capacity of IL in respect to $scCO_2$.

In parallel, Leitner performed the acylation of octan-1-ol by vinyl acetate catalyzed by CALB (Fig. 14.12) [77]. Working in batchwise mode (39°C, 9.5 MPa), they obtained a 92% product yield,

while in continuous flow mode they reached 93.9% conversion after 24 h (45°C, 10.5 MPa). In both works, the two groups also performed the kinetic resolution of *rac* − 1-phenylethanol by transesterification in the same IL/scCO$_2$ biphasic system (see below).

The new methodology found by Lozano's and Leitner's groups opened an innovative path in biocatalytic synthesis that circumvented the problems of separate ILs and scCO$_2$ solvents, and at the same time benefited from the additional advantages of each. ILs protect enzymes, but the methods of separation of enzymes and reaction reagents and products are, in general, not optimal in ILs. On the other hand, scCO$_2$ can dissolve by fine modulation of the variables pressure and, most importantly, temperature (and consequently density and parameters such as Hildebrandt's or water activity, A_w), numerous lipophilic reactants, and, by addition of cosolvents, also more hydrophilic ones. However, scCO$_2$ gradually deactivates the enzymes (see Table 14.2). In two-phase IL/scCO$_2$ systems, the enzymes are immersed in the ILs (working phase), the substrates and products dissolved in scCO$_2$ (extractive phase) and the reaction takes place at the interface. In these two-phase systems, the ILs protect the enzyme from the corrosive effect of scCO$_2$. The reaction proceeds rapidly due to the high mass transfer rate of the supercritical solvent, with both reagents diffusing fast from scCO$_2$ to the active center of the enzyme and products diffusing in the reverse direction. These products are obtained in isolation with a high degree of purity, simply by depressurization. Moreover, the biocatalyst, embedded in the IL, can be recycled for a very high number of catalytic cycles. And all this performed with optimum reaction yields.

As it will be seen in the following examples, classified based on similar reaction characteristics, the biphasic combination of scCO$_2$ with ILs has proven to be a clean and green pairing in the synthesis of many compounds of industrial interest.

14.8.2 Kinetic resolution

Kinetic resolution (KR) is crucial in many different fields of fine chemistry. It is particularly in the field of pharmaceutics, where it is very often necessary to obtain enantiomerically pure compounds, since it is often the case that only one enantiomer is pharmacologically relevant, while the other is often inactive or even toxic [78]. Enzymes are, by constitution and by their three-dimensional structure, optically active and their function is also stereochemically selective: in a racemic mixture, they distinguish each enantiomer and can specifically catalyze 50% of one of the two, the *R* or the *S* (Fig. 14.13). This KR is one of the tools in which biphasic IL/scCO$_2$ systems are most useful.

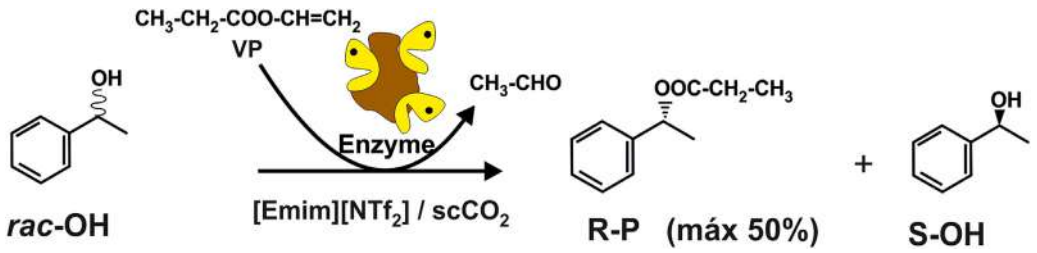

FIGURE 14.13

An example of a lipase-catalyzed the kinetic resolution (KR) of sec-1-phenylethanol. The enzyme mainly or exclusively reacts with an enantiomer, leaving the other stereoisomer unaltered.

In the two pioneering papers cited earlier, the second of the reactions by which they demonstrated the feasibility of the IL/scCO$_2$ biphasic systems involved KRs. Specifically, in the first work, *R* − 1-phenylethyl propionate was obtained from the transesterification of the *rac* − 1-phenylethanol racemic mixture with vinyl propionate (Fig. 14.13) [76]. In this case, a selectivity higher than 95% (100°C, 15 MPa) was obtained using the IL [Emim][NTf$_2$], with an enantiomeric excess higher than 99.9%. In a subsequent work, the same authors compared the activity and reusability of CALB in its free form (Novozym 525 L) and as an immobilized enzyme (Novozym 435). In the three ILs used, the immobilized enzyme was about 15 times more active than the free enzyme in the first cycle (in both cases the *ee* was greater than 99.9%) at 120°C and 10 MPa. However, the most interesting fact was to find that the yield of the immobilized enzyme in [Bmim][NTf$_2$] was not altered after 10 cycles, while, on the contrary, the half-life of the free enzyme was only 3 cycles [79].

Subsequently, the same group also demonstrated that it was possible to separate the enantiomeric esters resulting from the transesterification of *rac*-glycidol with butyl vinyl ester (Fig. 14.14) by the lipases, *C. antarctica* lipase A (CALA) and *C. antarctica* lipase B (CALB) (both from *C. antarctica*) and MML (from *Mucor miehei*), although the stereospecificity was not the same for each of them. While MML and CALA showed a preference for the *R*-stereoisomer, CALB was more effective in eliciting the *S*-stereoisomer [80]. The catalytic efficiency of CALB on IL/scCO$_2$ was also studied for the transesterification of *rac* − 1-phenylethanol by transesterification with vinyl propionate with five ILs all containing quaternary ammonium cations functionalized with different chemical groups. In all cases, the activity in the IL/scCO$_2$ biphasic systems was superior to that obtained in the hexane/scCO$_2$ system. Interestingly, there was a 2000-fold increase in the half-life of the enzyme in IL/scCO$_2$ in respect to hexane/scCO$_2$. The higher the hydrophobicity of the cation, the higher was the half-life of the biocatalyst, understood as the number of cycles in which it remained active. They also subsequently verified the relevance of the support on which CALB was immobilized in respect to the activity and most importantlythe half-life in cycles of the enzyme [81]. For this purpose, they compared the same transesterification reaction carried out by CALB, free (Novozyme 525 L) and modified enzyme on media with different chemical reactivity (alkyl,

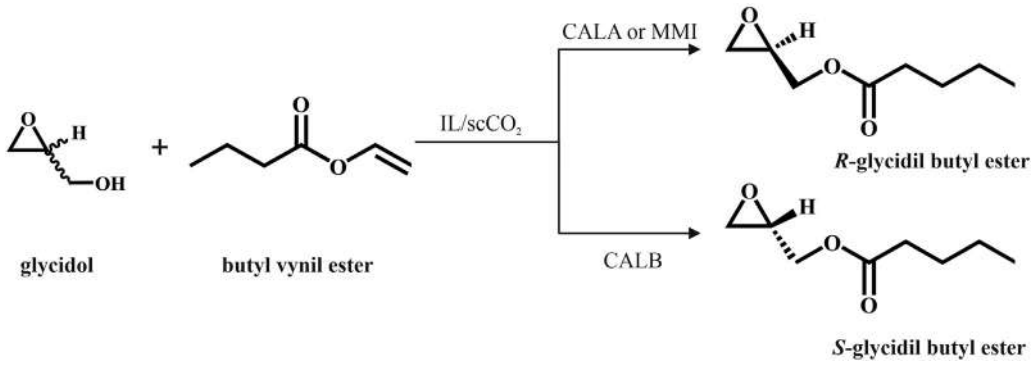

FIGURE 14.14

Transesterification of *rac*-glycidol with butyl vinyl ester to obtain the two corresponding enantiomeric esters catalyzed by *C. antarctica* lipase A (CALA), *C. antarctica* lipase B (CALB) of *Mucor miehei* lipase I (MMI).

amino, carboxylic, nitrile) and compared the activity both in hexane/$scCO_2$ and in IL/$scCO_2$. The best supports were those obtaining nonfunctionalized alkyl chains and those for which the water activity, A_w (i.e., the water vapor pressure of a solution in respect to the pure water, related to the ability to solve a solute in the solvent water) was increased up to 0.90, that is CALB/butyl silica, with an enzymatic conversion increased up to fivefold. Moreover, in IL/$scCO_2$ medium the biocatalyst yield was also up to sixfold higher than in hexane/$scCO_2$ medium. They also found that the half-lives of the coated enzymes were also much longer (up to fivefold) than that of the free enzyme. Consequently, the hydrophobicity of both the IL medium and the support on which the enzyme is immobilized are two key features for its higher yield and higher reusability.

In turn, Leitner's group, in his pioneer paper [76], performed the KR of *rac* − 1-phenylethanol by its transesterification with vinyl acetate to synthesize *R* − 1-phenylethyl acetate, in continuous flow, and with optimal conversion. They also studied this type of KR reactions in-depth in subsequent studies. For example, using CALB they studied the transesterification of five secondary alcohols (Fig. 14.15) with vinyl acyls, both in batchwise and continuous mode. They investigated the effect of the anion (PF_6^-, BF_4^- and NTf_2^-) while keeping the cation ([Bmim]) unaltered, concluding that the hydrophobic nature of the anion was decisive. With the best anion, [NTF_2], they obtained selectivities above 90% and *ee* between 98%−99%, depending on the alcohol and after 65 h of reaction [82]. Other groups have also worked on the resolution of biphasic IL/$scCO_2$ biphasic systems. For instance, da Ponte and coworkers [83] studied the reaction concentrations between succinic anhydride and the racemic mixture (*R,S*) − 2-octanol in the presence of a lipase in the vapor-liquid equilibrium of mixtures containing $scCO_2$ and ILs. Transesterification at 11 MPa and 35°C yielded almost exclusively the *R*-stereoisomer and recovered the unreacted *S* − 2-octanol enantiomer with an enantiomeric excess of 98.41%.

In short, all these studies demonstrated the suitability of coating immobilized enzymes on the Il/$scCO_2$ bi-phase as an excellent strategy to increase the half-life of the enzyme (higher number of cycles) and, in most cases, increase the yield.

14.8.3 Dynamic kinetic resolution

The KR allows obtain, in the best case, 50% conversion of a racemic mixture, the one corresponding to the enantiomer for which the enzyme is active. If, at the same time as the enzyme reaction

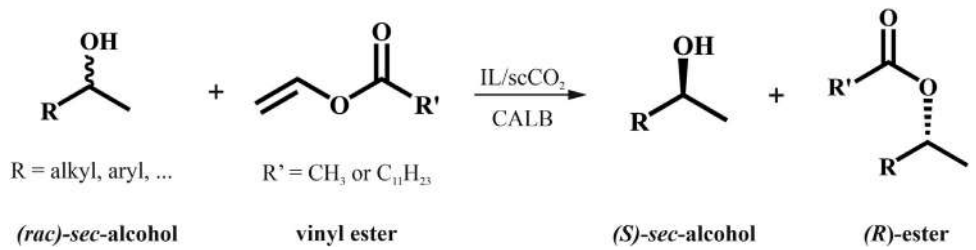

FIGURE 14.15

Lipase-catalyzed the transesterification of vinyl esters with several secondary alcohols to produce the corresponding enantiomeric esters.

takes place, there is a catalyst that balances the concentration of the two reacting enantiomers, then, as the enzyme active stereoisomer is consumed, the racemic mixture will be balanced until practically 100% conversion to the enantiomer under consideration is obtained (Fig. 14.16). This dynamic KR (DKR) has also been successfully carried out in biphasic IL/$scCO_2$ systems.

In one of the first examples of biphasic IL/$scCO_2$ systems published for this purpose, immobilized CALB particle and silica particles containing benzenesulphonic acid groups were used as catalysts for the DKR of *rac* − 1-phenylethanol in continuous flow under supercritical conditions (40°C and 10 MPa CO_2.). The process was carried out by placing each type of catalytic particles in separated layer of a continuous column reactor. Thus, the acid groups performed the *R/S* interconversion catalysis of the racemic mixture *rac* − 1-phenylethanol, while the immobilized enzyme exclusively synthesized the *R*-phenylethyl propionate ester [84]. Acceptable yields (70%, 92% *ee*) were achieved with good reaction times (1−6 days). However, direct contact of the enzyme with $scCO_2$ gradually denatured the catalyst.

Another approach to improve the catalytic system for continuous DKR was based on the combination of immobilized lipase with zeolites coated with ILs (e.g., [Btma][NTf_2], etc.), as the acid catalysts for in situ racemization of unreacted S-alcohol. Their suitability in a continuous DKR of *rac* − 1-phenylethanol when used as a combo-mixture with immobilized CALB particles under $scCO_2$ flow was successfully demonstrated (Fig. 14.17). The best results (98% yield, 96% ee) were obtained for a heterogeneous mixture between fajausite-type zeolite (CBV400) and Novozym particles, both coated with [Btma][NTf_2]. Based on the low acidity of the assayed zeolites, the packaging of the heterogeneous mixture of catalyst particles coated with IL did not result in any activity loss of the immobilized CALB during 14 days of continuous operation under different $scCO_2$ conditions, demonstrating the exciting potential of these multicatalytic (enzymatic or chemo-enzymatic) systems in ILs/$scCO_2$ medium for the green and clean synthesis of enantiopure drugs [85].

A further step with this system was achieved by using Supported Ionic Liquid-Like Phases (SILLPs), polymeric matrices modified with ILs, as support for enzyme immobilization [86]. These are nanostructures that generate a phase similar to the environments produced by ILs. In these SILLPs, the imidazolium groups are covalently bonded to the solid support so that a phase is generated that indeed acts as a "solid solvent" in which enzymes can be strongly retained in a

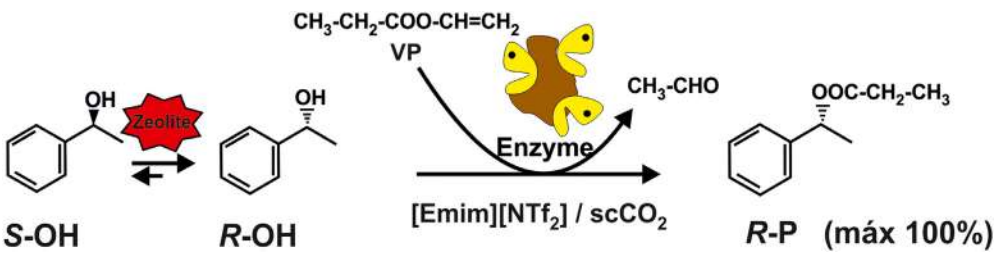

FIGURE 14.16

An example of a reaction of dynamic kinetic resolution (DKR). The enzyme exclusively reacts with an enantiomer, while another catalyst (zeolite) is continuously equilibrating the *S* and *R* stereoisomer concentrations, resulting in the full transformation of the initial racemic sample to a unique stereoisomer product.

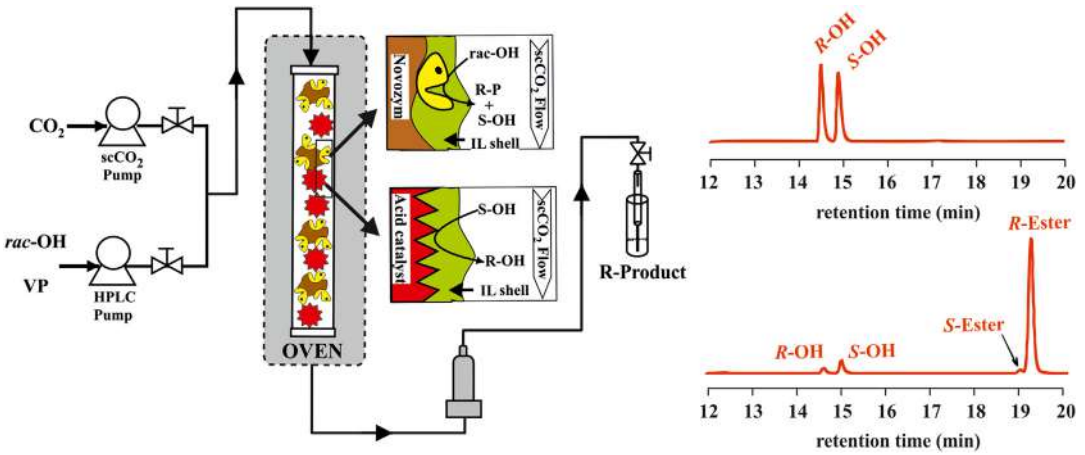

FIGURE 14.17

Left: Continuous flow reactor employed for the DKR of *rac* – 1-phenylethanol producing, almost exclusively the *R*-ester (adapted from reference P. Lozano, T. De Diego, M. Larnicol, M. Vaultier, J.L. Iborra, Chemoenzymatic dynamic kinetic resolution of rac-1-phenylethanol in ILs and ILs/supercritical carbon dioxide systems, Biotechnol. Lett. 28 (2006) 1559–1565, https://doi.org/10.1007/s10529-006-9130-7). *Right*: resulting chromatograms for the initial racemic sample (*up*) and the products of the reaction (*bottom*), where the *R*-ester was produced with ee higher than 97.3%.

noncovalent way, and avoid any loss of the IL phase by the continuous leaching. Excellent results were also obtained in this research (92% yield, *ee* 99.9% at 50°C, 10 MPa CO_2). This system, which was active for more than three weeks, was a more efficient approach compared to those performed in conventional solvents with homogeneous catalysis, in which anhydrous conditions and inert atmospheres are usually used.

14.8.4 Biodiesel synthesis

It has also been demonstrated that good results can be obtained in the transesterification of triglycerides to synthesize FAMEs (biodiesel, Fig. 14.5) using biocatalysis in two-phase IL/scCO$_2$ systems. Biodiesel is a renewable energy, although current methods of obtaining it are not [87]. Moreover, glycerol, the main waste product, is immiscible in the reaction mixtures in which it is obtained and pollutant, and is released into the wastewater. The biocatalytic synthesis of biodiesel has the problem that the enzymes are expensive and denature in contact with methanol, so processes in which these biocatalysts are reused and can be used in several cycles are needed. The ability to obtain biodiesel by biocatalysis in a biphasic scCO$_2$/IL mixture has been studied, both for enzymes coated by hydrophobic ILs [88], or adsorbed onto polymeric support having covalently attached IL, also named SILLPs [89]. This process in a biphasic IL/scCO$_2$ system allows obtaining pure products, which are entrained by scCO$_2$. maintaining the biocatalyst stable in the IL, that can be recovered and recycled. In the first work, CALB was immobilized with an IL layer. Fig. 14.18

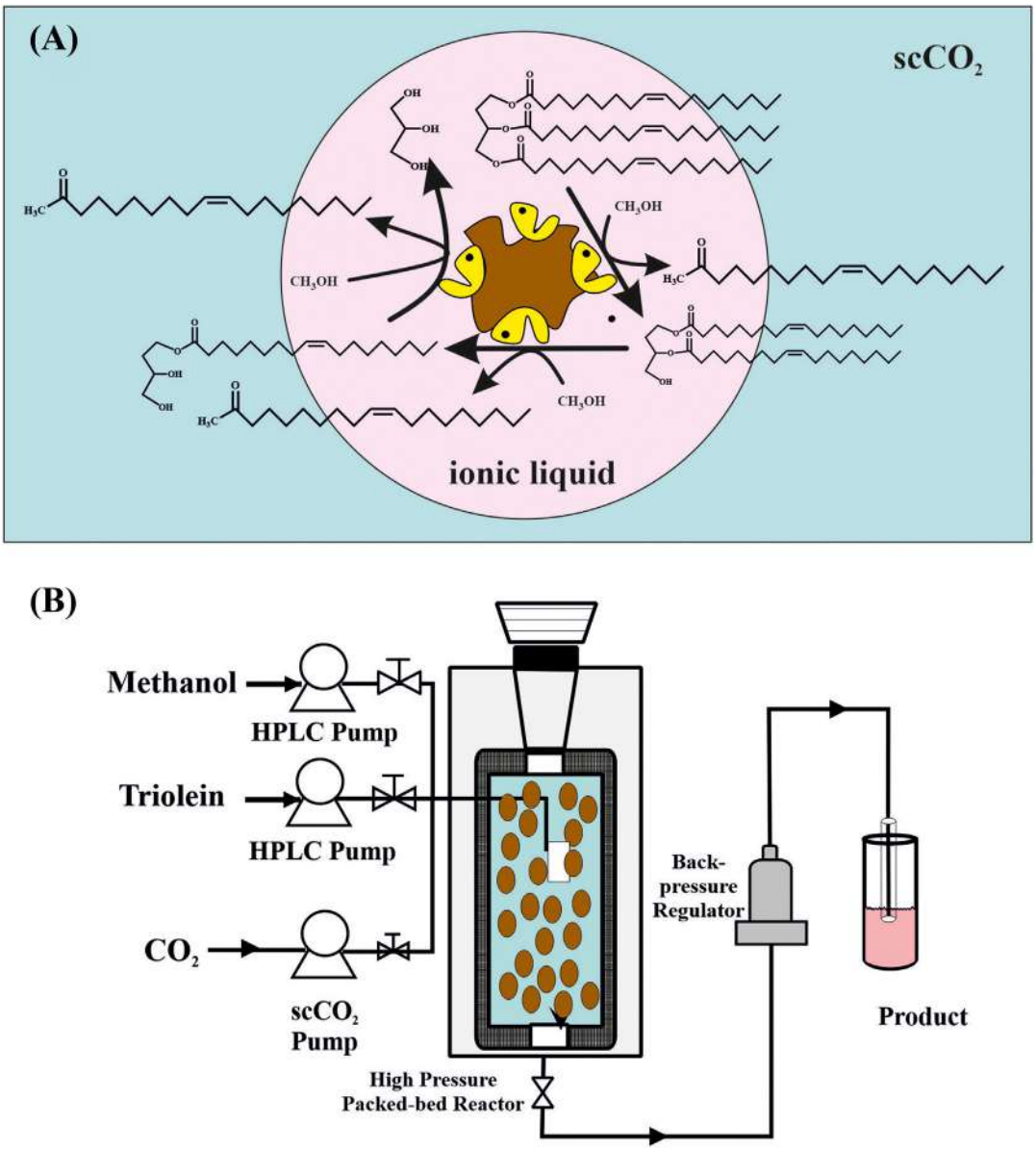

FIGURE 14.18

(A) Lipase-catalyzed three consecutive transesterification reactions on triolein for producing biodiesel and glycerol. (B) Schematic of the continuous flow reactor operating under supercritical conditions [88].

shows the enzymatic steps for obtaining biodiesel as well as the reactor design. The reactants (methanol and triolein triglyceride) were dissolved in scCO$_2$ and transported to the reactor, in which the biocatalyst particles were coated with the IL. The pure biodiesel generated was transported and released from the solvent by scCO$_2$ depressurization. Before the study of the reaction itself, the solubilization of the IL/triolein/methanol and IL/methyl oleate mixtures with various anions (NTf_2^-, PF_6^-, BF_4^-) and imidazolium cations of different chain length ($C_{12}^-C_{18}^-$mim) at 180 bar and 60°C was investigated. The best enzyme performance was obtained with the IL of formed by the most hydrophobic anion and the longest chain length cation ([C_{18}mim][NTf_2]). Under optimal conditions, a 95% yield of biodiesel production was obtained. The main problem of this approach concerns the hydrophilic nature of the by-product glycerol, which being retained onto the surface of the biocatalytic particles that poisons the enzyme action, because over the cycles this by-product avoid the entry of new hydrophobic triacylglycerol substrate molecules.

The use of SILLPs, based on covalently attached 1-decyl-2-methylimidazolium moieties onto a polystyrene divinylbenzene polymeric matrix, as support for the immobilization of *C. antarctic*a lipase B allowed the improvement of the productivity and especially the life-time of the enzyme in its reuse (Fig. 14.19) [88]. These SILLPs can, for example, modulate at will the amount and distribution of adsorbed enzyme, thus optimizing their catalytic properties. Using this innovative

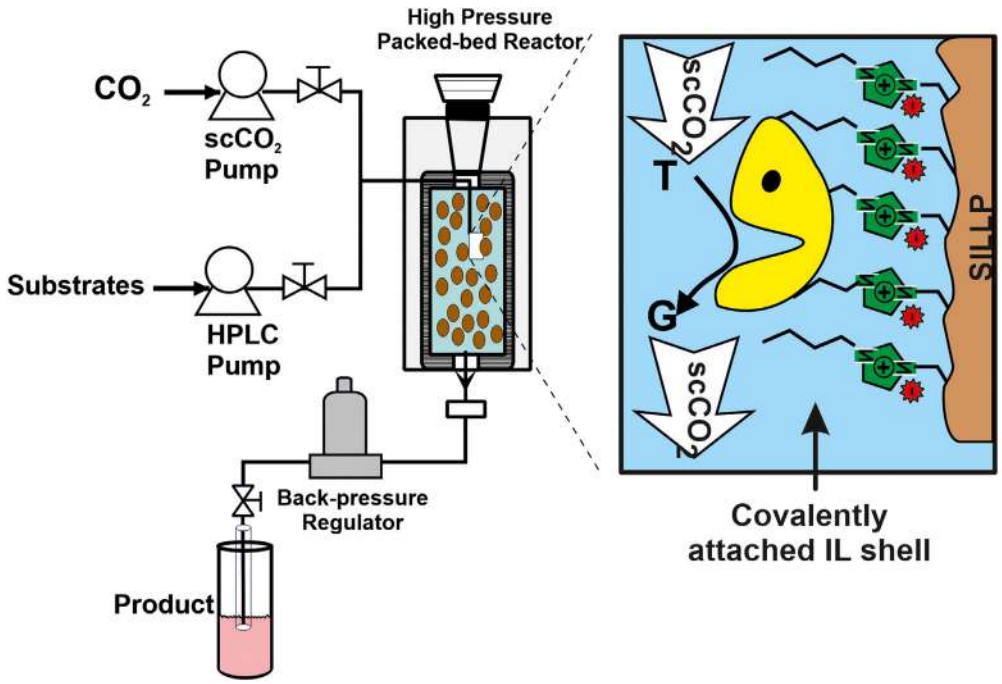

FIGURE 14.19

Reactor for obtaining biodiesel from triolein using a lipase adsorbed onto a supported ionic liquid-like phase (SILLPs) as catalyst [86].

methodology, yields of up to 95% (45°C, 18 MPa CO_2) were also obtained with optimum stability after 45 cycles of 4−8 h. However, the nonsolubility of glycerol in the $scCO_2$ mass-transport phase produces a continuous poisoning of the immobilized enzyme. This may be solved by washing the immobilized enzyme with t-butanol. The presence of a low amount of this solvent in the $scCO_2$ flow feed resulted in the maintenance of the enzyme activity for may operation days [89].

14.8.5 Other applications

The possibility of covalently immobilizing CALB on alpha-alumina membranes by cross-linking by glutaraldehyde and using the resulting membrane to produce butyl propionate synthesis by transesterification has also been demonstrated (Fig. 14.20) [90]. The reagents were transported in $scCO_2$ by tangential flow, the reaction occurred on the membrane and the product was dissolved in $scCO_2$. Three ILs were studied, all derived from the cation [Bmim] and with the same anion [PF_6], achieving a 99.5% product yield at 50°C and 80 bar CO_2. However, there was not any indication about the biocatalyst reuse for this process.

This methodology has also been applied to the synthesis of citronellol, an agent widely used in the flavor industry. The synthesis of citronellyl laurate by transesterification (Fig. 14.21) using immobilized CALB in a biphasic system of $scCO_2$/(organic solvents and IL as cosolvents) working in batchwise mode at different P and T was investigated [91]. The best yield (3.95 mmol/g substrate) was obtained when using ethyl methyl-acetone as substrate, and [Bmim][PF_6] (60°C and 10 MPa) as reaction medium, although the biocatalyst reuse was not studied.

Esters of sugar fatty acids are biosurfactants and are used in oil recycling, bioremediation, food, cosmetic, pharmaceutical and detergents. At the industrial level they are usually obtained in mixtures of acetone with *tert*-butanol, with the previous indicated environmental problems; while ILs have also been used, although here the typical problems of ILs alone are that it is not easy to recover the enzyme and the high viscosity of the medium. Recently, it has been shown that biphasic IL/$scCO_2$ systems are good for obtaining these sugar fatty acids. Specifically, they have been used

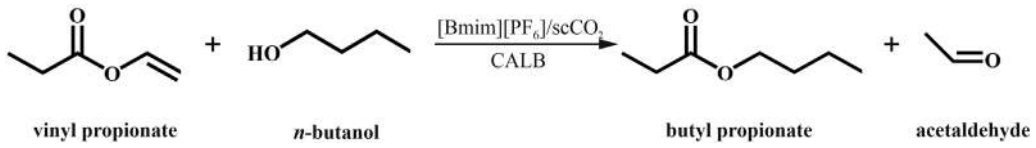

FIGURE 14.20

Lipase-catalyzed butyl propionate synthesis by transesterification of vinyl propionate with butanol.

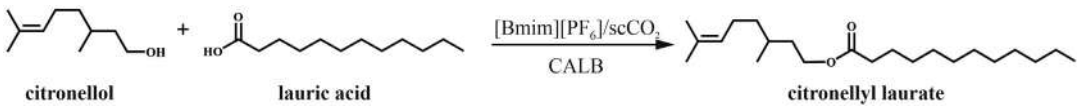

FIGURE 14.21

Lipase-catalyzed the synthesis of citronellyl laurate by direct esterification between citronellol and lauric acid.

for the synthesis of glucosyl laurate ester from glucose and vinyl laurate (Fig. 14.22), using 1-butyl-3-methylimidazolium tetrafluoroborate ([Bmim][BF$_4$]) and compared it with the analogous reaction carried out in IL alone [92]. At optimum conditions (50°C, 12 MPa) the yield, during 2 h of reaction time, was 95.5% in the biphasic mixture, whereas, also at optimum conditions for IL alone (50°C, 0.1 MPa), 81.19% was reached in 48 h. The drastic reduction in time is evident. It was also observed that the enzyme remained fully active for 10 h of continuous reaction.

The integrated reaction/separation approach based on IL/scCO$_2$ biphasic systems was also used to obtain the synthesis of omega-3 monoacylglycerides (MAGs) from omega-3 triacylglycerides in two consecutive catalytic processes (Fig. 14.23) [93]. Polyunsaturated fatty acid MAGs are used as dietary supplements since they are better absorbed than the natural triacylglycerides from which they are derived. In the first step, transesterification of raw fish or linseed oil with solketal was produced using the enzyme Novozym 435 immobilized in SLIL. In the second step, MAGs were obtained by the selective acid hydrolysis of solketal ring with the CBV720 acid zeolite using scCO$_2$ as both reaction and product extraction medium. The best results were obtained using *tert*-butanol as cosolvent. A study was carried out in both continuous and batchwise modes and, in both cases, 100% yield was obtained for transesterification, with a completely pure product. In continuous mode, the maximum yield of the reaction was reached after 2 h and was maintained at 100% for 4 h more. Thereafter, due to biocatalyst poisoning, the activity was reduced to less than half

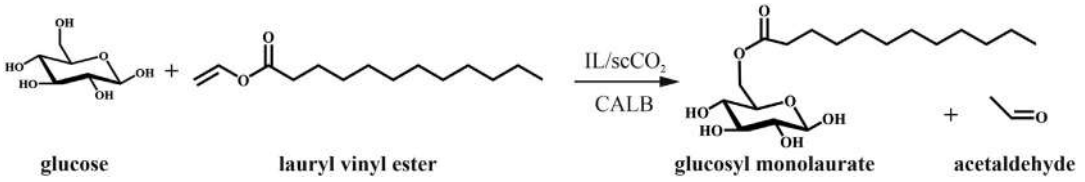

FIGURE 14.22

Lipase-catalyzed synthesis of glucosyl monolaurate by transesterification.

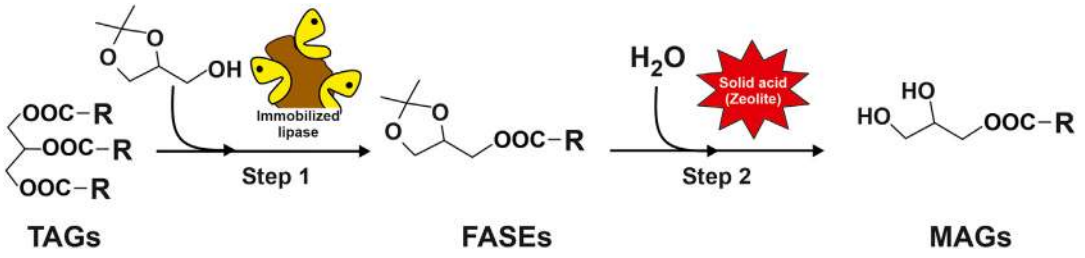

FIGURE 14.23

The two-step catalytic process for obtaining monoacylglycerides (MAGs) from triacylgycerides (TAGs) and solketal.

Adapted from reference R. Villa, E. Alvarez, S. Nieto, A. Donaire, E. Garcia-Verdugo, S. Luis et al., Chemo-enzymatic production of omega-3 monoacylglycerides using sponge-like ionic liquids and supercritical carbon dioxide, Green. Chem. 22 (2020) 5701–5710, https://doi.org/10.1039/d0gc02033h.

(45%) after a 4 h cycle. However, by washing the immobilized enzyme particles with *tert*-butanol, a biocatalyst reactivation was observed, which maintained the maximum activity for 14 h continuous operation. For both continuous and batchwise operational modes, the enzyme was shown to be active for extended periods. This coupled multiphase process is a demonstration of how omega-3 MAGs can be obtained from their natural derivatives in a facile, green medium and biocatalyst recovery manner.

14.9 Conclusions

Chemical synthesis is fundamental to human progress. In the last century, the vast majority of industrial synthesis processes have been carried out in organic solvents, which are harmful to health and the environment. In the 21st century, all scientific efforts are focused on the development of benign and environmentally friendly processes. Green chemistry is not an option, it is the inevitable consequence of the social awareness that resources are limited and so is our planet. In this context, neoteric solvents are a real alternative to organic solvents, and the use of biocatalysts in these media is one of the most efficient ways to take advantage of the resources that nature offers us in the synthesis of compounds. The biphasic combinations of two of these neoteric solvents, IL and $scCO_2$, and their reactions with biocatalysts are an example of a clean and green way of operating and where chemistry today can, should and does go. This chapter provides examples of these types of reactions. This field is fertile and still virgin. Undoubtedly, many more applications will be obtained in the coming years in these systems.

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CHAPTER

Enzymatic membrane reactors and nonconventional solvents

15

Jose Sanchez-Marcano and Marie-Pierre Belleville

European Membrane Institute, UMR 5635, CNRS, ENSCM, Montpellier University, Montpellier, France

15.1 Introduction

Enzymatic membrane reactors (EMRs) with nonconventional solvents, which constitute the topic of this chapter, are a special class of intensified reactors where a membrane separation process and an enzymatic reaction are integrated in a single device. The combination of a catalytic (or biocatalytic) reaction and a separation into a single unit called more generally catalytic membrane reactor (CMR) allows an increased control of transfer phenomena, presents compactness and modularity, leading to a reduced environmental footprint and cost. Moreover, synergies obtained result in higher conversion and/or yields compared to processes that are more conventional where the reaction and separation steps are not integrated. The first section provides a general overview on membranes and membrane-based separation processes with some of the basic concepts and definitions. This section includes a classification of membranes and a description of the main membrane separation processes for liquids: microfiltration (MF), ultrafiltration (UF), nanofiltration (NF), reverse osmosis (RO) and pervaporation (PV). In a second section, a classification of CMRs or EMRs according to the role of the membrane with respect to the removal or addition of species is given. Extractor, when the function of the membrane is to remove one or more reaction products, distributor, when one of the reactants is fed continuously through the entire length of the membrane, or contactor, when the role of the membrane is no longer to separate but to improve the contact between a reactive phase and the catalyst. Special sections are devoted to different immobilization methods of enzymes in solids and membranes, including adsorption, ionic forces, covalent bonds, encapsulation and entrapment. In the case of enzymes immobilization in membranes, the synergy between immobilization methods and membrane filtration process can be applied to place the biocatalyst in a specific location of the membrane structure: on the surface, inside the support or through all volume of the membrane. The final section provides a comprehensive and extensive description of the work reported in the literature on the main applications of EMRs with two main nonconventional and sustainable solvents: supercritical carbon dioxide (SCCO2) and ionic liquids (ILs). These enzymatic reactions include esterification and transesterification for synthesis or racemic resolutions with lipases as well as carbon dioxide separation with carbonic anhydrases (CAs).

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15.2 Membrane separation processes

A membrane is a filter or a permselective barrier able to carry out the separation of species in a fluid either by sieving or by regulating the velocity transfer of one or more solutes through its thickness (Fig. 15.1). Membranes are thin films manufactured from a diversity of materials such as metals, inorganic solids, and a large variety of polymers. Over the past 30 years, the development and applications of membrane separation processes have grown and continue to grow at a remarkably high rate on an industrial scale in two main areas: separation and purification of products and the treatment of contaminated effluents. These applications concern a wide range of industries, from the petroleum and petrochemical industries to pharmaceuticals as well as the treatment of domestic effluents. Compared to the more conventional separation processes such as distillation or adsorption, membrane processes present many advantages including relatively low energy consumption and initial investment and modularity; this latter characteristic is noteworthy because it facilitates the scaling up [1–4].

Membranes are thin separative layers and can have either symmetric or asymmetric structure; in the last case, the thin separative layer is deposited on a porous support to ensure mechanical strength while avoiding creating resistance to the fluid transfer (Fig. 15.1). They can be classified according to different factors: structure of the permselective layer (dense or porous), type of

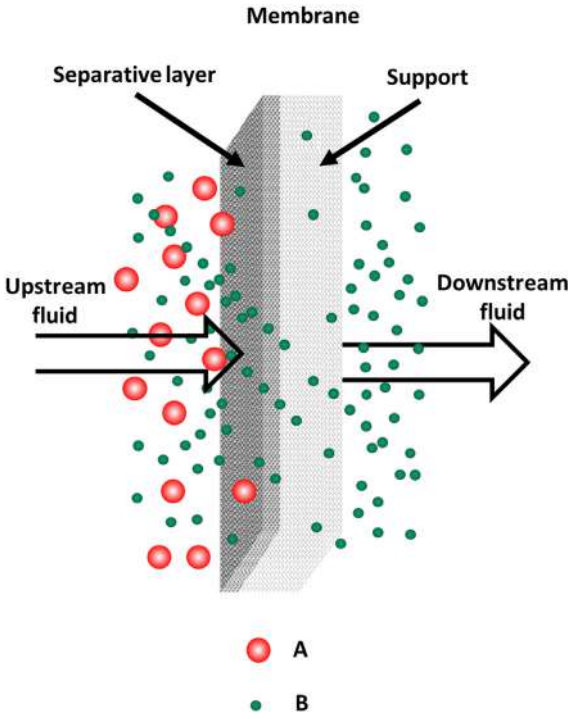

FIGURE 15.1

Principle of separation by membrane.

material used for their manufacture (organic, polymer, inorganic, metallic, etc.). The choice of material depends on the required chemical, thermal and mechanical stability, cost, and the ability to be shaped in special geometries. Polymeric membranes can be manufactured in almost all geometries including spiral-wound and hollow fibers whereas ceramic membranes can be only shaped in tubular, multitubular, and flat geometries [1,2].

The structure of the membrane (porous or dense) together with the driving force will define the size of species to be separated. Therefore, in membrane processes, a part of the fluid passes through the separating layer to obtain the filtrate or permeate, where some species have been removed; the phase enriched in rejected species constitutes the retentate. Transport processes through the membrane are the result of a driving force, which is characteristically associated with a gradient of pressure, concentration, temperature, etc. (Fig. 15.1).

Two main magnitudes describe membrane separation process: transmembrane flux or permeation rate and selectivity. The volumetric flow per unit area of the membrane and time defines the flux. In the case of gases and vapors is usual to report this magnitude as a molar flux (using the density and molecular weight and always reported in standard temperature and pressure (STP: 273K and 10^5 Pa). The SI of units of transmembrane flux are $m^3/m^2/s$ or $mol/m^2/s$. The selectivity of a membrane is the capacity of the membrane to separate species; it can be described by the retention (R) or the separation factor (α). R is more convenient for describing the selectivity or retention of a solute in diluted aqueous solutions. Retention is dimensionless and is calculated by: $R = (C_f - C_p)/C_f = 1 - C_p/C_f$, where C_f and C_p are the solute concentration in the feed and the permeate respectively. In the case of a binary mixture of gases or organic liquids ($A + B$), α is used to calculate the selectivity. It is defined by: $\alpha_{A/B} = (C_{Ap}/C_{Bp})/(C_{Af}/C_{Bf})$ where C_{ip} and C_{if} are the concentrations of component i in the permeate (p) and feed (f) respectively. It is also usual to characterize the membranes toward the permeability, a magnitude that depends on the couple fluid/membrane structure. It is defined as the ratio between the transmembrane flux, the membrane thickness and the driving force. In the case where the driving force is a pressure gradient (the most typical case) the units of permeability are mol (or m^3)/m^2/Pa/s [1–4].

Porous membranes achieve separation based on the average pore size and the molecular size of the species to be separated. According to the IUPAC, membranes can be classified corresponding to their mean pore size as macroporous, mesoporous and microporous for mean pore diameter greater than 50 nm, between 2 and 50 nm and less than 2 nm, respectively. In terms of membrane processes, MF is carried out with membranes with pores of the order of 0.1–10 μm, 10 nm to 0.1 μm for UF, a few nm for NF and a dense structure for RO and PV [1–4]. The cut-off point is defined as the molar mass of the smallest solute retained at 90%. In Fig. 15.2 is shown the comparison between the membrane characteristics and species size, in the figure is also indicated as a red rectangle the approximate range of size of enzymes (~10 to ~2000 kDa with a mean diameter in between ~3 and ~16 nm) to theoretically indicate membrane processes able to separate these biocatalysts. However, this comparison between the mean pore size and enzymes size is only an indication. Firstly, the pore size indicated is only a mean value (generally the pore size obeys to a more or less straight Gaussian distribution) and secondly, enzymes cannot be considered as spheres and in solution they can take different shapes according to the interactions between the solvent and chemical groups forming the biocatalyst. In fact, enzymes are usually separated with UF membranes with a cut-off between 1 and 100 kDa. Furthermore, enzymes are proteins and then amphiphilic (they are composed of hydrophobic and hydrophilic amino acids that can be responsible of interactions with the solvent and material interface). Depending on these interactions, enzymes can be adsorbed inside the pores or at the surface of the separative layer modifying the

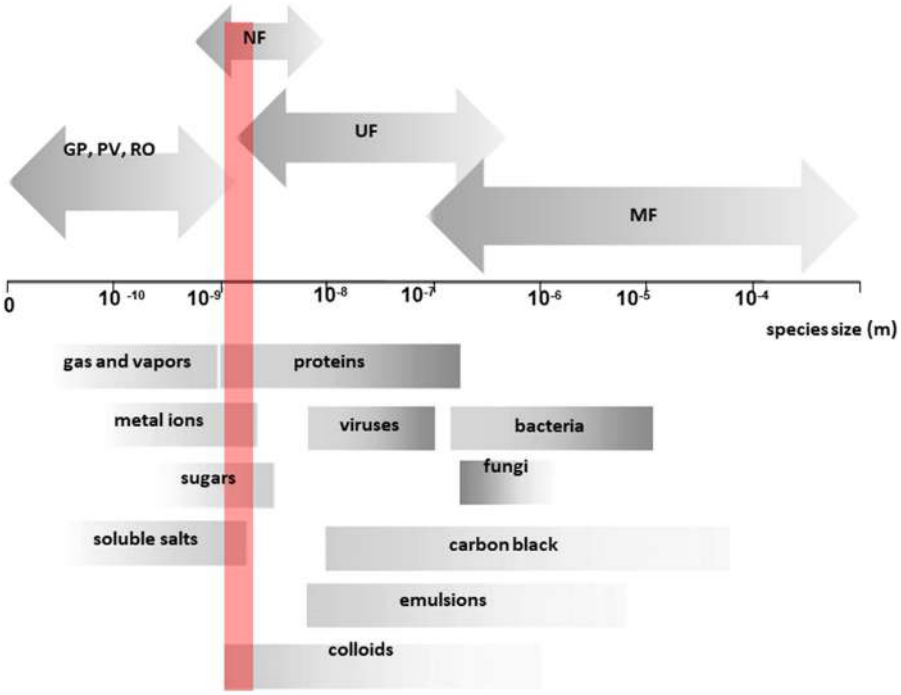

FIGURE 15.2

Different pore and particle sizes and related membrane processes (*GP*, gas permeation, *MF*, microfiltration, *NF*, nanofiltration, *PV*, pervaporation, *RO*, reverse osmosis, *UF*, ultrafiltration). The red rectangle indicates the approximate rank of enzymes size.

separation properties of the membrane. In conclusion, it is hazardous to make predictions about the separation of enzymes with membrane processes in terms of sieving properties, but in general, a deep experimental study is necessary to determine separation of enzymes with a specific membrane.

During the last 40 years, other types of membrane processes have been widely studied. In such processes, the membrane, which is not selective, separates two different fluids but only one of them wets the membrane material. The nature of the interaction between the membrane material (hydrophobic or hydrophilic) and fluids (water, organic solvents, ionic liquid, supercritical solvents, etc.) determine the position of the feed-solvent interface. Indeed, the membrane acts only a physical barrier between two different fluids and/or phases, creating a high and well-controlled interfacial exchange area. As explained previously, membranes can be manufactured in different geometries, but in membrane contact processes hollow fibers are the more extensively studied because they present a very high surface/volume ratio. Indeed, hollow fibers membrane contactors (HFMCs) allow an intensified contact between phases while avoiding emulsions formation. Furthermore, in HFMCs the velocity of each fluid (upstream and downstream) can be chosen independently allowing a better control of the species concentration on each side of the membrane [2,4,5].

15.3 Integration of a membrane separation and a catalytic reaction

Membrane reactors (MRs) are a class of intensified processes in which a single unit combines a catalytic or biocatalytic reaction and a separation step (through a polymer or inorganic membrane). Compared to processes that are more conventional where the reaction and separation steps are not integrated, the combination of these two unitary operations allows synergies resulting in higher conversion and/or yields. Numerous experimental and modeling studies have established the improved performance of MRs over conventional separated operations for a wide range of applications and operating conditions. Indeed, in MRs reactions can generally be performed under milder operating conditions and/or with higher product selectivity. Certainly, MRs have attracted significant technical importance during the last three decades, and even today there is still a continuous interest in these intensified processes because they present great compactness and modularity, leading to a reduced environmental footprint and cost (CAPEX) [2,4,6].

Clearly, MRs are appropriate for reactions where the conversion is limited by thermodynamic equilibrium. In such reactions, the continuous and selective extraction of one or more products through a selective membrane improves the yield by shifting the thermodynamic equilibrium. Esterification and dehydrogenation are the more obvious reactions of this category where the continuous separation of products (water and hydrogen respectively) results in improved yields. In addition to equilibrium-limited reactions, MRs have demonstrated to be interesting to carry out many other catalytic reactions including hydrogenation, partial and total oxidation, etc. In such reactions, the continuous separation of intermediates and products from the reaction zone can avoid the early deactivation of the catalyst and/or undesirable side reactions. Moreover, in some catalytic applications, MR principle has been coupled with membrane contactors, in such case the membrane does not even need to be selective, but only acts as a controlled reactive interface between reactants flowing on opposite sides of the membrane [2,4,6].

When a membrane is coupled with a bioreaction, MRs are called membrane bioreactors (MBR) or EMR if they are concerned with enzymatic conversions. Such MBRs have been applied to produce antibiotics, amino acids, and other fine chemicals. However, the major industrial application of MBR is wastewater treatment, which is now a proven and mature technology with many large-scale plants located all over the world [2,4,7].

MRs can be categorized according to different factors, and configuration is one of the most significant factors among these. The most common or conventional configuration is a MR with two devices (Fig. 15.3A), the reaction mixture is continuously recycled between both devices, in the first one the reaction takes place, the second one is a membrane module where a part of the fluid and the products of the reaction are accumulated or swept. In the second configuration (Fig. 15.3B) which is undoubtedly the authentic CMR, the membrane is itself catalytic, thus providing simultaneously the separation and reaction functions. In this second configuration, the membrane is made catalytically active by grafting, noncovalent bonding, entrapment, adsorption, etc. of the catalyst or biocatalyst on its surface or in the pores. This last configuration is currently called CMR. There are other MR configurations like packed bed or fluidized bed MRs. In such configurations, a membrane surrounds a packed or a fluidized bed where the catalyst or biocatalyst is immobilized [2,4,6].

MRs and CMRs can also be classified according to the role of the membrane with respect to the removal or addition of various species. MRs or CMRs can be considered as extractors when the

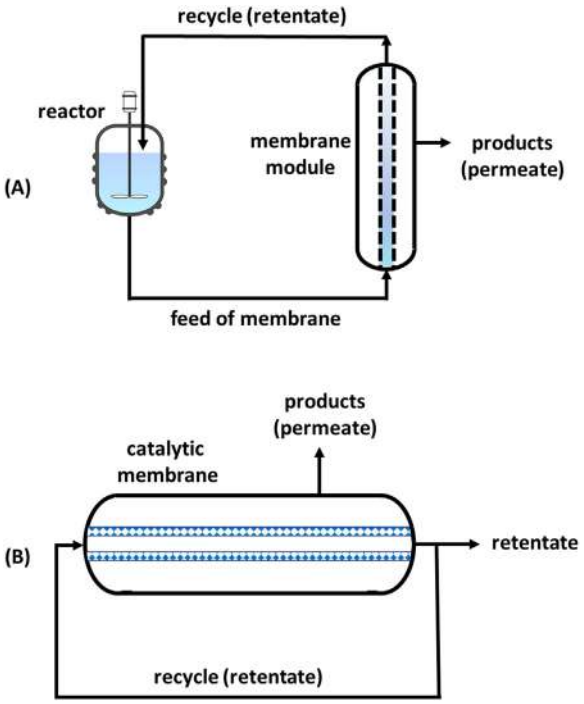

FIGURE 15.3

Types of MRs. (A) Conventional configuration with a reaction vessel connected in series with a membrane module, (B) Catalytic membrane reactor. *MRs*, Membrane reactors.

function of the membrane is to remove one or more reaction products (as the removal of water in esterification reactions). Moreover, they are distributors when one of the reactants is fed continuously through the entire length of the membrane (for example in partial oxidation reactions it allows maintaining a low oxygen partial pressure to avoid total oxidation). In CMRs, the membrane can also play the role of a contactor to improve the contact between a reactive phase and the catalyst. When contact is made by flowing the reagent through the porosity of the catalytic membrane, the CMRs are called flow through MRs (FTMRs). In Fig. 15.4 are illustrated the different types of CMRs described above.

15.4 Enzymatic membrane reactors

EMRs have in general the same characteristics and properties of MRs described earlier. Usually, enzymatic reactions are carried out in the classical configuration where a tank reactor is coupled with a membrane separation unit (Fig. 15.3A) [2,4]. In such EMR configuration free enzymes can be implemented if the separation unit includes a UF or NF membrane in such a way to separate the costly enzymes from the bioreaction products [2,4,8,9]. This configuration where reaction and

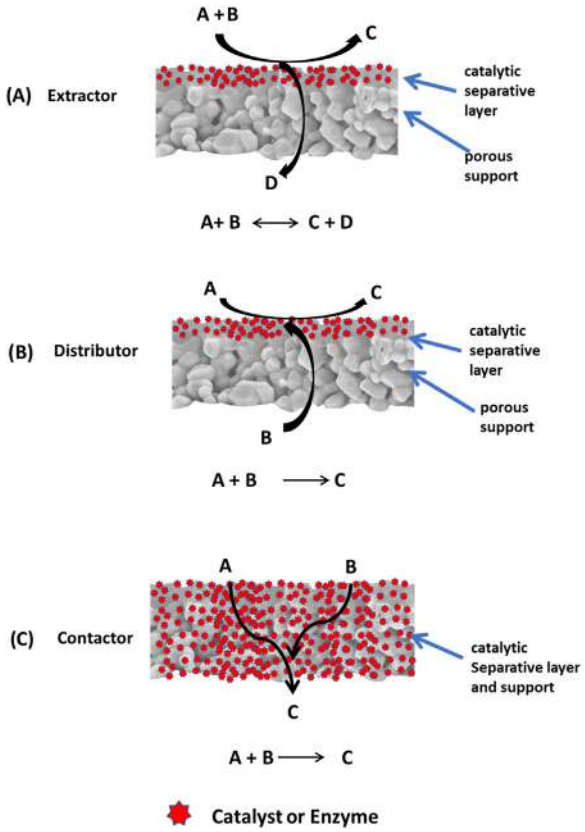

FIGURE 15.4

Types of CMRs according to the role of the membrane. *CMR*, Catalytic membrane reactor.

separation units are placed in series presents some advantages like the flexibility because both units can be controlled independently to optimize the whole process performance. Indeed, production may be adjusted by acting separately on process parameters like pH, temperature, fluid velocity, pressure, reactor volume and membrane surface. In the same manner, the enzyme concentration can also be varied in such a way to increase activity and productivity. Interesting results showing the interest of these EMRs have been reported for the hydrolysis of proteins, oligosaccharides, polysaccharides and oils, amino acids production, synthesis of sugar fatty acid esters, etc. [10–17].

Nevertheless, this EMR configuration also presents some drawbacks like the decay of enzyme activity caused by the shear stress produced by the agitation inside the tank and the recirculation pump. Moreover, under the effect of pressure and transmembrane flow, macromolecules or other compounds present in the solution (proteins, enzymes, polysaccharides, salts, etc.) can accumulate at the membrane surface and thus form a polarization layer or even a gel layer if macromolecules concentration exceeds solubility limits. These phenomena can be responsible of some problems

such as permeability decrease, clogging, selectivity changes and even inactivation of enzymes [18,19]. However, in some special cases, it can be taken advantage of the gel layer formation to immobilize enzymes at the membrane surface; this part will be described in the Section 15.6.

To decrease the effect of shear stress on enzymes activity as well as on polarization layer and gel formation, biocatalysts can be immobilized in solid particles, polymers, etc. Another advantage of using enzymes immobilized is the enhancement of the size of the species to be separated with the membrane. Indeed, this allows the use of UF membranes with larger pore size or even MF membranes. In the same way, softer process conditions can be applied, including lower transmembrane pressure and recirculation rate.

15.5 Immobilization of enzymes

In addition to the EMRs operation enhancements explained earlier, immobilization improves the thermal resistance and stability of enzymes. Additionally, biocatalysts immobilization also allows their reuse over several reaction cycles maintaining good catalytic efficiency and obviously reduces the cost of the process. Nevertheless, in many cases immobilization results on a reduction of the enzymatic activity because these biocatalysts often miss their native structure or function when they are in contact with a support surface [20–25].

Enzymes can be immobilized on different types of materials by adsorption (Van der Waals interactions, hydrophobic/hydrophilic balance, etc.), ionic forces, covalent bonds, encapsulation and entrapment. The immobilization materials are truly diverse; they include inorganic (active carbon, alumina, clay, magnetic nanoparticles, silica, etc.), organic (synthetic polymers, ion exchange resins and biopolymers) and hybrid/composite materials (silica-magnetite, chitosan-silica, etc.) [26–31]. Many of these materials can be shaped in form of pellets, porous particles, porous monoliths, membranes, etc. Consequently, all the techniques and methods explained below are also applicable to membranes.

Immobilization methods can be classified as reversible and irreversible: the first class includes adsorption, ionic forces, affinity binding and chelation. Adsorption is based on the creation of nonspecific interactions mainly via hydrogen bonds, ionic, hydrophobic or hydrophilic interactions. This type of immobilization results on limited changes of the enzyme structure and generally a catalytic activity close to that of free enzymes is obtained [32]. Immobilization by ionic charges is based on the properties of both enzymes and solid support, which can present different surface charge according to the pH, and ionic strength of the solution. For this, the isoelectric point of the enzyme and the solid must be quite different in order that both entities present opposite charge at the same pH. Furthermore, the pH must be in the range where the enzymatic reaction can take place. Affinity binding (by activation of a support material that contains a coupled affinity ligand) and chelation or metal binding (by coordination binding in between enzymes, transition metal salts or hydroxides and the surface of organic carriers) are also reversible methods of immobilization. Sometimes it is necessary to enhance the stability of the support-enzyme interaction. For this purpose, the enzyme can be modified, for example, polymer conjugation can be used to modify the surface of an enzyme providing additional points of hydrogen bonding with the surface, decrease dehydration, or supply thermodynamic barriers [33,34]. For this purpose, polymers or small molecules have been used (carbohydrates, proteins, biopolymers, polyethylene glycol, etc.) [34–36],

such immobilization methods are the result of weak links and then are reversible. This property allows the possibility to reuse the support when the enzymatic activity decays but present the disadvantage of a possible catalyst leaching during reaction.

Irreversible enzyme immobilization involves covalent binding, encapsulation, entrapment, and cross-linking (Fig. 15.5). Covalent binding is a traditional method for immobilization based on the reaction between functional groups of enzymes and support material. Indeed, $-NH_2$,$-SH$, and $-OH$ groups of enzymes are able to react with carbodiimides, cyanogen bromide, diazonium salts or reagents such like epichlorohydrin or glutaraldehyde to form covalent bonds. It is important to notice that sometimes previous preactivation treatments are necessary [i.e., irradiation or chemical treatments, silanization with 3-aminopropyltriethoxysilane (APTES), etc.] especially in the case of inorganic supports [37]. Among the coupling agents reported, glutaraldehyde has been extensively used because it generally gives excellent results in terms of enzymatic activity and stability [8,27,38–43]. However, covalent attachment is generally considered to be an expensive and nonenvironmentally friendly method of immobilization because it requires chemicals and preactivation treatments in the case of inorganic supports. Nevertheless, in spite of this inconvenient this method is widely used because it guarantees the absence of enzyme in the final product.

Encapsulation refers to the inclusion of enzymes within semipermeable microspheres made of polymers, biopolymers, liposomes or even inside the porosity of inorganic solids [27,36,44]; some of the more recent studies have reported the encapsulation in metal-organic frameworks [45]. Entrapment is based on the retention of the enzyme within the framework of polymers, biopolymers or gels as well as in polymeric grid or fibers. The main advantage of these two methods is that the material of the capsule or the framework maintains the integrity of enzymes by protecting them in a microenvironment that enhances their stability. Moreover, enzymes leaching is avoided whilst substrates and products molecules can diffuse across the permeable material. Furthermore, these

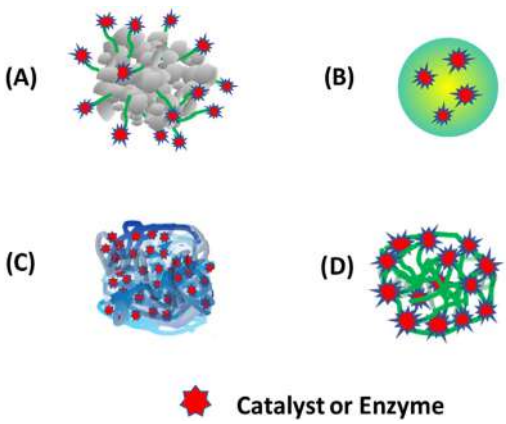

FIGURE 15.5

Schematic of irreversible immobilization methods of enzymes. (A) Immobilization on a solid or a polymer by a coupling agent, (B) encapsulation on a microsphere or liposome, (C) entrapment inside a porous structure or a framework, (D) enzymatic aggregates (cross-linking enzyme crystals or cross-linking enzyme aggregates).

techniques are interesting because they are generally cheap, rapid and are carried out in mild conditions [27,46–50].

In some cases, the stability of enzymes is improved by creating enzyme aggregates through cross-linking reaction between biocatalyst molecules without the use of any solid support. This reaction is carried out with bi-or multifunctional reagents such as glutaraldehyde or carbodiimides, leading to three-dimensional cross-linked aggregates, which are not soluble in aqueous medium. These self-supported enzymatic aggregates are called cross-linking enzyme crystals or cross-linking enzyme aggregates [51–53].

Despite all the advantages of the immobilization methods described earler, the immobilization of enzymes on porous solid particles can also present some drawbacks, such as diffusional limitations. Indeed, if enzymes are immobilized inside a microsphere or a framework of a permeable material or inside the porosity of solid particles or polymers, substrates and products must diffuse through these materials to reach the active sites. Moreover, in such cases, it is impossible to recover the carrier once the enzyme is inactivated. Table 15.1 resumes the main advantages and disadvantages of the different immobilization techniques.

Table 15.1 Advantages and drawbacks of different enzyme immobilization techniques.

Immobilization technique	Strength of interactions	Advantages	Drawbacks
Adsorption Ionic binding Affinity binding Chelation or Metal binding	Weak	Enzyme modification is not especially important. Simple, inexpensive, reversible, process: reusability of support when immobilized on the surface of the carrier	Level of immobilization depends on the force of interaction. Possible leaching of enzymes
Covalent bonds	Strong	Strong and stable immobilization. Multipoint attachment. Relatively simple method. Variety of supports available.	Poly-functional reagents are required. Loss of functional conformation of enzyme. Irreversible binding, no possibility to reuse the support. Expensive.
Encapsulation	Weak	No enzyme modification. Protection of enzyme. Cost effective method.	Less concentration of enzymes. Possible diffusion limitations.
Entrapment	Weak/ strong	No enzyme modification. Fast method. Low cost.	Possible enzyme leaking. Pore or framework diffusion limitations of substrates and products.
Cross-linking (cross-linking enzyme crystals, cross-linking enzyme aggregates)	Strong	No support needed. High strength of interactions.	Poly-functional reagents are required. Denaturation or structural modification.

15.6 Enzymes immobilized on membranes

As explained earlier, the second EMR configuration (Fig. 15.3B) involves enzymes immobilized on membranes, and this configuration is really the one where the two processes are coupled in a single unit. Indeed, the enzymes can be immobilized on the surface or within the internal membrane structure, depending on where the reaction is to take place. The choice of the immobilization method depends on the characteristics of the membrane (type of material and properties), the enzyme characteristics before and after immobilization (activity, stability, operation temperature and pH), the method itself (ease of implementation, desired bonding strength, etc.) as much as the cost (enzyme, products for immobilization process, possibility of membrane regeneration, product value, etc.). Moreover, the membrane process itself can be applied to carry out the immobilization to place the biocatalyst in specific locations of the membrane structure (surface, inside the support, through all volume of the structure) [2,4,47,54].

Fig. 15.6 provides an overview of the different types of processes for immobilizing enzymes in membranes. The first four methods concern immobilization on the membrane surface according to different types of attachment: dynamic gel layer of enzymes obtained by filtration (Fig. 15.6A), deposit of enzymes by static adsorption (Fig. 15.6B) by ionic or other type of weak attachment (Fig. 15.6C) and by covalent binding (Fig. 15.6D).

As explained previously, in EMR with free enzymes, it was observed that enzymes could be concentrated near the membrane surface. Thus, depending on the concentration reached and the membrane pores size, a continuous layer of enzymes can be formed at the membrane surface (Fig. 15.6A) [55,56]. In some cases, the gels formed are not stable due to the solubilization of proteins in the aqueous retentate as soon as the transmembrane pressure decreases [14,57]. This drawback can be settled by cross-linking the gels formed [37–39,58]. This property has been used as a simple method for the immobilization of enzymes on the surface of membranes especially when the membrane material is relatively inert. For example, enzymes were immobilized by covalent bonds on the surface of ceramic membranes previously coated with a thin gelatin layer obtained by tangential filtration [59]. The grafting agent used (i.e. glutaraldehyde) acted not only as a coupling agent between the gelatin and the biocatalysts but also as a cross-linking agent of the gelatin layer allowing to improve its stability. In addition, the gelatin also provides a favorable microenvironment for enzyme configuration allowing good reactivity. However, this method also has some disadvantages that may result either from the inactivation of some active sites of the enzymes or from the inaccessibility of an undetermined amount of these active sites to substrates. This last problem can be the result of steric limitations (binding near active sites, enhancement of the rigidity of the enzyme conformation, etc.) or diffusion limitations of substrates to the active sites. Similar methodology had been used to immobilize enzymes on a membrane surface by a previous modification of the separative layer with polydopamine or APTES and then carrying out the filtration of an enzymatic solution creating a kind of active fouling layer, in some other works enzymes are also cross-linked [60,61].

Fig. 15.6E–G concerns other different locations of enzymes on membranes. Fig. 15.6E shows the entrapment by reverse filtration, here the enzymes cannot cross the separative layer and remain immobilized inside the porosity of the membrane support. On the contrary, Fig. 15.6F and 15.6G show the dispersion of enzymes through all membrane structure (surface, separative layer and support). This immobilization can be carried out by different processes like impregnation or by flowing the enzyme solution through the membrane (with relatively large pores like macroporous membranes). Moreover,

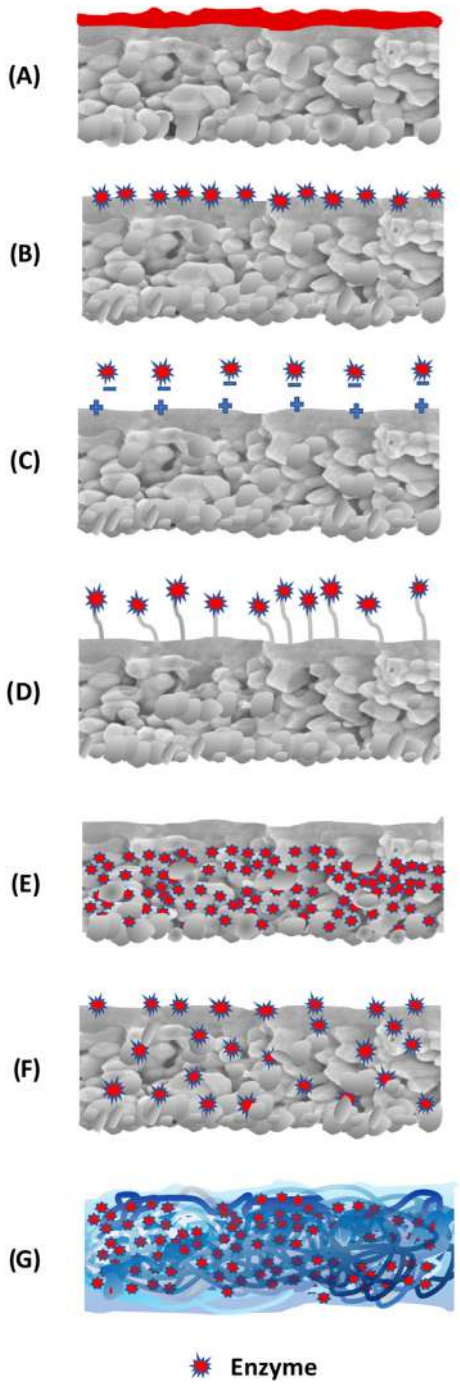

FIGURE 15.6

Different immobilization methods of enzymes on membranes. (A) Filtration and formation of enzymatic gel, (B) Adsorption at the membrane surface, (C) Attachment by weak interactions like ionic forces, (D) Immobilization by covalent bonds, (E) Entrapment inside the porosity of a membrane support, (F) Immobilization through all membrane porosity, (G) Entrapment in the framework of polymers or biopolymers.

as explained above these dynamic methods for the location of enzymes are generally combined with immobilization techniques like covalent attachment, entrapment, etc. described above.

Immobilization of enzymes by direct adsorption on a surface and/or inside of a membrane structure is an easy and inexpensive immobilization method because it can be carried out in one step (by immersion in enzyme solution or by filtration). This method has been applied to immobilize lipases and inulases on polymer hollow fibers (Fig. 15.6B) [62,63]. Nevertheless, as it has been explained above if the interactions between enzymes and membrane material are not strong enough, some enzyme leaching is possible.

The ionic immobilization of enzymes (Fig. 15.6C) has been successfully carried out by using a basic anion exchanger membrane [64] or by filters coated with poly(allylamine hydrochloride)/poly (sodium-4-styrene sulfonate) [65].

The covalent binding of enzymes on membranes (Fig. 15.6D) can be carried out through coupling agents after the preactivation of the material forming the membrane (silanization, carboxylation, CVD, etc.). Covalent binding generally results on very stable bonds avoiding enzyme leaching. The technique can be applied to immobilize enzymes on the surface of membranes but also through the membrane porosity [66–68]. As it has been described previously, covalent binding has also been applied to cross link gels formed on a surface of a membrane to stabilize the gel and immobilize the enzymes. In recent work, a laccase was immobilized with an interesting strategy by mixing the enzyme with ILs and polyvinyl chloride, after a casting process and cross-linking with glutaraldehyde a stable and reactive membrane was obtained [69].

Reverse filtration of an enzymatic solution (by feeding the enzymatic solution from the support to the separative layer) allows immobilizing enzymes inside the porosity of the membrane support (Fig. 15.6E). Here, enzymes are maintained inside the support structure because enzymes cannot flow through the thin separative layer. For example, polysulfone hollow-fiber membranes with perfect radial gradient pores have been activated by reverse filtration with a lipase from *Candida rugosa* which had been adsorbed on a phospholipid layer previously deposited inside the porosity of the support. These enzymatic membranes were successfully tested for the hydrolysis of glycerol triacetate [70]. In the case of macroporous membranes, enzymes can also be immobilized not only on the surface but also through all membrane porosity (Fig. 15.6F). This method had been successfully used to immobilize lipases or esterases; these enzymes behave as a surfactant and can therefore spontaneously adsorb onto many surfaces. Indeed, lipases had been immobilized inside the porous structure of tubular ceramic membranes or polymer hollow fibers [71–77]. Sometimes, enzymes can be immobilized on the surface of the grains forming a ceramic membrane in such a way as to allow the free passage of fluids through the porosity. This method had been applied to immobilize a lipase through a thin film of ionic liquid containing globules of a lipase [78] and a laccase by covalent grafting the enzyme on an extremely thin layer of gelatin [79]. The immobilization inside the membrane porosity enhances the amount of immobilized biocatalyst compared to the immobilization on the membrane surface. Moreover, it increases the possibility of reaction in between the biocatalyst and substrates by reducing the distance in between both species while having a better control of the contact time. Indeed, each pore of the membrane where the biocatalyst is immobilized can be considered as a microreactor through which the substrates are flowed. This configuration can also be assimilated to the "Flow Through Membrane Reactor" (FTMR) concept [2,4].

A wide variety of enzymatic reactions has been implemented in MRs in a multitude of different solvents, from classical organic solvents such as hexane to supercritical fluids or ILs. Obviously, the main solvent used is water, saline solutions and/or buffers where the enzymes have a structural

conformation in accordance with their reactivity. Indeed, water has been involved in most enzymatic reactions reported on the literature like hydrolysis of oligosaccharides or proteins [2,8,40]. Furthermore, in the last ten years, EMRs with laccases immobilized on polymer or ceramic membranes have been applied for water depollution and in particular, for the removal of dyes, phenolic compounds and micropollutants (residues of hormones and drugs) [69,79–85]. Even if there is still a lot of research to be done in this field, the literature of enzymatic reactions in water is very well known and extended. Consequently, in the following sections this chapter will be focused on green solvents but only the nonconventional ones, such as supercritical carbon dioxide and ILs.

15.7 Enzymatic membrane reactors and nonconventional solvents

15.7.1 Enzymatic membrane reactors and supercritical carbon dioxide

Carbon dioxide becomes SCCO2 when the temperature and pressure are higher than 304K and 7.4 10^6 Pa, respectively. SCCO2 is considered as a green solvent because unlike traditional solvents it is nontoxic, nonflammable and can be completely removed by depressurization once the reaction is finished and is easily recycled. SCCO2 presents many interesting properties like a viscosity and diffusivity close to those of gases which is an important asset for mass transfer (easier diffusion of solutes). At the same time SCCO2 has a solvent capacity close to that of liquids, these two properties result in the reduction of energy requirements and therefore costs. All these characteristics make SCCO2 a solvent of choice to replace traditional organic solvents, which are nonenvironmentally friendly for a wide range of applications such as extraction, chromatography, and chemical and biological synthesis reactions [86]. Furthermore, SCCO2 is a particularly interesting solvent for the implementation of enzymatic reactions because its low critical temperature (i.e. 304.1K) is well suited for the implementation of thermosensitive molecules. Moreover, it has been shown that SCCO2 at pressures lower than 200 kPa do not denature enzymes. It is important to notice that water as most polar compounds is not soluble in SCCO2, but enzymes need to keep their hydration spheres or an aqueous environment in their vicinity to maintain a structural conformation in agreement with the reactivity. Indeed, the presence of traces of water in SCCO2 promotes good stability of these hydration spheres and improve the reaction. It is important to notice that the solubilization of CO_2 in water leads to an acidification of the medium, which can compromise the enzymes stability if the pH in their vicinity becomes too low; in such case the addition of solutes such as sodium bicarbonate, glycerol, trehalose or even ILs allows to keep a good activity [87,88].

Despite all the factors described above, many lipases are stable and reactive under supercritical conditions. The potential of SCCO2 for the enzymatic synthesis of aromatic esters as well as optically active compounds has been extensively studied. All these reactions have been carried out with immobilized lipases (Novozym 435, Lipozyme TL IM, etc.) [86–89]. Most of the works concern studies conducted in stirred tank reactors. However, Couto et al. [90] have demonstrated that the use of a packed-bed reactor instead of a stirred tank reactor allowed a better control of the water activity of the reaction medium. For example, in esterification reactions the water produced is continuously removed from the reactor by the $SCCO_2$ limiting the risk of hydrolysis. Moreover, enzymatic conversions carried out in packed-bed reactors resulted in an increase of the enzyme stability and the conversion rate at steady state [90,91]. Many different esterification reactions and biodiesel synthesis have been achieved in packed bed reactors with the same types of immobilized lipases [90–93].

The studies carried out in EMRs are not very numerous and had been carried out mainly with lipase B from *Candida antartica* (Novozym 525L) covalently immobilized on a ceramic membrane according to the method described in Section 15.6 [59,94]. The first works carried out had for objective the validation of the efficiency of the immobilization method for a use in SCCO2 in a EMR operating in a fed-batch configuration for the synthesis of butyl butyrate from vinyl butyrate and 1-butanol (Fig. 15.7A) [95]. The reactor was fed continuously but without any output. At regular time intervals,

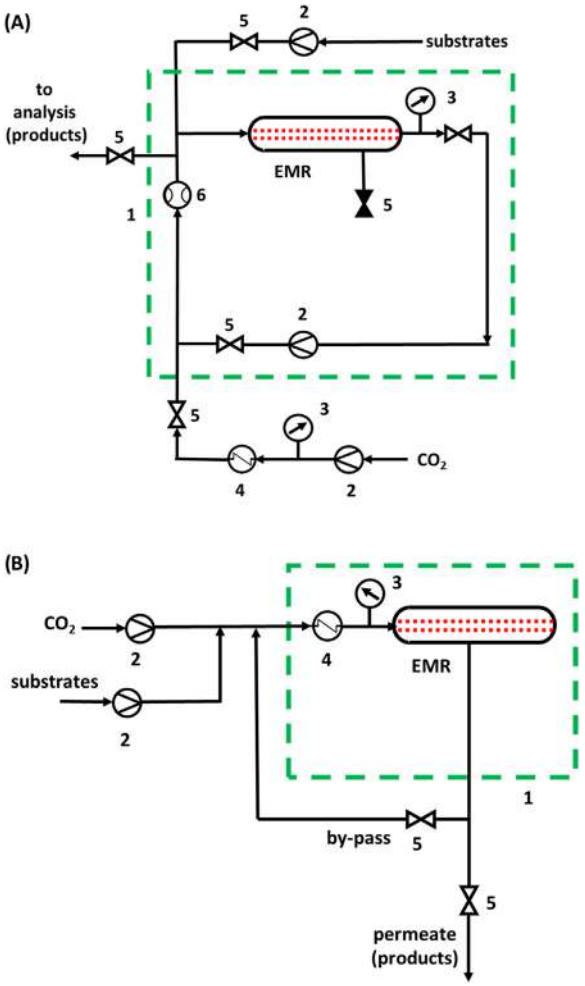

FIGURE 15.7

EMR in two different pilot configurations, (A) fed-batch, (B) continuous. 1: Temperature-controlled enclosure, 2: High-pressure pump, 3: Pressure transducer, 4: Heat exchanger, 5: Valves, 6: Flow controller. CO_2 was heated and pressurized with high-pressure pumps and heat exchangers to reach supercritical conditions at the EMR input. *EMRs*, Enzymatic membrane reactors.

the system was depressurized, the reaction medium was taken out for analysis from the recirculation loop, and then the whole system was re-pressurized and re-fed with the substrates. The results obtained showed a significant improvement on the performance of the enzymatic membrane when the synthesis of butyl butyrate was carried out in SCCO2 rather than in conventional organic solvents. Depending on the density of the SCCO2 (i.e. 0.31 to 0.2 kg/m^3), the catalytic activity of the membrane varied from 483 to 923 U/cm^2 while this parameter was respectively equal to 11, 26 and 28 U/cm^2 with acetonitrile, acetone and hexane. Concerning the selectivity, it was always >99% in SCCO2 while in conventional solvents this parameter was ranged between 90% and 94%. Furthermore, the most notable result was the excellent stability of the enzyme after different cycles of pressurization/depressurization. These authors showed that under optimal operating conditions (333K, 8 MPa), the residual activity of the membrane remained close to 100% after 7 cycles of continuous use of 6 h/day, which corresponds to 360 cycles half-life time.

The synthesis of butyl laurate from butyl acetate and lauric acid in SCCO2 was performed with similar enzymatic membranes [96]. The experiments were carried out in the pilot unit shown in Fig. 15.7B. It was designed to provide several modes of operation: batch, fed-batch and continuous. Experiments in batch configuration, were started by the continuous filtration of the feed mixture (2 mL/min) keeping the permeate valve opened. After one hour, the feed was stopped and the permeate valve was closed for 2 h. Finally, the totality of the reaction mixture filling the pilot was taken out for analysis. This configuration allowed to establish the optimal operating conditions (333K and 8 MPa) and to confirm the good stability of the enzymatic membrane after several cycles of pressurization/depressurization. Nevertheless, the average productivities reached with this mode of operation are low (2 to 11 μmol/min/m) and according to the authors, probably limited by lack of substrate. In fed-batch operation, feed is kept constant throughout the experiment but permeate was continuously recycled, the permeate outlet valve being opened only occasionally to take samples for analysis. However, despite a continuous supply of substrate, this configuration did not overcome substrate limitations and the productivity remained in the same range as obtained in batch configuration. Finally, in continuous mode of operation SCCO2 and substrates are feed continuously and permeate is constantly recovered. The best productivity obtained was 61 $\mu mol/min/m^2$ after 6 h of operation (substrate flow rate of 7.5 mL/min at 323K and 10 MPa). This result validates the superiority of the continuous operation over the batch or fed-batch configuration.

The potentiality and the interest of this EMR configuration were highlighted on a larger scale by the same research group through the synthesis of anisyl acetate from anisic alcohol and vinyl acetate [97]. For this purpose, industrial scale ceramic and multichannel membranes (total surface area of 0.5 m^2) were used in a pilot unit able to operate in continuous mode (dead end filtration) or with recycle (Fig. 15.8). Experiments were carried out in continuous operation with 2 types of enzymatic membranes, one being obtained by covalent immobilization [94], the other by simple adsorption of lipase B from *C. antartica* (Novozym 525 L) [76,97].

The temperature and the working pressure were respectively fixed at 318K and 10 MPa. A period in between 3 and 4 h was necessary to reach the steady state and an average productivity of 19 $mmol/h/m^2$ corresponding to a conversion rate of 40% was obtained when the lipase was covalently immobilized. Nevertheless, although the conversion obtained with the enzymatic membrane prepared by simple adsorption is slightly lower (only 35%), this immobilization method seems preferable to implement on an industrial scale due to the simplicity of its preparation protocol (Fig. 15.9). However, it should be noted that the impact of the immobilization protocol depends on

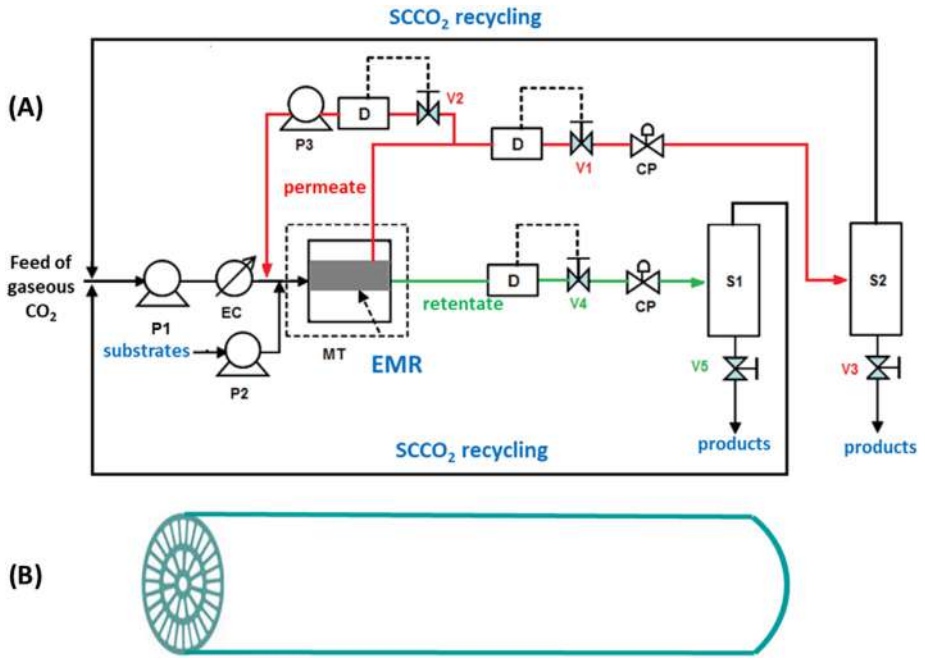

FIGURE 15.8

(A) Pilot unit and multichannel membrane utilized for the synthesis of anisyl acetate from anisic alcohol and vinyl acetate at large scale, *CP*: pressure controller, *EC*, heat exchanger, *D*, flowmeter, *MT*, temperature-controlled enclosure, *S*, valves, *S*, cyclones for gas separation. (B) Draw of ceramic multichannel membrane (39 channels, 1.2-m length, 2.5×10^{-2} m of external diameter, 0.5 m^2) [97].

Adapted from S. Ben Ameur Villain, Development of a Multichannel Monolith Enzymatic Membrane for Aroma Compounds Synthesis in Supercritical Carbon Dioxide (PhD dissertation (in French)), Ecole Nationale Supérieure de Chimie de Montpellier, Montpellier, France, 2012. https://theses.enscm.fr/ENSCM_2012_BEN-AMEUR.pdf.

the nature of the reaction medium. In the case of butyl acetate hydrolysis carried out in aqueous medium, the membrane prepared by covalent bonding showed a specific activity 35% higher than the one obtained with the membrane obtained by simple adsorption (7.2 ± 0.4 mmol/min/m^2 versus 4.6 ± 0.4 mmol/min/m^2).

Other research teams have also been interested in the potential of coupling EMRs with SCCO2. The work reported by Habulin et al. [98] concerns two different EMRs operating in continuous mode, one with flat sheet membrane and a second one with a tubular one. The flat EMR was applied to the hydrolysis of sunflower oil in SCCO2, catalyzed by free lipases from *Candida cylindracea* and *Aspergillus niger*. In this study the role of the membrane was to retain the biocatalysts in the reaction medium. The hydrolysis of carboxy-methyl cellulose was studied in the EMR with cylindrical geometry. For this purpose, a cellulase from *Humicola insolens* was immobilized by covalent bonding according to the method described by Lozano et al. [94]. For the two EMRs operating with SCCO2, it was shown that the membranes were stable and that the enzymes were active.

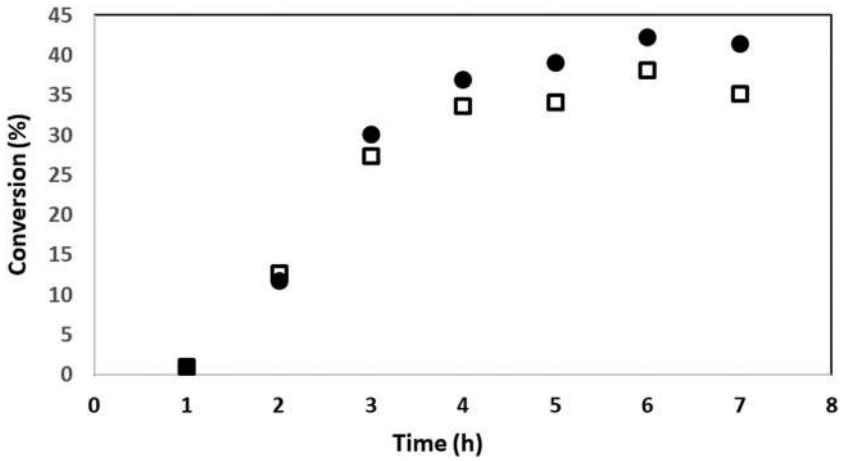

FIGURE 15.9

Conversion achieved for the synthesis of anisyl acetate with a EMR in SCCO2 with 39 channels membrane and lipase immobilized by covalent bonding (closed cercles), adsorption (open squares). Substrate flow rate = 19 g/h, CO_2 flow rate = 1.8 kg/h, T = 318K, P = 10 MPa. *EMRs*, Enzymatic membrane reactors.

Adapted from S. Ben Ameur Villain, Development of a Multichannel Monolith Enzymatic Membrane for Aroma Compounds Synthesis in Supercritical Carbon Dioxide (PhD dissertation (in French)), Ecole Nationale Supérieure de Chimie de Montpellier, Montpellier, France, 2012. https://theses.enscm.fr/ENSCM_2012_BEN-AMEUR.pdf.

Furthermore, the performance of the reactors in supercritical media was shown to be superior to that obtained with conventional solvents.

EMRs with immobilized lipases have also been applied to the interesterification reaction between castor oil triglycerides, which are relatively viscous, and methyl oleate. Here the SCCO2 by its fluidizing properties allows to enhance the transfer through the enzymatic membrane. An interesting and stable conversion of 30% was reached in the permeate during 20 h. In this work was also noticed that the conversion decreased with the simultaneous enhancement of the transmembrane pressure and flow. In fact, increasing the flow resulted on a decrease of the residence time and then part of the substrates was removed from the EMR before being transformed [99,100]. Nevertheless, the consumption of methyl oleate increased with flow rate enhancement, so it seems that the system was in limitation of substrate. In conclusion, the transmembrane pressure was the key parameter of the process, when it was low the conversion was maximized, which favored quality, whereas when it was high, production was maximized at the expense of quality [100]. Lozano et al. [101] have reported the racemic resolution of ketoprofen by esterification with 1-butanol in an EMR combining both sustainable nonconventional solvents: SCCO2 and ILs. The EMR was composed of two compartments separated by a membrane. The reaction took place in a reaction tank containing ILs and an immobilized lipase (Novozym 435). The reaction mixture was then recirculated between the tank and the upstream compartment of the EMR, whereas the downstream compartment was swept with SCCO2 to extract continuously the R-ketoprofen butyl ester with an enantiomeric excess varying from 100% to 60% in 24 h.

15.7.2 Enzymatic membrane reactors and ionic liquids

ILs are salts, which are in the liquid state at room temperature. Indeed, an IL is any ionic salt that whose melting point is below the arbitrary temperature of 373K. ILs have many interesting characteristics like high stability, low volatility and high conductivity. Moreover, the range of temperatures in which they are found in the liquid state is relatively large. ILs can be formed from many different cations and anions allowing a very large variety of chemical structures (for example cations derived from 1-methylimidazole, pyridine, alkylammoniums, phosphoniums, etc., and anions like tetrafluoroborate, hexafluorophosphate, bis-trifluoromethanesulfonimide, etc.). Given the very large variety of existing anions and cations, from a theoretical point of view it is possible to design more 10^{18} ILs, however, at present are known just near 2000 different ILs. This feature allows a vast modulation and variability of the above-mentioned characteristics according to the selected cation and anion. Indeed, ILs with different properties can be chosen according to the desired application (alkalinity or acidity, aprotic or protic, hydrophobicity or hydrophilicity, solvent power, achiral or chiral, etc.). The applications of ILs as solvent are very diverse; they include catalytic and enzymatic reactions, biopolymer processing, carbon dioxide capture, etc. [102,103].

Since the beginning of the year 2000, the research on the implementation of enzymatic reactions in ILs has been widely reported. Indeed, the research in this field has been the subject of more than 31,500 publications in the last 10 years [104]. It is well known that some polar organic solvents denature certain enzymes such as lipases, which does not seem to be the case with ILs. Indeed, during enzymatic reactions with lipases in ILs, the enzymes do not solubilize; thus, their three-dimensional structure as well as "the essential hydration shell" are preserved even under low water activity conditions. In these adapted ILs, the enzymes can then present exceptional levels of activity and stability and even for some lipases or proteases their enantioselectivity is improved [105,106]. Moreover, in some cases, the ILs make possible many enzymatic reactions thanks to the dissolution of organic substrates (i.e. nonpolar substrates) which are not soluble in water or conventional polar organic solvents [107]. Thanks to their modular solubility and miscibility, ILs are good candidates for the generation of multiphase (bio)catalytic systems: the biocatalyst is immobilized in an IL phase with eventual substrates and the products are extracted in another immiscible phase. Moreover, in the case of esterification reactions, as the ILs are nonvolatile, the application of vacuum to the reactor allows to carry away the water and the volatile compounds; the reaction equilibrium is then shifted in favor of the production of compounds of interest. Moreover, the nonvolatility of ILs permits their full recovery for further reuse [106].

While the ability to recover and recycle ILs is critical to the design of a sustainable process, the ability to limit the volumes of ILs needed is also critical to reduce process costs. In this perspective, different types of systems coupling a membrane process with ILs have been developed; they include membranes where ILs are immobilized, membrane contactors using ILs, composite ILs-polymer membranes, composite ILs-mixed matrix membranes, polymerized-ILs membranes and ionic gel-ILs membranes. In some cases, it has allowed to reduce the volumes and make the process more sustainable [108–110].

The coupling of EMRs with ILs has been studied by few research groups, and most works are concerned with esterification reactions in EMRs. In fact, ILs are excellent solvents and replace advantageously conventional organic solvents for the implementation of esterification reactions. However, the conversion of these reactions remains limited because of the accumulation of water

in the medium [111]. This problem has been solved by dehydrating the IL by coupling the esterification reactor with a pervaporation unit [112]. Gubicza et al. [113] studied the enantioselective esterification of (R,S) − 2-chloropropanoic acid with butan-1-ol using *C. rugosa* lipase in free form in different ILs and organic solvents. They showed that the 1-butyl-3-methylimidazolium hexafluorophosphate ([bmim]PF6) allowed to obtain production yields like those obtained in hexane (about 50%). In addition, they noted that the lipase was more stable in the IL than in Hexane; the enzyme kept 100% of its activity after 5 cycles of use against only 60% of residual activity in hexane. By coupling of the reactor with a PV unit equipped with a hydrophilic membrane the amount of water was maintained at about 1% in the reaction medium; this percentage allowed preserving the activity of the enzyme at a maximum level promoting the esterification reaction. They also showed that due to the nonvolatility of IL, it was possible to work at relatively high temperatures (333K) to maximize the conversion yields but also to facilitate the transfer of water in the PV unit. The same research group showed also that it was possible to couple the enzymatic reactor with two PV units equipped with hydrophilic and hydrophobic membranes respectively, allowing the simultaneous extraction of water with the hydrophilic membrane and the ester with the hydrophobic one, thus facilitating the separation of the product of interest [114]. In a later study on the enzymatic synthesis of isoamyl acetate, they also considered the use of biphasic medium (alcohol/IL) in view to improve the product separation with a simultaneous reduction of the quantities of ILs required to reduce the process cost [115]. Enzymes suspended in a limited volume of IL were put into contact by stirring with a light phase containing the substrates (acid + alcohol + water). During the reaction, the light phase was continuously dried by percolation over a zeolite bed (thus allowing enhancing the reaction yield without risk of denaturation of the enzymes, which were not in contact with the zeolites). At the end of the reaction, the light phase was sent to a membrane contactor and then to a PV unit placed in series, which allowed removing water, and separate the unreacted isoamyl alcohol which was recycled. The heavy phase IL + enzyme could then be reused (Fig. 15.10).

The coupling of ILs and porous membranes in EMRs has been also applied with a view to limiting IL volumes. This objective can be achieved by covering porous enzymatic membranes with a thin layer of IL. In such case IL is not plugging the pores but covering the grains of the ceramic material keeping the porosity opened, a schematic of the surface of the grains of the ceramic membrane covered by IL and enzymes is shown in Fig. 15.11. This configuration corresponds to the membrane contactor shown in Section 15.6 (Fig. 15.6F). As explained previously in this chapter, this configuration is very interesting because it enhances the possibility of reaction between the biocatalyst and the substrates by reducing the distance between both species while having a better control of the contact time. Indeed, the enzymatic membrane can be considered as an assembly of microreactors.

Such principle of flow through enzymatic membrane reactor has been applied by Mori et al. [78], who have immobilized a lipase B from *Candida antarctica* inside the pores of a ceramic membrane with a mean pore size of 0.2 μm. For this purpose, three ILs were synthetized (1-Ethyl-3-methylimidazolium triflimide ([emim][Tf2N]), 1-Butyl-3-methylimidazolium hexafluorphosphate ([bmim][PF6]), 1-Butyl-3-methylimidazolium triflimide ([bmim][Tf2N])) and then mixed under agitation with CH_2Cl_2 and an enzyme solution in a phosphate buffer. Afterward, this suspension (5 mL) was permeated through the membrane porosity until the complete adsorption of the liquid. After drying the enzymatic membranes were used for the synthesis of butyl laurate by the acidolysis between butyl acetate and lauric acid in a hexane/water mixture (98:2, v/v). The reaction occurred during the transfer through the membrane. The results showed that membranes were

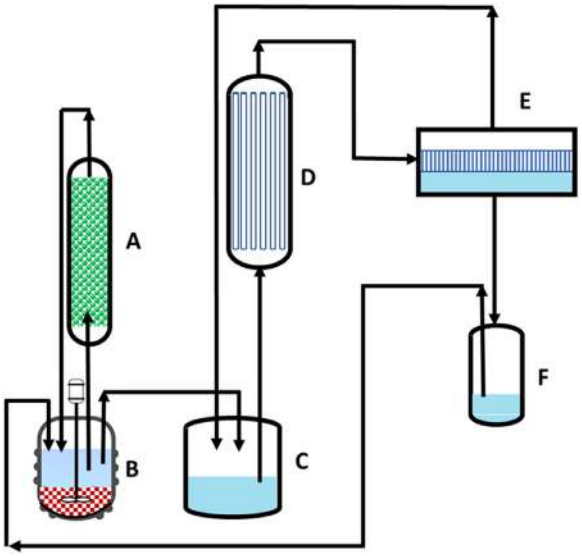

FIGURE 15.10

Simplified draw of the pilot unit for the enzymatic production of isoamyl acetate and separation of the reaction mixture (A, adsorption by packed bed of zeolite; B, bioreactor; C, tank for upper phase; D, membrane contactor, E, PV unit, F, trap for isoamyl alcohol).

Adapted from E. Fehér, B. Major, K. Bélafi-Bakó, L. Gubicza, Semi-continuous enzymatic production and membrane assisted separation of isoamyl acetate in alcohol -ionic liquid biphasic system, Desalination 241 (2009) 8–13. https://doi.org/10.1016/j.desal.2007.11.080.

active and maintained their activity after several runs and for four months. Other authors have reported the positive effect of ILs on the selectivity of enzymatic reactions. Hernandez et al. [116] have demonstrated the positive impact of the presence of IL in the vicinity of the lipase for the synthesis of butyl propionate from vinyl propionate in SCCO2 in a recycling EMR. In this case, the enzymes were previously immobilized by covalent bonding via a composite layer of gelatin deposited on a ceramic membrane as reported by Lozano et al. [94]. Then, enzymatic membranes were impregnated with three different ILs based on dialkylimidazolium. The results showed that with the biphasic systems (IL/SCCO2) the process selectivity increased up to $>99.5\%$ compared to using SCCO2 alone but enzymatic activity (U/cm^2) was lower. Mori et al. [78] also observed this activity loss, in both cases this decrease of enzymatic activity may be due to mass transfer limitations through the IL layer around the biocatalyst.

As previously described, other types of supported IL membranes including membrane contactors have also been used for bioconversion. These membranes are prepared by the trapping ILs inside the porosity of a porous membrane. As the IL plugs the separative layer porosity, this layer must be as thin as possible to keep interesting mass transfer properties. Miyako et al. [117] reported the preparation of supported liquid membrane by immersion of a macroporous polypropylene membrane in [bmim][TF2N] which is insoluble in aqueous solutions. This membrane was used for the

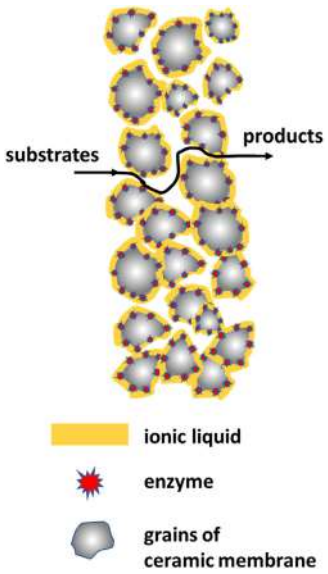

FIGURE 15.11

Schematic of EMR with IL covering the grains of the ceramic support. *EMRs*, Enzymatic membrane reactors.

separation of the (S)-enantiomer from a racemic mixture of ibuprofen. The membrane was placed between the feed containing the racemic ibuprofen in a water/ethanol solution and an aqueous receptor phase. Each one of the compartments also contained a specific lipase. In the feed compartment, a lipase from *C. rugosa* catalyzed the esterification of ibuprofen to ethyl-ibuprofen, which was preferentially absorbed in the IL and diffused through to the downstream compartment where a lipase from porcine pancreas catalyzed the reverse reaction releasing the S enantiomer. Thanks to an appropriate choice of the IL and the lipases used, it was possible to optimize the flow of the enantiomer (S) at the expense of the enantiomer (R) to obtain in the receptor phase an enantiomeric excess of 75% from a 50/50 mixture (Fig. 15.12).

Hernández-Fernández et al. [118] have also carried out a racemic resolution in the case of 1-phenylethanol by transesterification with vinyl esters catalyzed by an immobilized *Candida antarctica* lipase B in a EMR. For this purpose, they filled the pores of a hydrophilic Nylon-polyamide-membrane (0.45 μm of mean pore size) with ILs and placed the membrane in a diffusion cell separating two compartments filled with hexane. In the feed compartment they introduced the immobilized enzyme and the substrates whereas in the downstream compartment only the fresh solvent. There are two main differences when compared with the case studied by Miyako et al. [117], first, the ester was not hydrolyzed due to the absence of enzyme in the downstream compartment and secondly it was less sustainable process because it involved an organic solvent. These authors highlighted the impact of the nature of the IL and concluded that among the six ILs studied the [bmim][BF4] presented the best transport properties. They also showed that the efficiency of the reactor was improved with the rise of the enzyme concentration and the increase of the alkyl chain length of the vinyl ester [118,119].

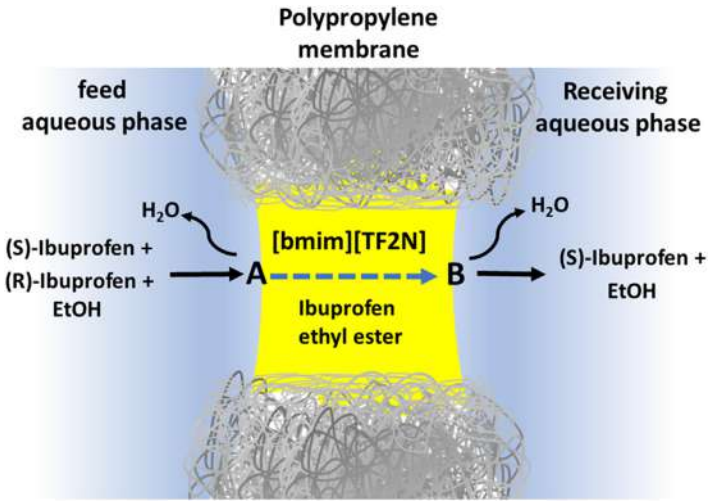

FIGURE 15.12

Schematic of enantioselective transport of (S)-ibuprofen through a [bmim][TF2N] (*yellow*) in an enzymatic membrane contactor. **A** Lipase from *Candida rugosa*, **B** porcine pancreas lipase.

Adapted from E. Miyako, T. Maruyama, N. Kamiya, M. Goto, Enzyme-facilitated enantioselective transport of (S)-ibuprofen through a supported liquid membrane based on ionic liquids, Chem. Commun. (2003) 2926–2927. https://doi.org/10.1039/B310990A.

Some works have been based on the good solubility of CO_2 in some ILs, this characteristic has been applied to enhance the gaseous separation or the enzymatic conversion of CO_2. Zhang et al. [120] carried out the CO_2 conversion into methanol with an enzymatic membrane prepared by immobilizing by adsorption three enzymes in the porosity of a polymer membrane: formate dehydrogenase (from *Candida boidinii*), formaldehyde dehydrogenase (from *Pseudomonas* sp.), and alcohol dehydrogenase (from *Saccharomyces cerevisiae*). The upstream compartment of the EMR was filled with a mixture of an IL (choline-amino-acids) and NaDH in a buffer under a CO_2 atmosphere, while a buffer was present in the downstream compartment. The cascade enzymatic reaction was possible by adding NaDH which was stoichiometrically consumed to produce one mole of methanol and NaD^+. The best methanol yields (70%) were reached when cholinium glutamate was used as IL.

Bednár et al. [121] studied the facilitated transport of CO_2 through an enzymatic membrane prepared by filling the porosity of a hydrophobic Durapore PVDF MF membrane with a suspension of [bmim][Tf2N], water and a CA (prepared and purified from spinach). The role of the CA was to catalyze CO_2 hydrating into HCO_3^- [122]. This transformation allows maintaining a high gradient of CO_2 at the interface, which is favorable to the absorption and mass transfer [123]. The membrane developed by Bednár et al. [121] was inserted in a gas permeation module and a differential gas pressure was applied between the upstream and downstream compartments. The pressure increase in the downstream compartment allowed calculating the gas permeation. These authors reported that the CO_2 permeability obtained with the IL alone was two times lower than when it contained the CA.

More recently and in response to the need for new sustainable technologies for greenhouse gas reduction, a new application of ionic liquid-based membrane contactor has emerged. To facilitate the absorption of CO_2 gas by a liquid phase, Kim et al. [124] developed a system with a composite polypropylene-PVDF membrane, which separates a N_2 + CO_2 gas mixture from liquid absorbents: water and monoethanolamine or N-methyldiethanolamine (MDEA). To boost the transfer and absorption, they had the idea to couple the contactor with an enzymatic reaction. They added CA (Novozymes AS) in the liquid phase (1%) to maintain the driving force through the transformation of CO_2 into HCO_3^- and for further improvement of the transfer; they added blends of poly-ionic-liquids and zinc salts tailored for CO_2 transport inside the membrane porosity. It is important to notice that the mixture of water, amines and CA was unable to enhance the CO_2 transport. In fact, to improve the gas transport it was necessary to have the synergistic effect of the membrane impregnated with poly ILs and the liquid mixture described above. The most interesting results were reached by using a commercial blend of a specific poly-ionic liquid and zinc salts combined with CA (1 wt.%) in 30 wt.% MDEA (in water). In such conditions enzymes enhanced the absorption rate by a factor of >2 in aqueous MDEA. Malankowska et al. [125] have developed a microfluidic device for CO_2 separation from anesthesia gas, containing Xe. The microdevice contained a dense, permeable polydimethylsiloxane (PDMS) membrane separating the gas stream from a mixture of water, cholinium propionate (*CP*) as IL and CA. Preliminary experiments were conducted to measure the permeability to CO_2 and Xe in the microfluidic device and compare them to those obtained with free PDMS membranes mounted on a standard permeation cell.

The rest of the experiments were carried out by flowing a fresh mixture solution continuously in downstream compartment. The upstream compartment containing the gas mixture was closed and the pressure decay was continuously recorded. The results showed that the *CP* with or without the CA has no effect on the Xe transport, but remarkably enhances the CO_2 transport by a factor up to 1.9 in the presence of 0.1 mg CA/gIL.

15.8 Conclusions

EMRs with nonconventional solvents are intensified and sustainable processes that can potentially be applied to a wide range of reactions from fine chemical synthesis to carbon dioxide separation. Indeed, laboratory-scale studies have been carried out for the application of EMRs to improve the conversion of reactions that are limited by thermodynamic equilibrium through the selective removal of products such as in esterification reactions. The interest of EMRs in enzymatic transformations for the production of value-added chemicals such as aromatic compounds or optical resolution of pharmaceuticals has already been proven. In the case of ILs, these processes allow the use of polymer membranes and hollow-fiber configuration having a very high surface-to-volume ratio leading to an excellent intensification factor. In addition, these membranes are commercial and readily available materials and do not have stringent requirements in terms of membrane housing and sealing. However, for SCCO2, ceramic membranes are certainly the best option so far. Further progress is expected for large-scale applications in terms of the cost of ILs and inorganic membranes; this consideration probably remains the main factor delaying progress.

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CHAPTER

Applied biocatalysis in deep eutectic solvents

16

Caroline Emilie Paul[1] **and Vicente Gotor-Fernández**[2]

[1]*Department of Biotechnology, Delft University of Technology, Delft, The Netherlands* [2]*Organic and Inorganic Chemistry Department, University of Oviedo, Oviedo, Spain*

16.1 Introduction

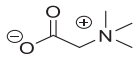
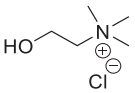
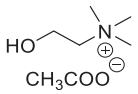
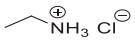
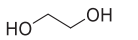
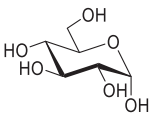
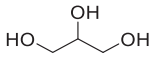
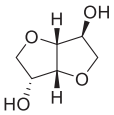
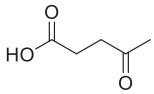
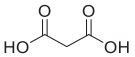
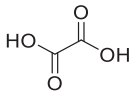
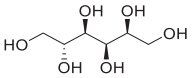
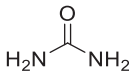
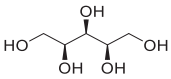
Deep eutectic solvents (DES) have emerged over the last two decades as nonconventional solvents. For sustainability, natural DES (NADES) have been designed and are considered to mimic the aqueous environment for an enzyme [1,2]. DES are attractive for their inexpensive and simple preparation (mixing and stirring), tunable properties, and inherent biodegradability. DES are composed of a mixture of quaternary ammonium salts acting as a hydrogen bond acceptor (HBA, Table 16.1), and a counter compound, such as a sugar, performing as hydrogen bond donor (HBD). The properties of a DES depends on the intermolecular interactions between its HBA and HBD components, with the nature of its hydrogen-bonding lowering its overall melting point, leading to a liquid eutectic solvent mixture without further processing or purification needed [3]. With this hydrogen-bonding network, varying the molar ratio of the HBA and HBD enables tuning of the freezing point, such that the solid salt choline chloride (ChCl, Table 16.1) mixed with urea (U) with a molar ratio of 1:2 ChCl:U leads to a freezing point of 12°C. On the other hand, a molar ratio of 1:2 for ChCl:glycerol (Gly) gives a freezing point of −40°C.

DES can reproduce in vivo cell environments [4], such that a protein structure may be better preserved than in organic solvents. DES have thus been increasingly applied in biocatalysis [5−7].

This chapter essentially focuses on the use of DES in relevant biocatalytic reactions, with an emphasis on performance measured by reaction conversions or yields that have clear synthetic applications. The reader is directed to further extensive literature that already addresses other aspects such as changes in activity and stability [8−13]. We begin our overview by showcasing interesting single reactions catalyzed by hydrolases in DES and DES-aqueous medium mixtures, in particular, lipases, which have been fully explored due to their easy handling and lack of cofactor requirements. Next, the use of oxidoreductases, lyases and transferases will be discussed offering a wide number of possibilities for the production of organic compounds in a selective and sustainable manner. Finally, the development of multicatalytic transformations will be covered by combining enzymes with organocatalysts or metal species, which allow the design of multistep chemoenzymatic cascades.

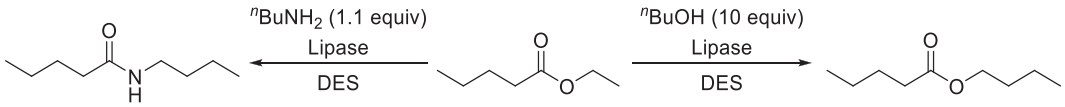
Biocatalysis in Green Solvents. DOI: https://doi.org/10.1016/B978-0-323-91306-5.00011-X

Table 16.1 Selected list of hydrogen bond acceptor (HBAs) and hydrogen bond donor (HBDs) leading to deep eutectic solvents described in this chapter, including their abbreviation, name and structure.

HBAs					
Be	Betaine		ChCl	Choline chloride	
ChAc	Choline acetate		EACl	Ethylammonium chloride	
HBDs					
EG	Ethylene glycol		Glc	Glucose	
Gly	Glycerol		Iso	Isosorbide	
LA	Levulinic acid		MA	Malonic acid	
Ox	Oxalic acid		So	Sorbitol	
U	Urea		Xyl	Xylitol	

16.2 Hydrolases

Their simplicity of use and lack of cofactor requirements for their correct action has motivated the great interest for the development of hydrolase-catalyzed reactions in organic synthesis, including a wide variety of enzymes such as lipases, esterases, amidases, nitrilases, proteases and epoxide hydrolases (EHs), among others. While most of these biocatalysts have been employed for hydrolytic processes, lipases have attracted especial attention due to their ability to react with very different nucleophiles (alcohols, amines, ammonia, hydrazines, thiols or hydrogen peroxide) to favor synthetic reactions over the competitive hydrolytic processes. Without any doubt, the advances on enzyme immobilization techniques have paved the way for the production of multiple organic compounds including for instances esters, amides, hydrazides, thioesters and peracids. Since the pioneer work developed by Kazlauskas and coworkers in 2008 [14], the use of DES has grown in an exponential manner [15,16]. This research group reported for the first time the use of lipases for the transesterification between ethyl valerate and 1-butanol using DES as solvents (Scheme 16.1),



SCHEME 16.1

Lipase-catalyzed transesterification and aminolysis of ethyl valerate with *n*-butanol and *n*-butylamine, respectively, in DES.

finding, in some cases and depending on the DES components, similar or superior conversion values with *Candida antarctica* lipase B (CAL-B), *C. antarctica* lipase A and *Pseudomonas cepacia* lipase, in comparison with the reactions carried out in a hydrophobic solvent such as toluene, traditionally used in lipase-catalyzed reactions. Remarkably, competitive reactions were observed in the transesterification reaction when the DES contained alcohol components, while the aminolysis side reaction with ethylammonium chloride (EACl) was not found in any extension. Additionally, the benefits of using DES as additives in lipase- and EH-catalyzed reactions were demonstrated when using *p*-nitrophenyl acetate and styrene oxide as substrates, respectively, achievements that have been widely expanded in recent years for synthetic goals as described in a recent review by Erol and Hollmann [17].

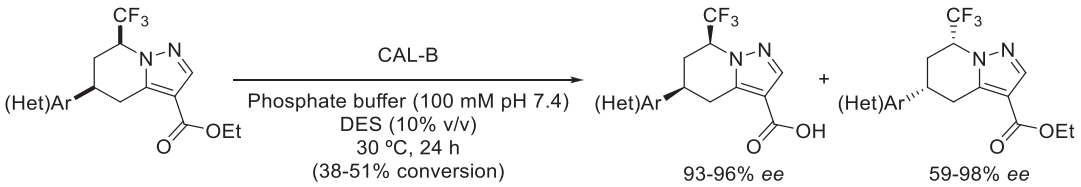
From this starting point, the use of DES as reaction media in biotransformations have exponentially grown, presenting these neoteric solvents as environmentally friendly solvents for multiple applications. Next, the use of hydrolases in DES will be described, dividing their applications depending on the reaction types, which means starting from classical hydrolytic reactions to later move toward synthetic application using a series of nucleophiles.

16.2.1 Hydrolases and deep eutectic solvents in hydrolytic reactions

Hydrolysis is the natural reaction of the hydrolase family, and the ability of multiple enzymes were demonstrated in conventional aqueous media, but also when using organic solvents as solvents and water as reactive nucleophile. In this case, the use of DES as cosolvents helps the solubility of organic compounds and are compatible with the use of a wide family of hydrolases including cellulases, EHs, glucosidases or lipases. Next, practical examples will be discussed where conversion and/or yields have been reported.

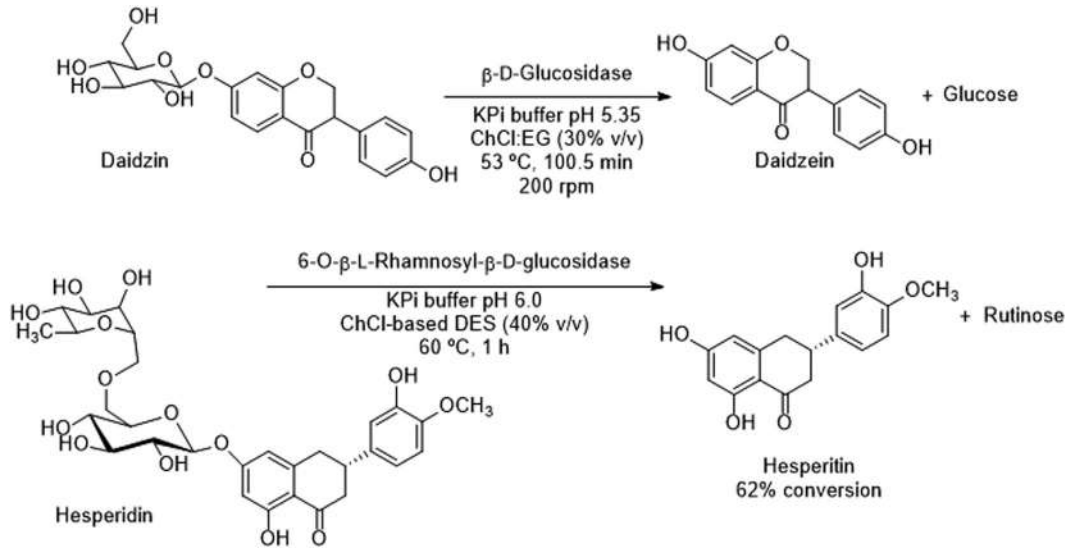
The stereoselective hydrolysis of racemic ethyl *cis* − 5-substituted-7-(trifluoromethyl) − 4,5,6,7-tetrahydropyrazolo[1,5-a]pyridine-3-carboxylates was developed using ChCl-based DES (Gly, U and Xyl, 10% vol) as additives (Scheme 16.2) [18]. Despite the DES did not fully dissolve the substrates, in contrast with the perfect solubility achieved when employing dimethyl sulfoxide, the kinetic resolution of three substrates was conducted with excellent enantioselectivities using CAL-B (38%−51% conversion, $E > 82$).

The flavonoid chemistry is usually challenging due to the low solubility of this type of substrates in traditional organic solvents, so the use of DES might be a useful solution to solve this problem. Cheng and Zhang studied the hydrolysis of daidzin to prepare daidzein using the almond β-D-glucosidase (Scheme 16.3 *top*), and after optimization of different reaction parameters such as DES components, reaction time, temperature and solvent system, the best conversion was found



SCHEME 16.2

Kinetic resolution of racemic tetrahydropyrazolo[1,5-α]pyrimidine derivatives in buffer using DES as cosolvents.



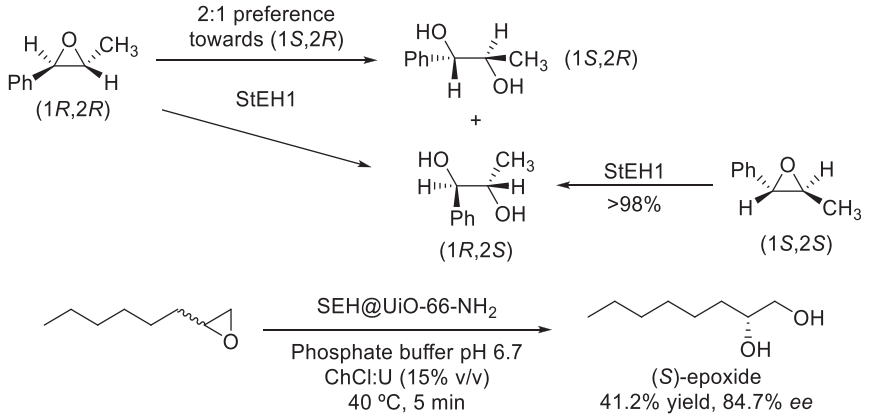
SCHEME 16.3

Enzymatic-catalyzed hydrolysis of flavonoid substrates in DES/buffer media.

using the ChCl:EG (1:2 mol/mol) in a 30% v/v, yielding a 97.5% of daizden with good purity [19]. Interestingly, the enzyme was recycled and reused six times with a final conversion above 50%.

Following with flavonoid chemistry, the enzymatic hydrolysis of hesperidin to produce hesperitin was achieved using 6-*O*-α-L-rhamnosyl-β-D-glucosidase as biocatalysts (Scheme 16.3 *bottom*) [20]. The use of DES helped in the solubilization of hesperidin in up to 90 mM, employing ChCl-based solvents containing U, Gly and EG as hydrogen-bond donors in different ratios, observing a complete deactivation of the enzyme when the DES content was over 80% v/v. Satisfyingly, the conversion reached a 62% after 1 h at 60°C using the ChCl:Gly-buffer (40:60 v/v) system, which was a better result than the reaction in pure aqueous medium (26%).

EHs allow the production of vicinal diols and epoxides with excellent regio- and stereoselective levels in aqueous medium, with the use of DES improving enzymatic activities. In 2010, Widersten



SCHEME 16.4

Epoxide hydrolase-catalyzed hydrolysis reactions using buffer/DES systems.

and coworkers described the combination of buffer and DES for the hydrolysis of 1,2-*trans* − 2-methylstyrene oxide enantiomers as substrates using potato EH from *Solanum tuberosum* (StEH1) as biocatalyst (Scheme 16.4) [21]. Interestingly, the use of certain DES as cosolvents such as ChCl:EG, ChCl:Gly and ChCl:U (1:2 mol/mol) improved the reaction kinetics and the regioselectivity of the process toward the epoxide ring opening at the benzylic carbon. In addition, it was calculated that all DES dissolved around 1.5 times more substrate than when using the pure buffer, achieving complete conversion after only 4 h for the hydrolysis of the (1*S*,2*S*)-epoxide. Lou and coworkers have also reported the applicability of using a buffer/DES mixture containing of 15% v/v ChCl:U, achieving the stereoselective hydrolysis of 1,2-epoxyoctane to (*R*) − 1,2-octanediol (81% *ee*) in 41% yield through immobilization of the soybean EH [22].

16.2.2 Hydrolase-catalyzed nonhydrolytic conventional reactions

The use of lipases for synthetic applications has allowed the production of several families of compounds with high chemo-, regio- and stereoselectivity. Among the different biotransformations catalyzed by lipases, probably esterification, aminolysis and transesterifications processes have gained the major attention, the use of DES appearing as an alternative to traditional conventional organic solvents.

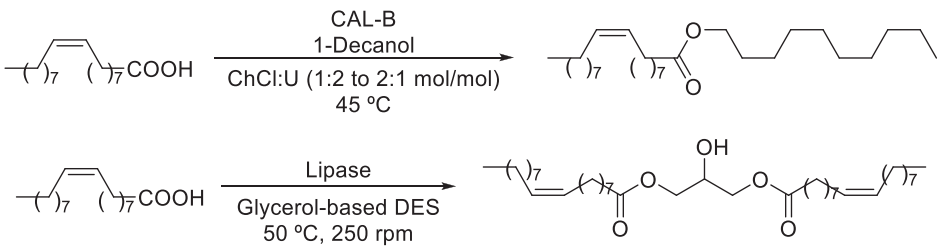
16.2.2.1 Esterification reactions

The reaction between carboxylic acids and alcohols represents the more straightforward approach to produce esters as main products, forming water as by-product. Generally, this reaction can be easily developed using nonenzymatic approaches involving the formation of reaction chloride acid intermediates, although the use of lipases allows the development of environmentally friendly reactions. In this context, the use of DES has been described, for instance in the esterification of oleic acid with 1-decanol as nucleophile as reported by Kleiner and Schörken using CAL-B as soluble

biocatalyst (Scheme 16.5 *top*) [23]. A two-phase reaction system was employed, the CAL-B catalyzed esterification occurring in the interface and shifting the equilibrium toward decyl oleate production by entrapment of the water molecules formed as reaction by-product, the use of DES such as ChCl:Gly and ChCl:U, the latest leading to the desired ester with excellent selectivity.

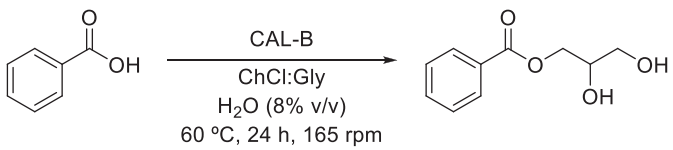
Zeng and coworkers reported later the lipase-catalyzed esterification of oleic acid with glycerol [24]. Five lipases from *C. antarctica*, *Thermomyces lanuginosus*, *Rhizomucor miehei*, *Penicillium camemberti* and *Rhizopus oryzae* (ROL) were tested using the glycerol as substrate but also as part of the DES solvent in combination with ChCl and betaine as quaternary ammonium salts (hydrogen-bond acceptors, HBAs), and the influence of the HBA:HBD ratio (1 to 1−2.5, HBD defined as hydrogen-bond donor), water content (0%−4%) and reaction time were explored. Best results were found with the ChCl:Gly (1:2 mol/mol) in the presence of molecular sieves and immobilized CAL-B (Novozyme 435) at 50°C, achieving a 43% conversion to the corresponding 1,3-diacylglycerol after only 1 h (Scheme 16.5 *bottom*).

The use of water has a key influence in enzymatic and process, and recently the synthesis of α-monobenzoate glycerol has been reported starting from benzoic acid and glycerol using commercially available CAL-B (Novozyme 435). Four different DES were tested (ChCl:HBD 1:2 mol/mol), satisfyingly the use of ChCl:Gly and water (8%−20% v/v) leading to full conversions and complete selectivity after 24 h at 60°C (Scheme 16.6) [25]. The water has a key role in the reaction medium, highly decreasing its viscosity so interestingly, percentages of water below 5% led to lower conversion values to the α-monobenzoate glycerol (40%−80%), while the use of a 30% water content stop the ester formation around 40% due to the appearance of byproducts caused by the hydrolytic action of the enzyme. In spite of the observed significant decrease of the conversion



SCHEME 16.5

Lipase-catalyzed esterification of oleic acid with glycerol-based DES.



SCHEME 16.6

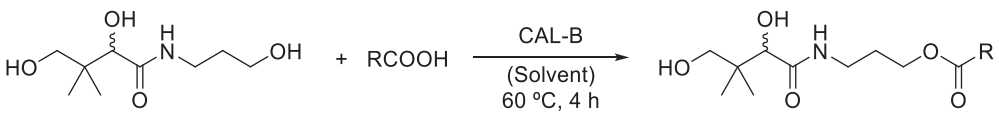
CAL-B catalyzed esterification of benzoic acid with glycerol in DES-water binary mixtures.

during the first recycling experiment, from 100% to 63%, later reuses do not lead to a dramatic loss of activity (52%, 48% and 47% conversion, respectively) in the reactions at 60°C for 24 h.

Compared to the 13 g α-monobenzoate glycerol/Ld achieved with Novozyme 435, the same authors reported the use of different immobilized CAL-B preparations, for example, via cross-linked enzyme aggregates (CAL-B CLEA), describing a more stable enzyme that can be reused for six cycles with any loss of activity, and attaining a higher productivity under similar reaction conditions (35 g α-monobenzoate glycerol/Ld) [26]. In addition, the double immobilization of CAL-B by encapsulation of the CAL-B CLEA in Lentikats provided a stable and active catalyst, successfully producing α-monobenzoate glycerol in DES by using batch and continuous mode processes [27], which expand the possibilities of CAL-B in the monobenzoylation of glycerol in flow mode using DES-water mixtures [28]. More recently, the use of *Pseudomonas stutzeri* lipase immobilized as CLEAs has been reported as an alternative to CAL-B for the production of α-monobenzoate glycerol, although attaining a low yield, around 20% conversion, after 24 h at 60°C when using ChCl: Gly (1:2 mol/mol) and 10% of water as phosphate buffer pH 7.0 [29].

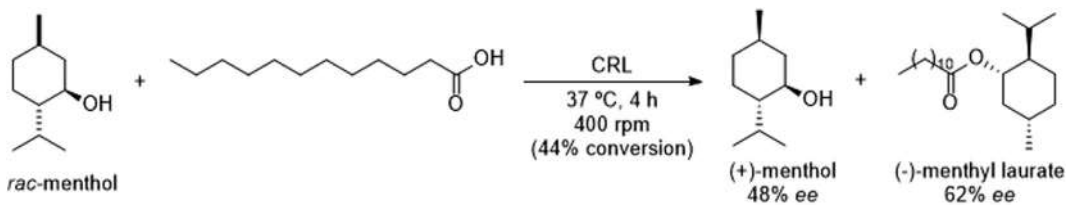
Lozano and coworkers have expanded the potential of ionic liquids (ILs) and DES by describing the esterification of six fatty acids (capric, lauric, linoleic, myristic, palmitic and linoleic acids) to prepare the corresponding panthenyl monoacyl esters (Scheme 16.7) [30]. Reactions were carried out by stirring the fatty acids with solid panthenol (1−3 equiv) for 4 h at 60°C in the presence of CAL-B and molecular sieves, selecting the production of the panthenyl monolaurate for the study of the enzyme recycling that was possible for seven operation cycles with conversions over 80% and excellent selectivities.

Finally in this section, the unique example of a stereoselective transformation through an esterification reaction in DES is described [31], this is the case of the esterification of lauric acid with racemic menthol by simply mixed and stirred both components in the presence of *Candida rugosa* lipase (Scheme 16.8). After optimization of the menthol: acid ratio, the best conditions were found



SCHEME 16.7

CAL-B catalyzed esterification of panthenol with fatty acids through in situ DES formation.



SCHEME 16.8

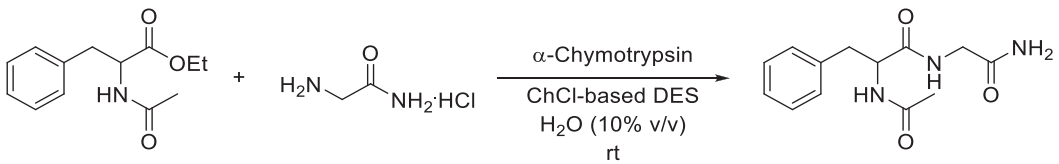
Kinetic resolution of racemic menthol by reaction with lauric acid.

when using 0.5 equiv of the lauric acid, reaching a 44% conversion after 3 h at 37°C, and recovering substrate and product with moderate optical purity: (−)-menthyl laurate (62% *ee*) and (+)-menthol (48% *ee*).

16.2.2.2 Aminolysis reactions and peptide synthesis

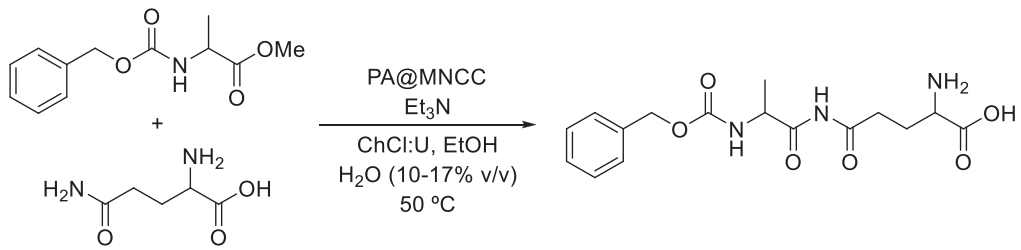
The replacement of alcohols by amines as nucleophiles give direct access to amides when starting from carboxylic acid or esters, the use of lipases and proteases in hydrophobic solvents being highly explored in the last three decades. However, DES is a good candidate as the reaction solvent, and this is the case of the reaction between racemic *N*-acetyl-L-phenylalanine ethyl ester with glycinamide hydrochloride (0.5 equiv) using α-chymotrypsin and DES solvents (Scheme 16.9) [32]. The influence of the water content, enzyme loading and reagent concentrations was explored employing four different ChCl-based DES, finding ChCl:Gly (1:2 mol/mol) as ideal media for the production of the corresponding peptide with excellent conversions at 10%−25% water percentage, while increasing to a 50% water content led to a significant formation of the corresponding carboxylic acid due to a competitive hydrolysis reaction. Interestingly, the reusability of the enzyme was studied, observing a significant loss of activity after the four cycle, moving from quantitative conversion to a value under 40% of the reaction with 10% water content and after 2 h at room temperature.

Cao and coworkers reported the synthesis of *N*-(benzyloxycarbonyl)-alanyl-glutamine dipeptide (Z-Ala-Gln) using the Z-L-alanine methyl ester (Z-Ala-OMe) as acyl donor and glutamine (Gln, 2.06 equiv) as nucleophile in ChCl:U (1.2 mol/mol), while a papain (PA) immobilized onto magnetic nanocrystalline cellulose (MNCC) was selected as enzyme (Scheme 16.10) [33]. The authors



SCHEME 16.9

Chymotrypsin-catalyzed peptide synthesis between *N*-acetyl-L-phenylalanine ethyl ester with glycinamide hydrochloride in a water/ChCl-based DES mixture.



SCHEME 16.10

Enzymatic synthesis of *N*-(benzyloxycarbonyl)-alanyl-glutamine in DES catalyzed by a PA@MNCC nanobiocatalyst.

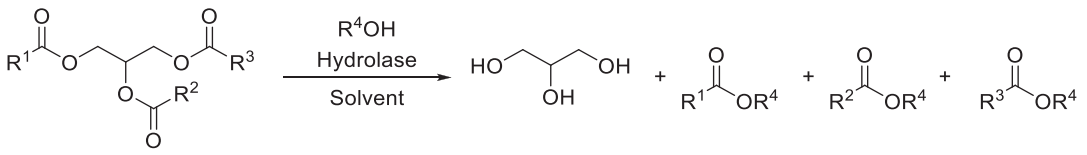
explored different reaction variables including the influence of water content, ester:amide ratio, temperature and the comparison between the use of free or immobilized papain, disclosing a selective peptide synthesis with a maximum 71.5% conversion value. The use of an organic base such as triethylamine was a key item for the complete solubility of the reagents, its presence not causing a detriment in the operational stability of the biocatalyst since more than 88% of its activity was retained after reusing it for five times.

16.2.2.3 Transesterification and transphosphatidylation reactions

Excluding the development of hydrolytic processes, the transesterification reaction between esters and alcohols is probably the most widely explored transformation using lipases as biocatalysts. In this context, the use of hydrophobic solvents has received great attention, appearing in the last two decades ILs as alternative sustainable solvents including more recently the use of DES. The use of these neoteric solvents in transesterification reactions can be mainly accomplished in three different ways: (1) as unique solvents; (2) in combination with lower amounts of water or isopropanol (<10% v/v) as cosolvent that interferes in the hydrogen-bond network between the DES and the reactants, normally increasing the reactivity of the catalytic system but without preferentially favoring the concomitant hydrolytic reaction; (3) in combination with traditional organic solvents to provide a higher enzyme stability.

Biocatalyzed biodiesel production consists in the reaction between natural triglycerides and aliphatic alcohols such as methanol or ethanol (Scheme 16.11), the use of hydrolases in different reaction media (organic solvents and neoteric solvents) being extremely helpful for synthetic purposes [34]. Yang and coworkers reported the reaction between the crude oil extracted from *Millettia pinnata* seeds with methanol in ChAc:Gly (1:2 mol/mol) at 50°C, finding immobilized CAL-B and *T. lanuginosus* lipase (TLL) as the more suitable enzymes reaching after 48 h 55% and 45% conversion, respectively [35] Yellow horn seed oil has also served for the biodiesel production using CAL-B under microwave irradiation [36]. From the eleven tested DES, ChCl:Gly (1:2 mol/mol) gave the best results and after studying the influence of the enzyme loading, methanol/oil ratio and microwave parameters, a 95% conversion was achieved after 120 h using MeOH (6 equiv) at 50°C. Interestingly, the enzyme was successfully reused with gradual deactivation of the enzyme until 70% conversion after the sixth cycle.

The use of low water contents has been demonstrated to have a beneficial impact in the production of biodiesel, as described by Zhao and coworkers in 2011 [37]. Therefore, the transesterification of the triglyceride Miglyol oil 812 with methanol and CAL-B in DES formed by ChAc or ChCl as ABD and glycerol as HBD led to high conversions (82%–97%) in short reaction times (1–3 h) at low contents of water (1% v/v) and under optimized reaction conditions, these are 50°C,

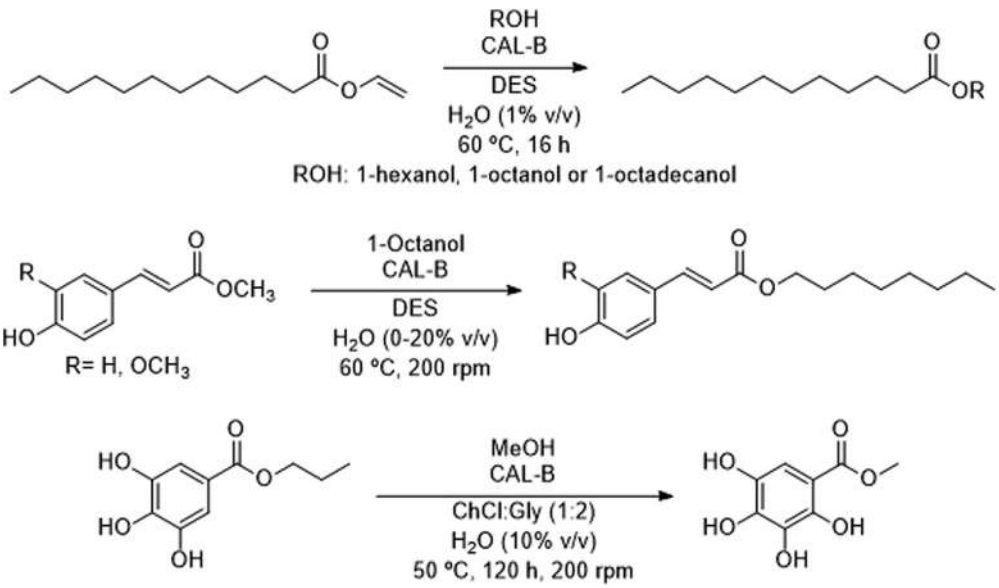


SCHEME 16.11

Lipase-catalyzed production of biodiesel through transesterification reactions.

ChAc:Gly (1:1.5 mol/mol) and MeOH (20% v/v). Under similar conditions with methanol as nucleophile, but starting from soybean oil [38], CAL-B has also been found as a suitable enzyme to produce biodiesel. In this case, after 24 h at 50°C the use of ChCl:Gly (1:2 mol/mol) allowed 81 or 88% conversion when using 1% or 0.2% of water, respectively. At the lowest water concentration, the reusability of the enzyme was successfully demonstrated for four cycles (79%−88% conversion).

The transesterification of vinyl laurate with different alcohols such as 1-butanol, 1-octanol or 1-octadecanol (6 equiv) have been successfully achieved using CAL-B and DES at lower water contents (1%, Scheme 16.12 *top*) [39]. The influence of the DES HBD was analyzed, finding that with ChCl:U and ChCl:Gly quantitative conversions to the desired esters were attained in all cases after 16 h at 60°C. However, the DES containing malonic acid (MA), oxalic acid (Ox), or ethylene glycol (EG) led to lower conversions (5%−41%) and selectivities (25%−100%) due to the reactivity of these components and the formation of byproducts with the concomitant destruction of the DES over the time. The same research group reported the use of DES (ChCl:U and ChCl:Gly) in the transesterification of phenolic esters such as methyl *p*-coumarate and methyl ferulate with CAL-B (Scheme 16.12 *middle*) [40]. For instance, the reaction between methyl *p*-coumarate and 1-octanol (6 equiv) was scaled-up to 3 g of substrate, yielding after 72 h a 93% isolated yield of the octyl ester (97% conversion). In these transesterification reactions, a great benefit was observed when using water as cosolvent, moving from very low conversions (<2% after 4 days) in pure DES to quantitative conversions at 8%−10% of water, without observing significant ester hydrolysis. The



SCHEME 16.12

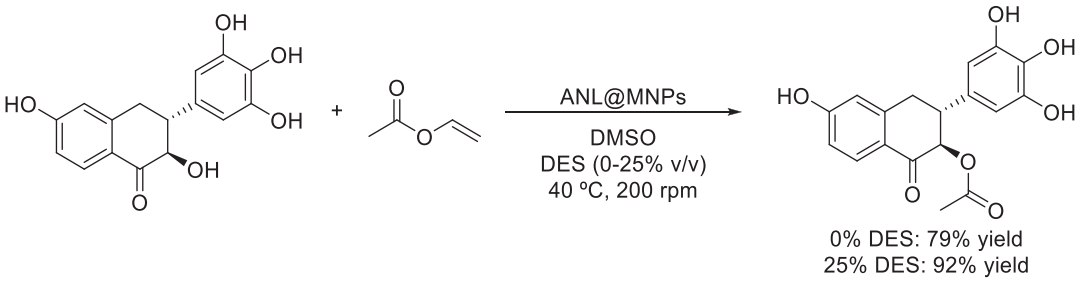
Transesterification of vinyl laurate and phenolic esters with aliphatic alcohols in the presence of CAL-B and low water contents.

competitive hydrolytic reaction occurred with significant extension when higher water contents were used (10%−20%).

CAL-B has also efficiently catalyzed the transesterification of propyl gallate using methanol as nucleophile (Scheme 16.12 *bottom*), overcoming the negligible conversion attained when exploring the corresponding esterification of gallic acid in methanol, 2-butanone or DES, probably caused by phenolic acid inhibition [41]. The transesterification process was studied at different enzyme concentrations (10−80 g/L), methanol concentrations (1−8 equiv), temperatures (35°C−60°C) and agitation speeds (75−250 rpm), finding around 60% conversion at 50°C and 55°C with almost exclusive ester formation when using 40 mM substrate concentration, 6 equiv of methanol, ChCl:Gly (1:2 mol/mol), water (10%) and 200 rpm. The reactivity seriously decreased at lower (35°C and 45°C) and higher temperatures (60°C).

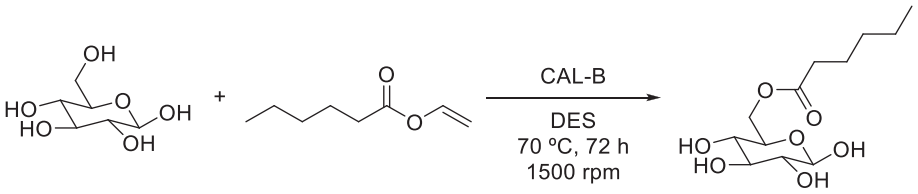
Interestingly, these transesterification reactions can be performed in a chemo-, regio- and/or stereoselective manner, the use of DES helping to favor the solubility of highly polar compounds or improving the stability of the enzyme in the reaction medium. This is the case of the monoacetylation of dihydromyricetin (DMY, 20 mM), a natural flavanol displaying antibacterial, antiinflammatory and antitumoral activities, that was reacted with vinyl acetate (10 equiv) using the *Aspergillus niger* lipase (ANL) immobilized onto magnetic nanoparticles in a high polar solvent such as dimethylsulfoxide (DMSO) [42]. The monoacetate at the C-16 position was obtained in 79% yield in the absence of DES, while the use of a 25% of ChCl:Gly improved the conversion until 92% (Scheme 16.13).

Regarding the development of regioselective transformations, Pöhnlein and coworkers reported in 2015 the selective acylation of glucose using vinyl hexanoate (2 equiv) and CAL-B in pure DES (Scheme 16.14) [43]. The best results were found in ChCl:U (1:2 mol/mol) and ChCl:Glc (1:1 mol/mol), obtaining glucose-6-*O*-hexanoate as the major product after 3 days at 70°C. Hollenbach and coworkers reported the synthesis of glucose monodecanoate in a hydrophobic DES formed by (−)-menthol and decanoic acid catalyzed by CAL-B [44], attaining superior results to the ones previously obtained in the same reaction but using a traditional hydrophilic DES such as ChCl:U (1:2 mol/mol) with a 5% of water (11% yield after 24 h vs <1%) [45]. More recently, Ortega and coworkers reported the use of a ChCl:Gly/DMSO/*tert*-butanol (1:1:3 v/v/v) system to overcome the mass transfer limitations in pure DES, yielding almost a 25% conversion in the regioselective reaction between glucose and vinyl laurate using different hydrolases [46].



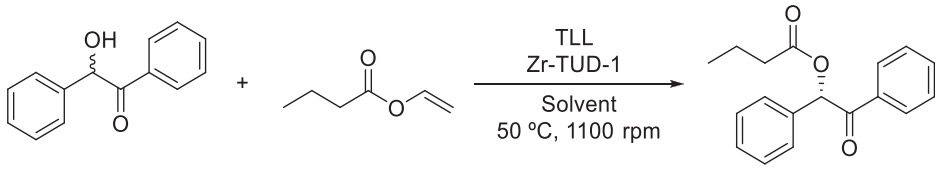
SCHEME 16.13

Lipase-catalyzed acetylation of DMY using a DMSO/DES (3:1 v/v) mixture.



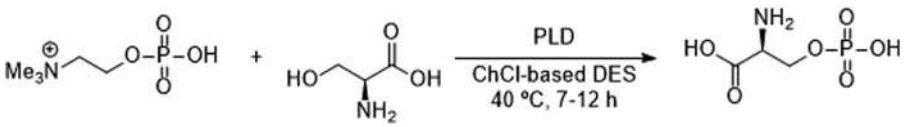
SCHEME 16.14

CAL-B catalyzed acylation of glucose with vinyl hexanoate in DES.



SCHEME 16.15

Lipase-catalyzed dynamic kinetic resolution of racemic benzoin in organic solvents and DES using vinyl butyrate as acyl donor.



SCHEME 16.16

Phospholipase-catalyzed transphosphatidylation reaction between phosphatidylcholine and L-serine for the synthesis of phosphatidylserine.

Regarding the use of hydrolases in stereoselective processes, Kara and coworkers studied the dynamic kinetic resolution of racemic benzoin (Scheme 16.15), using vinyl butanoate (6 equiv) as acyl donor in both organic solvents (toluene, 2-methyltetrahydrofuran and cyclopentyl methyl ether) and DES (EACl:Gly, ChCl:Gly, ChCl:Iso, ChCl:LA, ChCl:Ox and ChCl:U; 1:1.5−2.0 mol/mol) [47]. TLL was selected as stereoselective enzyme and combined with a heterogeneous zirconium-based catalyst (Zr-TUD-1) to achieve the racemization of the unreacted benzoin. Although the use of organic solvents led to much better results in terms of conversion, the use of ChCl:Iso (1:2 mol/mol) provides an elegant alternative for downstream processing, improving also the poor solubility of the benzoin, especially when some amount of isopropanol (10% v/v) was added as cosolvent.

Finally, the unique example of a transphosphatidylation reaction is covered in this section, which occurred between phosphatidylcholine and L-serine (6 equiv) in DES to produce phosphatidylserine, which is a phospholipid with interesting properties to rejuvenate brain cell membranes and increase acetylcholine brain levels (Scheme 16.16) [48]. The reaction was catalyzed by the

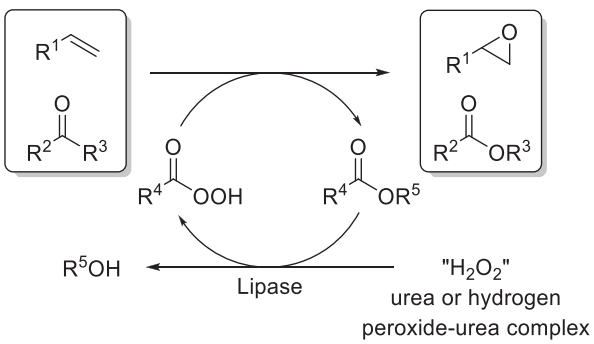
phospholipase D (PLD) using a 0.5% of water in both ChCl:Gly (1:2 mol/mol) and ChCl:Gly (1:2 mol) at 40°C, obtaining the phosphatidylserine in 90% and 92% yield after 7 and 12 h, respectively. Remarkably, the PLD seemed to be highly stable and active along the time, conserving 81% of its original activity after 9 reuses under optimized conditions in the ChCl:EG system.

16.2.3 Nonconventional biotransformations using hydrolases as catalysts

The ability of the enzyme active site to catalyze unexpected reactions, different from its natural reaction, is known as biocatalytic promiscuity, and from the wide number of biocatalysts, lipases have appeared in the last two decades as versatile catalysts for synthetic transformations, for instance for the formation of C−C and C−heteroatom bonds. This is an area of relevant interest since it can provide synthetic alternatives usually in a simple manner. However, it is important to investigate the reaction pathway since the catalytic center of the enzyme is not always responsible for the studied reaction, and this approach is known as pseudopromiscuity [49,50]. Classical examples are the lipase-mediated epoxidations or the Baeyer−Villiger reactions where the enzyme is only responsible of the formation of a reactive peracid intermediate, which subsequently reacts with the corresponding epoxide or ketone, respectively (Scheme 16.17). Therefore, the global reaction occurs in a tandem mode, first the lipase-catalyzed ester perhydrolysis of the starting carboxylic acid or ester using a hydrogen peroxide source to form the peracid, and then the chemical reaction of this active specie with the olefin or carbonyl compound to form the desired oxygenated compound.

16.2.3.1 Tandem oxidative reactions mediated by lipases in deep eutectic solvents

Without any doubt, CAL-B has been the most employed enzyme for this type of transformations, displaying a good reactivity in DES as described in previous sections of this contribution with more conventional reactions. Among the epoxidation reactions, a series of examples can be highlighted, for instance Wang and coworkers described the epoxidation of aliphatic alkenes and styrenes using an aqueous solution of H_2O_2 (2 equiv) and octatonic acid as peracid precursor (1 equiv) in phosphate

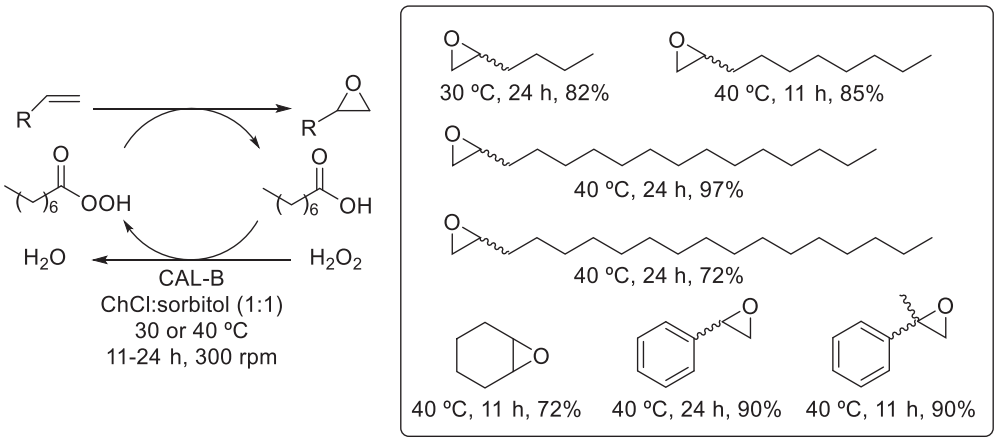


SCHEME 16.17

General scheme for lipase-mediated epoxidation or Baeyer−Villiger reactions via formation of peracid intermediates.

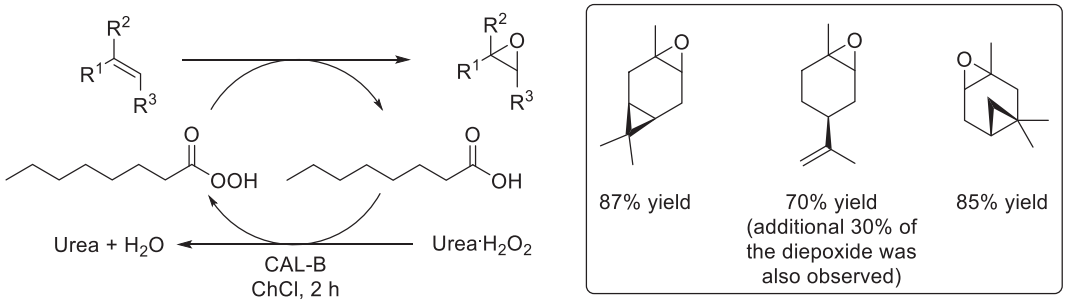
buffer pH 6.0 and nine different ChCl-based DES (Scheme 16.18) [51]. The best results were found in ChCl:So (1:1 mol/mol) leading to the corresponding epoxides in 72%−97% conversion by GC analyses after 11−24 h at 30°C or 40°C.

CAL-B and octanoic acid have resulted to be also a good system for the epoxidation of monoterpenes such as 3-carene, limonene and α-pinene in DES (Scheme 16.19) [52]. The DES mixtures were prepared in situ by mixing ChCl and the urea-hydrogen peroxide (2 equiv), achieving quantitative conversions to the epoxides after 2 or 3 h, which were easily recovered with good to high yields (77%−87%) after a simple extraction protocol with water and ethyl acetate. Interestingly, the valorization of agricultural wastes has been recently demonstrated through the epoxidation of limonene from orange peels using a ChCl:1,2-propanediol:H_2O in an equimolar ratio or ChCl:EG (1:1 mol/mol) and a series of enzymes including lipases (CAL-B, RML or TLL) or the choline



SCHEME 16.18

CAL-B-mediated epoxidation of alkenes and styrene using octanoic acid and hydrogen peroxide.



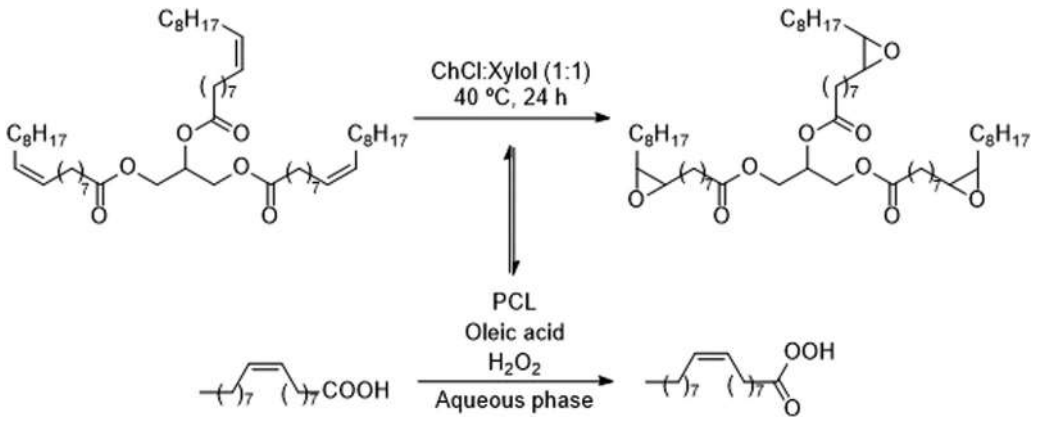
SCHEME 16.19

CAL-B-mediated epoxidation of monoterpenes using in situ generated ChCl-based DES.

oxidase isolated from the soil bacterium *Arthrobacter nicotianae*, obtaining mixtures of the mono- and diepoxide as also occurred in the previous examples [53].

The potential of other lipases in epoxidation reactions in DES has also been demonstrated. This is the case of the lipase G from *P. camembertii* (PCL), which displayed even better results than CAL-B in the oxidation of glyceryl trioleate, since PCL avoids the formation of side hydrolytic products observed in the reaction with CAL-B (Scheme 16.20) [54]. Reactions were carried out in buffer and five different DES, finding the best conditions with the ChCl:Xyl (1:1 mol/mol) when using hydrogen peroxide (3 equiv) and 40°C. In this case, the external addition of the peracid precursor was not needed since it was present in the vegetable oil samples used as starting materials. PCL has also been found to be a suitable enzyme for the formation of epoxides using the soybean oil as source of triglycerides, finding in this case the best results using a biphasic system composed by the oil and water, while the ChCl:So (1:1 mol/mol) served to form a micro emulsion enabling the surface tension lowering of hydrophobic organic phases in aqueous medium [55].

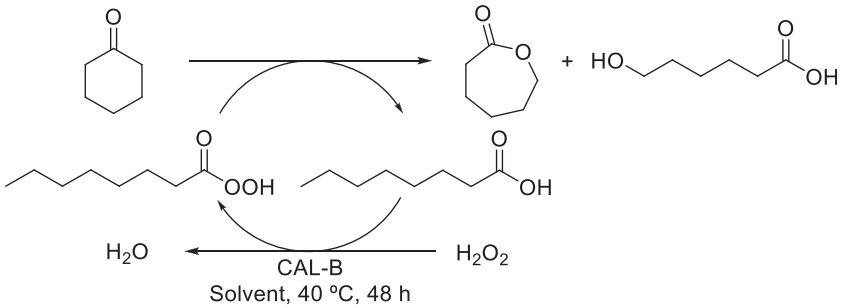
CAL-B-mediated Baeyer–Villiger have also attracted recent attention, such as the case of the transformation of cyclobutanone, cyclopentanone, cyclohexanone and 4-pentanone in the corresponding lactones [56]. In this case, the reactivity of the CAL-B wild-type and the engineered Ser105Ala mutant was compared using an aqueous solution of hydrogen peroxide (30% H_2O_2) and octanoic acid as peracid precursor using cyclohexanone as benchmark substrate (Table 16.2). In hexane-water (2:1 v/v) as solvent, the CAL-B wild-type highly favored the formation of the hydroxyl acid formed through lipase-catalyzed hydrolysis of the lactone (2:1 hydroxy acid vs lactone, 55% conversion), while the mutant predominantly led to the formation of the ε-caprolactone (1:2.5 ratio, 24% conversion). From the five DES tested, higher selectivities toward the Baeyer–Villiger products were found when the reactions were carried out in a DES systems such as ChCl:So (1:1 mol/mol), attaining for the Baeyer–Villiger oxidation of cyclohexanone a 92% conversion (46% selectivity) with the wild-type and a 47% conversion (99% selectivity) with the mutant.



SCHEME 16.20

Lipase G from PCL-mediated epoxidation of glyceryl trioleate.

Table 16.2 Baeyer–Villiger oxidation of ketones using CAL-B wild type or its Ser105Ala mutant and H_2O_2 (2 equiv) in ChCl:So (1:1) after 48 h at 40°C.

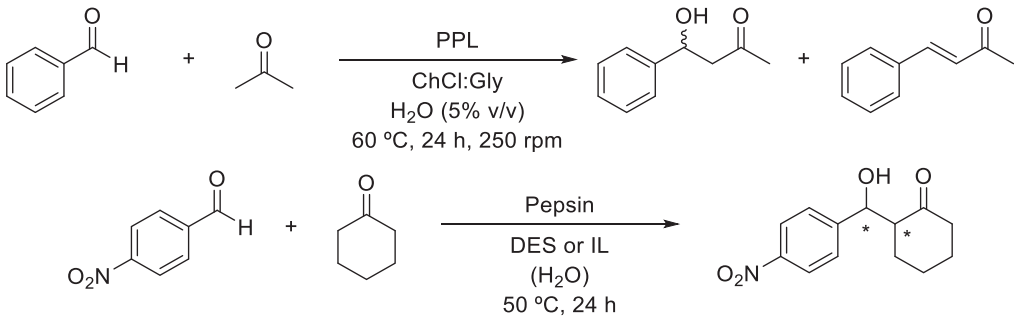


		CAL-B wild type		CAL-B Ser105Ala mutant	
Entry	**Ketone**	**Conv. (%)**	**Selectivity (%)a**	**Conv. (%)**	**Selectivity (%)a**
1	Cyclobutanone	99	93	99	100
2	Cyclopentanone	95	48	51	97
3	Cyclohexanone	92	46	47	99

[a]*The selectivity consists in the percentage between the lactone (Baeyer–Villiger product) and the hydroxyl acid formation (lactone hydrolysis).*

Among the C–C bond formation transformations, aldol reactions represent elegant examples to provide access to hydroxy carbonyl compounds with high selectivity. Traditionally, aldolases have efficiently catalyzed these biotransformations in a highly stereoselective manner, although in recent years the nonconventional action of lipases has been reported. For instance, the use of porcine pancreas lipase (PPL) could catalyze the aldol reaction between a series of substituted benzaldehydes and aliphatic aldehydes with acetone and cyclic ketones in DES [57]. Selecting as model reaction the one between benzaldehyde and acetone, and after optimization of the reaction conditions including aldehyde:ketone ratio, PPL loading and ChCl:Gly composition, full conversions were achieved toward the formation of the 4-hydroxy-4-phenylbutan-2-one after 24 h at 60°C (Scheme 16.21 *top*). The addition of some water to the DES (5%–20%) allowed the minimization of the side-product formation derived from the aldol-dehydration sequence, being able to develop synthetically useful reaction at high benzaldehyde concentrations (1–6 M) using 5 equiv of acetone. Finally, the enzyme recycling was studied for the reaction between 4-nitrobenzaldehyde and cyclohexanone (2 equiv), finding a significant drop of activity after the first recycling (around 33% of the enzyme activity) probably caused by the severe reaction conditions (60°C). The asymmetric version of this latest reaction has been described by Wang et al. using pepsin in ILs and DES (Scheme 16.21 *bottom*), optimizing the reaction conditions in DES in terms of deionized water composition (20%–95%) for the reaction in ChCl:Gly (1:2 mol/mol), and achieving the best results with the system ChCl:Gly/H_2O (3:7 v/v) for a 79.7% yield, 28:72 *anti*/*syn* diastereoselectivity and 60% *ee* of the major *syn* enantiomer [58].

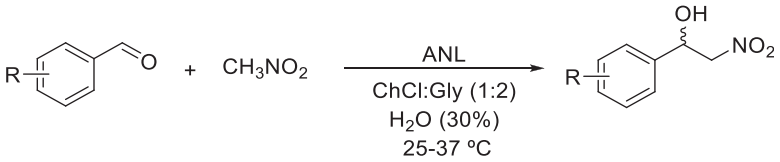
Another interesting C–C bond formation transformation is the Henry reaction, the ANL being able to catalyze the process between nitromethane and different benzaldehydes in pure DES [59].



SCHEME 16.21

Hydrolase-catalyzed aldol reactions in neoteric solvents.

Table 16.3 ANL-catalyzed Henry reaction between a series of benzaldehydes (214 mM) and nitromethane (15 equiv).



		100% H_2O		ChCl:Gly (1:2 mol/mol)/30% H_2O	
1	**R**	**Time (h)**	**Yield (%)**	**Time (h)**	**Yield (%)**
1	H	120	–	48	–
2	2-NO_2	10	81	4	87.7
3	3-NO_2	10	85	4	88.5
4	4-NO_2	10	87	4	92.2
5	2,4-$(NO_2)_2$	10	70	4	91.7
6	4-F	120	46[a]	48	12.3
7	4-Cl	120	80[a]	24	62
8	4-Br	120	91	24	89
9	4-OMe	120	37[a]	48	9.6
10	4-Me	120	14[a]	48	––

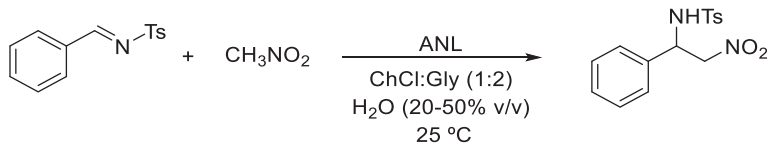
[a]*Reactions carried out at 37°C instead of 25°C.*

The biotransformation between 4-nitrobenzaldehyde and nitromethane was studied as benchmark reaction using various ChCl-based DES (Gly, EG and U as HBD in different ratios), water contents, reagents concentrations, enzyme loadings and temperatures, finding the highest conversion values for the those developed in ChCl:Gly/water (7:3 v/v, Table 16.3). Then, the study was extended to other substituted benzaldehydes comparing its reactivity with the reactions carried out in aqueous medium, observing a high influence of the pattern substitution at the phenyl ring.

In the same contribution, ANL was able to catalyze the aza-Henry reaction between (*E*)-*N*-[(4-methylbenzene-1-sulfonyl)oxy] − 1-phenylmethanimine and nitromethane (15 equiv) in ChCl:Gly (1:2 mol/mol), leading to the aza-Henry product in 35.5%−38.7% yield after 40 h at 25°C using a 20%−50% water content (Scheme 16.22), while lower amounts of water or the reaction in pure water led to poorer results.

Moving to C−heteroatom bond formation, Dandekar and coworkers reported the methylation of chitosan using dimethyl carbonate as methylating agent, and the lipase from *Burkholderia* species (Amano lipase PS) as biocatalyst (Fig. 16.1) [60], representing an environmentally friendly strategy to replace traditional transformation with methyl iodide in alkaline conditions. ChCl:Gly and ChCl:U (1:2 mol/mol) were used in combination with aqueous systems or/and dimethylformamide (DMF), finding PS as a highly active enzyme for the synthesis of *N*- and *O*-methylated chitosan derivatives, while no reaction was observed with CAL-B.

Multicomponent reactions are straightforward strategies to provide access to complex molecules, the use of lipases representing a clean an efficient way to perform this type of transformations. For instance, the Biginelli reaction has been successfully reported for the synthesis of pyrimidine derivatives combining the lipase from ROL with the use of aqueous, organic (methanol, 1,4-dioxane or DMF) and DES (ChCl:U (1:2 mol/mol)) solvents [61]. Seven dihydropyrimidin-2(1*H*)-one derivatives were prepared starting from an aromatic aldehyde, a dicarbonyl compound (ethyl acetoacetate or pentane-2,4-dione, 1.05 equiv) and urea or thiourea (1.1. equiv) using the ChCl:U (Scheme 16.23). In particular, the acceleration of the reaction between



SCHEME 16.22

ANL-catalyzed aza-Henry reaction between a *N*-tosyl imine and nitromethane in a DES/water system.

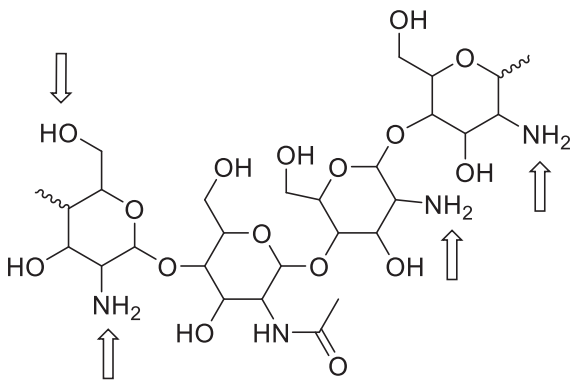
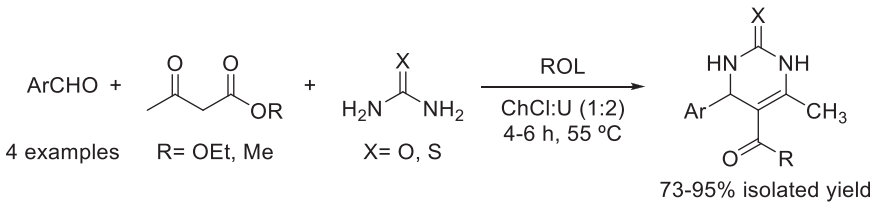


FIGURE 16.1

Chitosan positions susceptible of reaction through lipase-catalyzed methylation.



SCHEME 16.23

Lipase-catalyzed multicomponent Biginelli reaction accelerated by ROL in DES.

6-methoxynaphthalene-2-carbaldehyde, ethyl acetoacetate, and urea due to the enzyme action was significant, since in the absence of the ROL only 20% yield was reached after 7.5 h, while a 95% after 4 h was attained in its presence. The recyclability of the DES and lipase system was also demonstrated in this reaction, obtaining 75%–95% yields after five consecutive experiments.

16.3 Redox enzymes

The use of oxidoreductases (EC 1) in DES-water systems have been widely explored in the last decade, whether to enhance conversion or selectivity [62–64]. From this class of enzymes, alcohol dehydrogenases (ADHs) in reductive transformations, laccases and peroxidases for oxidation purposes, and flavin-dependent monooxygenases in the selective oxygenation of organic molecules, are without a doubt the biocatalysts of choice when considering DES as cosolvents. The main differences with previously studied hydrolases reside in their action mechanism and cofactor dependency, whether on inorganic metals such as copper for laccases, or on relatively small organic molecules such as nicotinamide adenine dinucleotides (NAD or NADP derivatives) for ADHs. This section has been classified depending on the type of reaction, moving from stereoselective bioreductions to nonselective oxidations and oxygenation transformations.

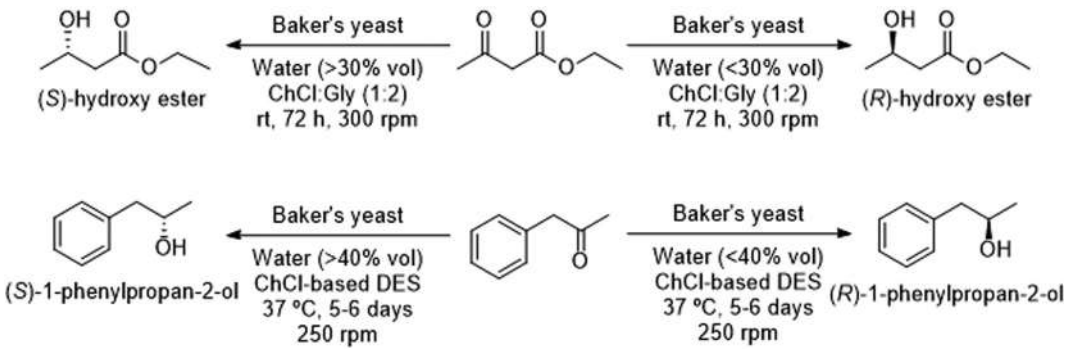
16.3.1 Reductions

The biocatalytic reduction of carbonyl groups is the most studied redox reaction in DES-water systems, employing mainly whole cell biocatalysts for stereoselective transformation [62,63]. The use of these enzyme preparations provide some advantages over isolated purified enzymes, such as the lack of external cofactor addition and the reduced of the system complexity since cofactor recycling is not required, which leads to a reduction of the overall cost of the process. In this context, the use of DES as cosolvents enable the work under higher substrate concentration compared to total aqueous medium, at the same time that the enzyme preserves its integrity. Nevertheless, the use of whole cells is sometimes limited by the presence of other enzymatic activities, which can produce a decrease in the yield and optical purity of the products caused by the occurrence of competitive enzymatic reactions.

Baker's yeast (*Saccharomyces cerevisiae*) has been the enzyme most commonly employed for reduction processes in mixtures of water and DES. Thus, Maugeri and Domínguez de María

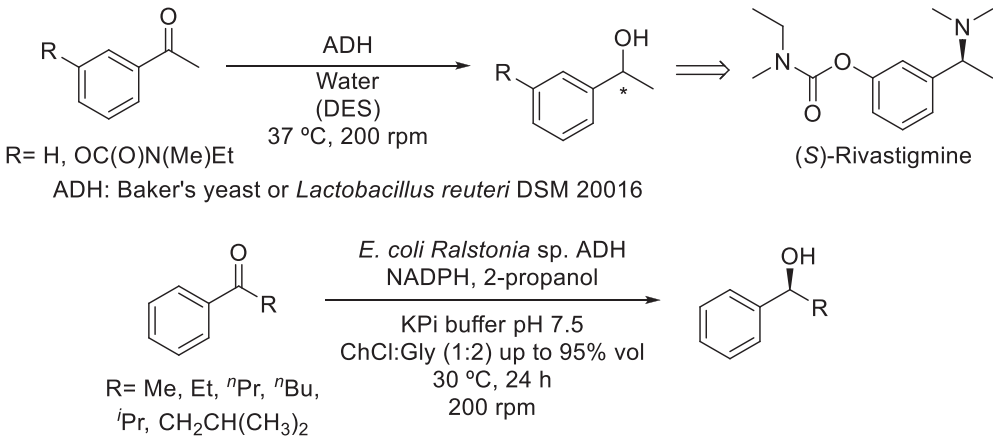
reported a pioneer work exploring the bioreduction of ethyl acetoacetate (Scheme 16.24 *top*) [65]. Complete conversion toward the formation of the ethyl (*S*) − 3-hydroxybutanoate was achieved in pure water after 72 h, while the addition of DES invert the selectivity toward the (*R*)-hydroxy ester, obtaining a virtually racemic alcohol at 30% v/v of DES and a total enantioselectivity in neat ChCl (1:2 mol/mol and remnant 1% weight of water determined by Karl-Fischer method). The achievement of this stereoinversion can be rationalized attending to the potential inhibitory effect of DES on (*S*)-oxidoreductases present in the BY whole cell preparation. Interestingly, the enzyme was still active for reaction times over 200 h when attempting reactions at both 10% and 50% of water. Redovnioković and coworkers deeply explored the same reaction employing a series of ChCl-based DES with different HBDs and ratios compared to the HBAs such as Gly, EG, Glc, Fru, Xyl, MA, Ox or U. [66] Selectivities and conversion values were highly depending on the type of HBD and the DES:water ratio, finding NADES involving sugars as HBDs (ChCl:Glc and Xyl) as suitable cosolvents (10% vol) for synthetic purposes, leading to the ethyl (*S*) − 3-hydroxybutanoate in high yield and selectivity (>90% conversion and >90% *ee*) as occur in pure water.

The inhibition of the Baker's yeast (*S*)-selectivity was also observed by Capriati and coworkers in the bioreduction of 1-phenylpropan-2-one (Scheme 16.24 *bottom*) [67]. The preferentially formation of the corresponding (*R*)-alcohol was observed at high DES contents (over 60% vol), the structure of the DES (Gly, Fru, Glc and U) playing a key role in the enantioselectivity value. For instance, the use of a mixture composed by 90% ChCl:Gly (1:2 mol/mol) and 10% water led to the (*R*) − 1-phenylpropan-2-ol in 96% *ee*, while the (S)-alcohol was obtained in also 96% *ee* in the absence of any DES. The use of other arylpropanones demonstrated the high influence of the substitution patterns at the aromatic ring in the product yield and selectivity. The same authors described the use of Baker's yeast resting cells in the bioreduction of acetophenone and 3-acetylphenyl ethyl(methyl)carbamate to the corresponding (*S*)-alcohols, the latter a precursor of the active pharmaceutical ingredient (*S*)-rivastigmine, a cholinesterase inhibitor used for the treatment of patients with moderate Alzheimer's disease (Scheme 16.25 *top*) [68]. However, better results were found when using the (*R*)-selective *Lactobacillus reuteri* DSM 20016 whole cells, although the use of DES was not reported in these reactions. Other aromatic ketones have also been



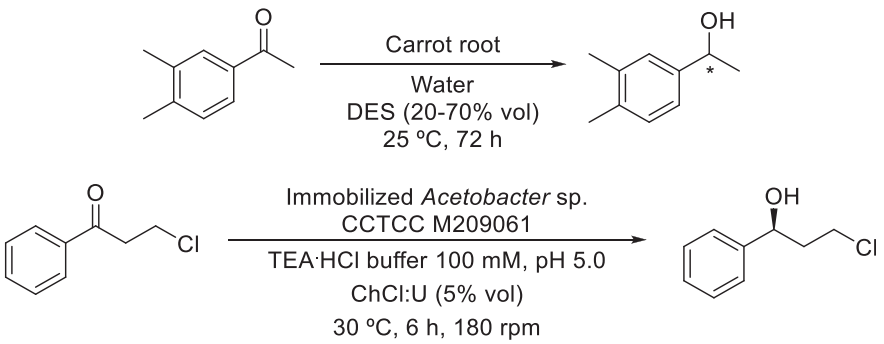
SCHEME 16.24

Change in the stereoselectivity in the bioreduction of prochiral ketones with Baker's yeast whole cells due to the presence of DES.



SCHEME 16.25

Stereoselective bioreduction of aromatic ketones in DES-water media.



SCHEME 16.26

Stereoselective bioreduction of aromatic ketones in DES-water media.

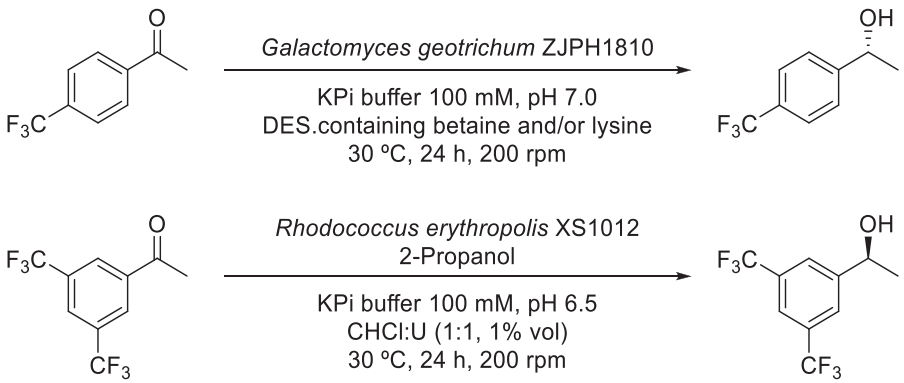
effectively reduced using recombinant overexpressing whole cells ADHs such as horse liver ADH (HLADH) and *Ralstonia* sp. (*Ras*ADH) using isopropanol for nicotinamide cofactor recycling purposes (Scheme 16.25 *bottom*) [69]. Special attention was paid to the use of *Ras*ADH that still remain active at 95% vol of ChCl:Gly (1:2 mol/mol), observing the highest enantiomeric excess at 90%−95% DES ratio.

Following with the reduction of aromatic ketones, 1-(3,4-dimethylphenyl)ethanone was used as substrate by Redovniković and coworkers with carrot roots as biocatalyst (Scheme 16.26 *top*) [70]. Starting from a 91% conversion and 96% *ee* of the (*S*)-alcohol in pure water, the use of five different DES composed by ChCl with EG (1:2), Glc (1:1) Gly (1:2), Xyl (2:1) and Xylol (5:2) were attempted finding. A dramatic decrease in activity was observed at higher DES contents

(20%−70% of DES), since conversion did not overcome 55%, observing an inversion of the enantioselectivity to the preferentially formation of the (*R*)-alcohol when only 30% of water was employed (33%−75% *ee*). Unfortunately, lower percentages of water led to poor conversion values (<10%) due to the high viscosity of the DES.

Enzyme immobilization can provide some advantages as occurred in the reduction of 3-chloropropiophenone to (*S*) − 3-chloro-1-phenylpropanol using *Acetobacter* sp. CCTCC M209061 (Scheme 16.26 *bottom*) [71]. The whole cells enzyme was immobilized on polyvinyl alcohol-sodium sulfate, studying its activity by combining a series of DES with TEA.HCl buffer pH 5.0. The ChCl:U (1:2 mol/mol) provided the best results, achieving the enantiopure (*S*)-alcohol in 82% isolated yield after 6 h when developing a 500 mL-scale reaction under optimal reaction conditions.

DES with variable HBAs, such as betaine, L-proline or L-carnitine, were prepared and used in the bioreduction of 1-[4-(trifluoromethyl)phenyl]ethanone (Scheme 16.27 *top*) [72]. These novel cosolvents (1% vol) enhanced the catalytic efficiency of *Galactomyces geotrichum* ZJPH1810 compared with traditional ChCl-based DES. In addition, the synthesis and application of lysine-based NADES was described, displaying also certain ability to enhance the regeneration of coenzyme were reported. Interestingly, the 500 mL-scale reactions at 400 mM substrate concentration using the betaine:Lys DES, occurred in 92.4% yield compared with the 78.4% yield achieved in phosphate buffer system, and maintaining the complete selectivity. The same research group has explored the reduction of 3,5-bis(trifluoromethyl)acetophenone using *Rhodococcus erythropolis* XS1012 for the synthesis of (*S*) − 3,5-bis(trifluoromethyl)phenylethanol, a key pharmaceutical intermediate of the NK-1 receptor antagonist (Scheme 16.27 *bottom*) [73]. In this case, ChCl:U (1:1 mol/mol) gave the best results with only a 1% vol, yielding the enantiopure alcohol with 91.9% conversion at 150 mM substrate concentration after 24 h at 30°C, which overcome the results obtained in buffer (82.6% yield). Similarly, the bioreduction of 2-chloro-1-(3,4-difluorophenyl)ethanone to produce (*S*) − 2-chloro-1-(3,4-difluorophenyl)ethanol was successfully achieved in a choline acetate/lysine (ChAc:Lys)-containing medium [74]. This DES strengthen coenzyme regeneration and improving cell membrane permeability during the bioreduction, leading to 87.0%



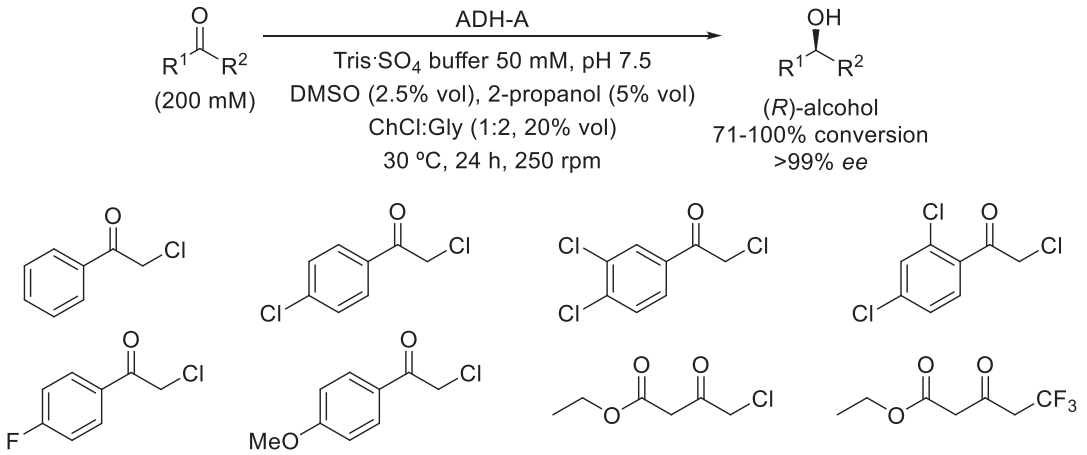
SCHEME 16.27

Bioreduction of trifluoromethyl ketones using DES as cosolvents.

conversion with complete selectivity, and increasing in 3.threefold the ketone concentration compared to the reaction in aqueous medium.

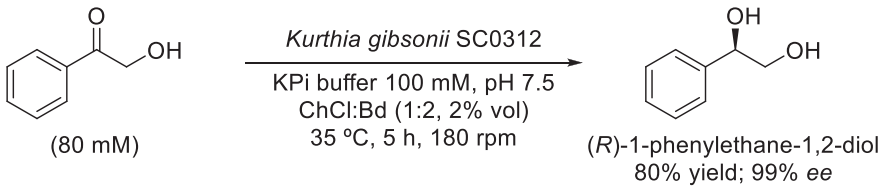
α-Haloketones are versatile precursors in organic synthesis because they can produce chiral halohydrins through bioreduction products, which in turn are immediate precursors of epoxides and azidoalcohols, among other interesting organic compounds. The use of DES in the bioreduction of α-haloketones have been recently reported using two stereocomplementary enzymes, the ADH from *Lactobacillus brevis* (*Lb*ADH) and the one from *Rhodococcus ruber* (ADH-A, Scheme 16.28) [75]. The main advantage of using DES up to 50% v/v, for instance ChCl:Gly (1:2 mol/mol), resides in the possibility to work at very high substrate concentrations (300−400 mM).

(*R*) − 1-Phenylethane-1,2-diol is a synthetic precursor of chiral drugs such as β-adrenergic blocking agents, and it can be straightforward accessed by stereoselective reduction of 2-hydroxyacetophenone (Scheme 16.29). The effect of five ChCl-based DESs on the reaction catalyzed by *Kurthia gibsonii* SC0312 were investigated by Lou and coworkers [76], the use of ChCl:1,4-butanediol (ChCl:Bd 1:4 mol/mol) in 2% vol increasing the catalytic rate of the enzyme



SCHEME 16.28

Bioreduction of α-haloketones in buffer-DES mixtures.



SCHEME 16.29

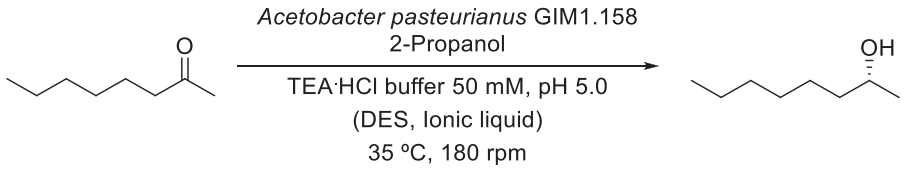
Asymmetric bioreduction of 2-hydroxyacetophenone in the presence of ChCl:Bd (2% vol).

by 22%. The reaction at 80 mM 2-hydroxyacetophenone concentration allowed the recovery of the (*R*)-alcohol in 80% yield and enantiomerically pure form.

The bioreduction of aliphatic ketones has also been reported in the literature, for instance 2-octanone was reduced by *Acetobacter pasteurianus* GIM1.158 cells (Scheme 16.30) using TEA. HCl buffer-ChCl-based DES mixtures (HBDs: U, Gly, EG, Ox, MA or Im) [77]. ChCl:EG (1:2 mol/mol) gave the best results in terms of reaction yield, finding a 40% ChCl:EG (1:2 mol/mol) ratio as optimal to obtain (*R*) − 2-octanol. The use of a biphasic system including the DES, the buffer and an imidazole-based IL (1-butyl-3-methylimidazolium hexafluorophosphate) was found to be an ideal media for the reduction of 2-octanone at high substrate concentration (1.5 M).

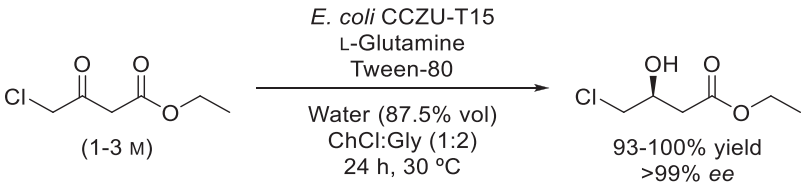
Ketoesters are also good substrates for bioreduction processes to obtain optically active hydroxyesters, as described earlier in this chapter [65,66]. Ethyl 4-chloro-3-oxobutanoate was converted to ethyl (*S*) − 4-chloro-3-hydroxybutyrate, a versatile precursor of chiral pharmaceuticals, using whole cells of *Escherichia coli* CCZU-T15 in a mixture of ChCl:Gly (1:2 mol/mol, 15% vol) and water (Scheme 16.31) [78]. Reaction conditions were optimized, finding a great improvement by adding: (1) the Tween-80 surfactant for a better dispersion of the substrate in the reaction medium; (2) L-glutamine to facilitate the cofactor synthesis. Thus the (*S*)-hydroxyester was obtained in enantiopure form when developing the reaction at a 2 M substrate concentration.

Structurally related to hydroxyesters, α-acetylbutyrolactone was reduced to α' − 1'-hydroxyethyl-γ-butyrolactone antistereoisomers (Scheme 16.32) using both growing and resting culture of seven different yeast strains [79]. After testing organic solvents (ethanol, glycerol, hexane and isopropanol) and ChCl:Gly (1:2 mol/mol) to help the substrate solubility, the DES (10%−50% vol) in combination with *Candida viswanathi* AM120, led to faster processes, also slightly improving the enantio- and diastereoselectivity in comparison with the reaction in buffer pH 7.0, especially when using 10 or 25% of DES.



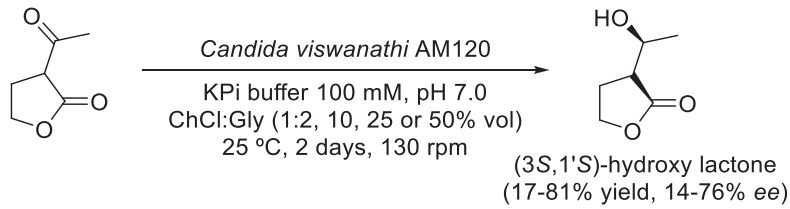
SCHEME 16.30

Stereoselective bioreduction of aliphatic ketones using ADHs in DES-water media.



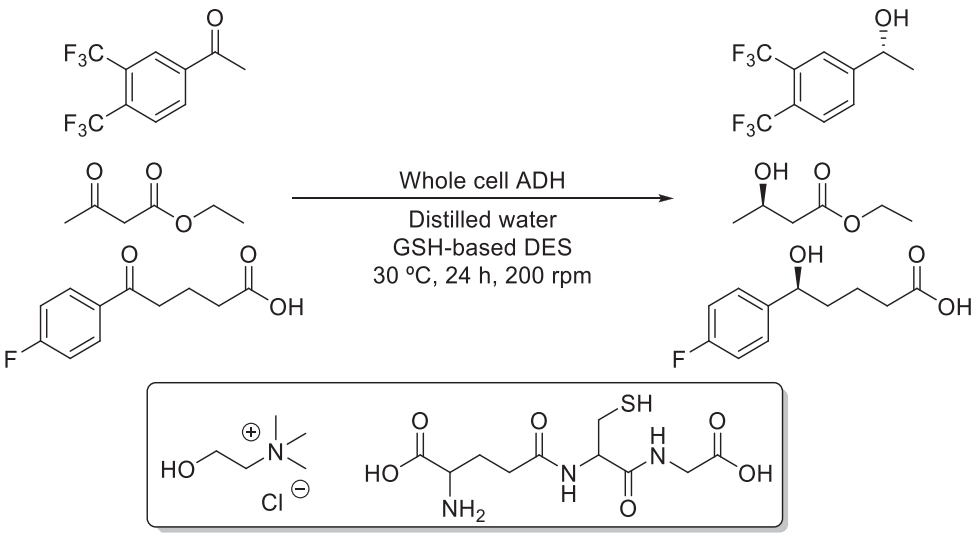
SCHEME 16.31

Stereoselective bioreduction of 4-chloro-3-oxobutanoate at high substrate concentrations using a water-DES mixture in the presence of a surfactant.



SCHEME 16.32

Stereoselective bioreduction of α-acetylbutyrolactone in DES-aqueous medium.



SCHEME 16.33

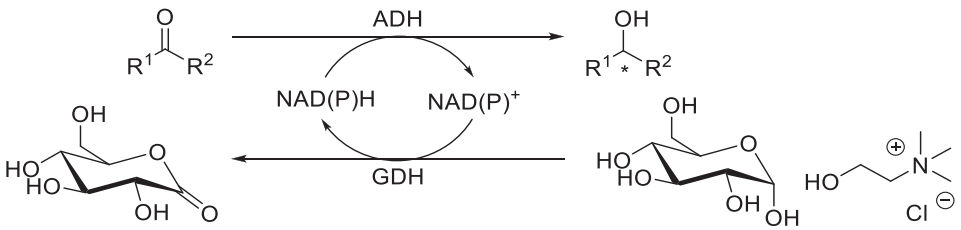
Bioreductions of carbonyl compounds using DES using GSH-based DES as cosolvents.

In the search for more environmentally friendly solvents, the use of a novel oligopeptide-based DES as cosolvent has been reported in the stereoselective enzyme-catalyzed reductions of thee selected substrates, including an arylketone, a ketoester and a ketoacid [80]. This DES contains ChCl and glutathione (GSH, comprised of Glu, Cys, and Gly, Scheme 16.33) and helped in the reduction of 3,5-bis(trifluoromethyl)acetophenone, 5-(4-fluorophenyl) − 5-oxopentanoic acid and ethyl acetoacetate, especially at higher substrate concentrations. Although it was only used in low ratios (0.5%−1.5% vol), the use of DES allowed the achievement of higher conversions in shorter reactions times, maintaining the enzyme selectivity. These achievements were explained based on the fact that the GSH forms hydrogen bonds with ChCl, as well as with the carbonyl group oxygen of the ketone, increasing its electrophilicity, thus facilitating proton acceptance from the reduced cofactor and promoting coenzyme regeneration.

Glucose dehydrogenase is an enzyme usually employed for reduced nicotinamide cofactor purposes at the expense of D-glucose oxidation into D-glucono-1,5-lactone that later spontaneously

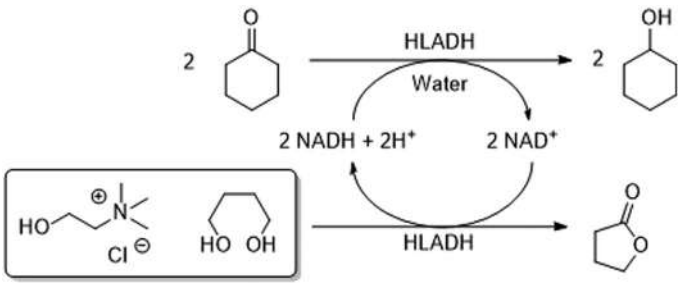
hydrolyzes toward the formation of D-gluconic acid. The possibility to use D-glucose as a HBD in a designer NADES has been reported, offering solutions for the bioreduction of ketones (Scheme 16.34) [81]. Thus, a higher solubility of ketones and a more efficient cofactor regeneration system is possible when combining an aqueous buffer (50 mM Tris.HCl, pH 7.5) and ChCl:Glc (1.5:1 mol/mol), Glc serving as a cosubstrate for several ADHs including *Lb*ADH, *Ras*ADH and ADH from *Thermoanaerobacter* sp. (ADH-T) in the bioreduction acetophenone, propiophenone and 2-octanone, respectively. The beneficial effect of this system was demonstrated at variable DES concentrations (10%−30%) and substrate concentrations (25−200 mM), finding great improvements at 50 and 100 mM ketones concentration compared to the reactions in buffer (83%−100% vs 59%−81% conversion), and maintaining the complete stereoselectivity.

Kara and coworkers were very active in the design of designer DES for bioreduction process, going through molecular dynamics simulations to quantify the molecular flexibility, hydration layer, and intraprotein hydrogen bonds of the HLADH [82]. The benchmark reaction considered was the transformation of cyclohexanone into cyclohexanol coupled with butane-1,4-diol (BD) as "smart cosubstrate" for cofactor regeneration (Scheme 16.35), which allows shifting the thermodynamic equilibrium to the alcohol side. The synthetic application was expanded to other substrates, and although benzaldehyde and ethyl 4-chloro-3-oxobutanoate did not lead to any conversion due to solubility issues, cinnamaldehyde was reduced to cinnamyl alcohol, a relevant compound in the aroma industry, in low extension (25%) after 48 h [83].



SCHEME 16.34

Use of glucose-based DES as designer cosolvents in the bioreduction of ketones.



SCHEME 16.35

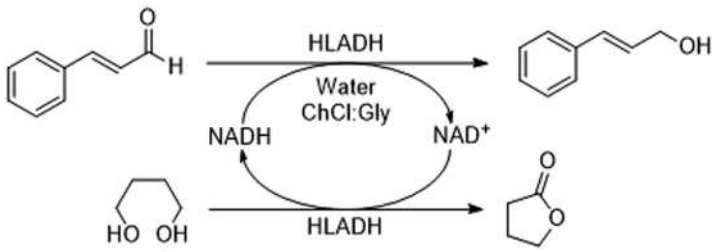
Use of 1,4-butanediol-based DES as designer cosolvents in the bioreduction of ketones.

The reduction of cinnamaldehyde to cinnamyl alcohol catalyzed by HLADH using 1,4-butanediol as cosubstrate for NADH recycling in varying mixtures of ChCl:Gly-water has been recently studied in depth (Scheme 16.36) [84]. Conversions led to up to 40 mM cinnamyl alcohol in the ChCl:Gly (1:9 mol/mol) mixture containing 20% vol of water within 8 h, compared with less than 10 mM in the when ChCl:Gly (1:2 mol/mol) was attempted, the higher glycerol content seemed to promoted higher conversions for HLADH.

Much less explored is the reduction of carbon−carbon double bonds using ene-reductases, this is the case of the Δ1,2-dehydrogeneration of cortisone acetate to prednisone acetate by using an immobilized version of the *Arthrobacter simplex* (Scheme 16.37) [85]. Three ChCl-based DES were used as cosolvents (4%−6% vol, HBD: Gly, EG and U, 32%−92% conversion), ChCl:U providing a higher conversion in comparison with the reaction in buffer (68%). The main advantages of using DES are: (1) the achievement of a better solubility of the cortisone acetate, as occurred with ethanol as cosolvent, running the reaction at 5 g/L substrate concentration; and (2) the possibility to reuse the enzyme, which was possible during 5 cycles, leading to 82%−93% conversion range.

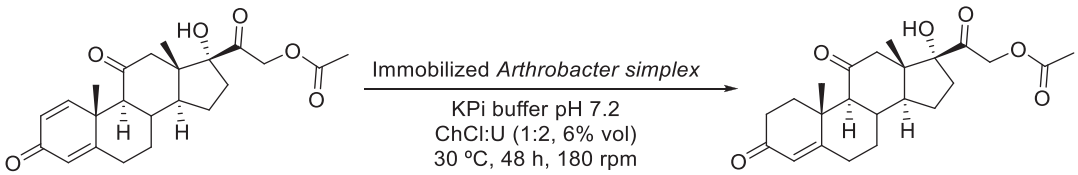
16.3.2 Oxidations

Reduction processes have been previously presented as ideal transformations to afford chiral alcohols in aqueous-DES mixtures. Next, their reverse reactions, alcohol oxidation for the formation of carbonyl compounds, are here described involving ADHs, catalases, laccases and oxidases.



SCHEME 16.36

Reduction of cinnamaldehyde catalyzed by HLADH using 1,4-butanediol as cosubstrate for NADH recycling in varying mixtures of DES-water.



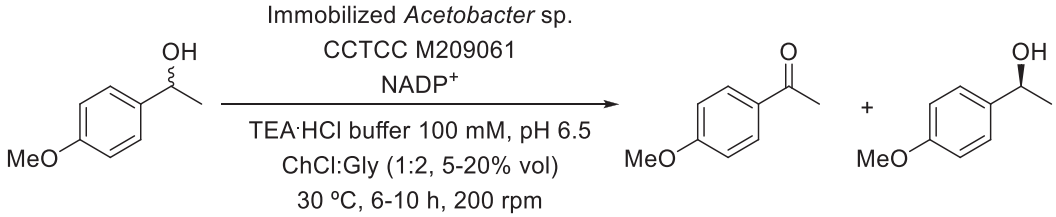
SCHEME 16.37

Bioreduction of cortisone acetate to prednisone acetate using DES as cosolvents.

However, despite the increasing literature in the field of oxidative transformations in neoteric solvents [86,87], the oxidation of alcohol in DES media remains nowadays in a premature stage because most of the reported works lacks organic synthetic applications in terms of reaction yields and product isolation, just focusing on kinetic and stability studies.

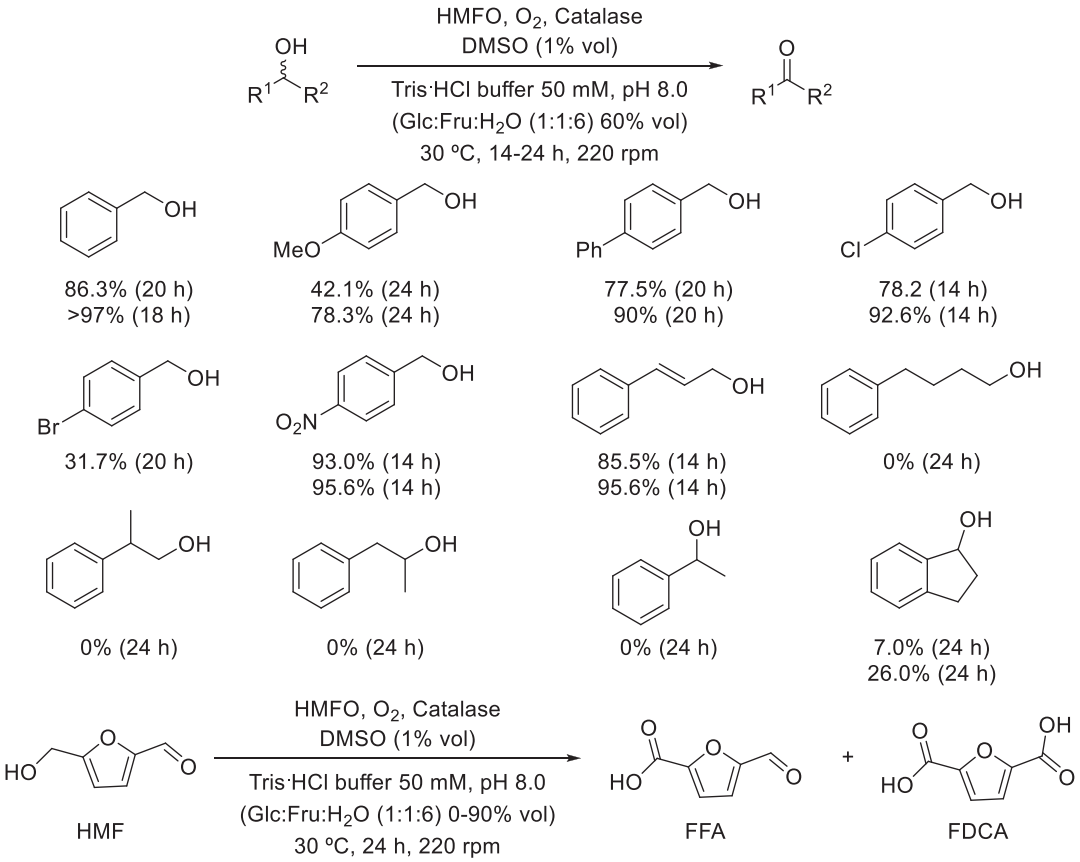
Kinetic resolution of secondary alcohols using oxidative enzymes constitutes an elegant manner to access to optically active alcohols, although the maximum conversion into an enantiomerically pure product is limited to 50%. Free and immobilized whole cells of *Acetobacter* sp. CCTCC M209061 were used in the resolution of 1-(4-methoxyphenyl)ethanol using a combination of TEA. HCl buffer and six different DES (Scheme 16.38) [88]. Selecting the immobilized biocatalysts, ChCl:Gly (1:2 mol/mol) in a 20% vol led to the best results, reaching a 49.4% conversion into the 1-(4-methoxyphenyl)ethanone, and recovering the (*S*) − 1-(4-methoxyphenyl)ethanol in 98.7% *ee*. Optimization of the reaction conditions were explored in terms of: (1) DES concentration, finding the optimal conditions at 5% vol for a 50% conversion after 9 h at 30°C; and (2) substrate concentration, increasing from 30 to 55 mM for the 500 mL preparative scale transformation, obtaining a 51.5% conversion after 7 h, and isolating the (*S*)-alcohol in enantiomerically pure form, while higher alcohol concentrations (60 and 65 mM) led to a decrease in the conversion (48.3−49.2%) and substrate enantiomeric excess (94.5%−97.8%). The same authors further investigated this oxidative resolution mixing the buffer with different ILs (12%−30% vol) to improve the productivity of the system [89]. Best results were found with 1-butyl-3-methylimidazolium hexafluorophosphate [bmimPF_6, 20% vol], obtaining a significant improvement when also the ChCl:Gly (8% vol) was also added, especially in terms of substrate concentration that was successfully increased until 80 mM (51.3% conversion, >99% *ee* of (*S*)-alcohol after 7 h at 30°C and 220 rpm).

The beneficial effect of DES as cosolvents has been recently reported in the nonselective oxidation of a series of primary and secondary alcohols using the 5-hydroxymethylfurfural oxidase (HMFO) in comparison with the use of plain buffer or in the presence of DMSO (1% vol) [90]. After testing a series of (natural) DES, in some cases higher conversions into the corresponding carbonyl compounds were obtained (Scheme 16.39). Best results were found using a Glc:Fru:H_2O (1:1:6 v/v/v) mixture, particularly studying the oxidation of 5′-hydroxymethyl furfural (HMF) into 5-formylfuran-2-carboxylic acid (FFA) and furan-2,5-dicarboxylic acid (FDCA), the latter platform molecule for the preparation of biobased polymers. Interestingly, when the reaction was performed in buffer only, the formation of FFA was highly favored (84%), whereas 16% of the FDCA was obtained. Complete conversions were also found at 30% and 60% of DES, finding the highest formation of FDCA (31%) at 60% DES after 24 h.



SCHEME 16.38

Kinetic oxidative resolution of racemic 1-(4-methoxyphenyl)ethanol.

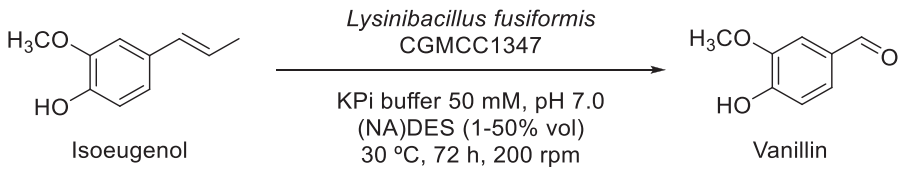


SCHEME 16.39

HMFO-catalyzed oxidation or primary and secondary alcohols in buffer-NADES mixtures. Top row corresponds with the reaction in the absence of DES, while in those cases reported, the bottom row corresponds to the reaction in the presence of DES.

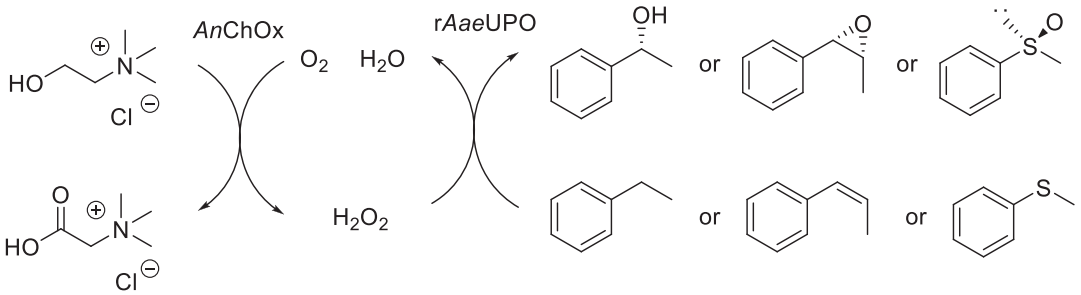
16.3.3 Hydroxyfunctionalization and dioxygenation reactions

The last section of the use of redox enzymes in DES media deals with the insertion of oxygen atoms or molecules using different enzyme classes. Enzymatic alkene oxidative-cleavage has been reported using *Lysinibacillus fusiformis* cells CGMCC1347 in whole cells form, converting isoeugenol to vanillin (Scheme 16.40) [91]. A total of 24 DES and 21 NADES were employed as cosolvents, finding some trends, for instance: (1) ChAc-based DES reached higher conversions than the ones attained with ChCl-based ones; and (2) Sugar DES provided better results than alcohol and organic acid HBD components. A 20% vol of NADES provide a beneficial effect, improving the yields especially with ChCl:lactose (4:1) and ChCl:raffinose (11:2), affording 132% and 131% higher conversions when compared to buffer only. Immobilization of the cells on poly(vinyl



SCHEME 16.40

Enzymatic alkene oxidative-cleavage of isoeugenol to vanillin.



SCHEME 16.41

Hydroxyfunctionalization of benzenes and alkenes using designers DES.

alcohol)-alginate beads led to higher conversion in both DES and NADES., observing the best results with ChAc-based DES that retained for at least 13 cycles its activity in 72 h reactions, displaying good operational stability. The same authors reported an experimental and mechanistic study of the same transformation using a lipoxygenase (EC 1.13.11.12), non heme, iron-containing dioxygenase [92]. The biotransformation was developed in borate buffer (200 mM, pH 9.0) and the presence of different additives including surfactants (6), organic solvents (10), DES (8) and NADES (13). Best results were obtained in DES and NADES, obtaining slightly better results in the reaction with 20% of ChCl:So (5:2 mol/mol) than in the reaction with pure buffer.

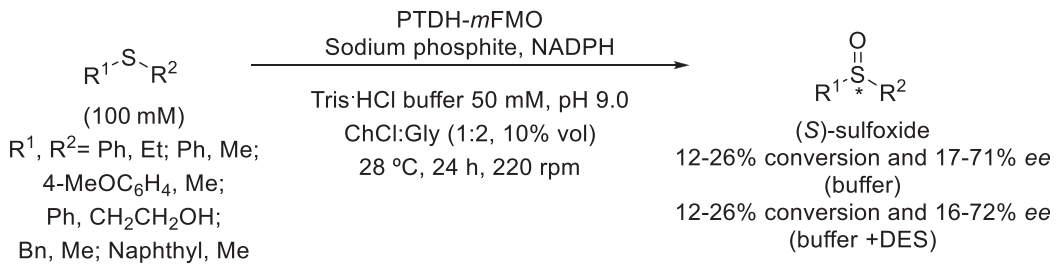
As occurred with the use of designer DES for bioreduction processes, their application as more than cosolvents and stabilizers have been also recently reported in oxygenation reactions, where the ChCl-based DES act as sacrificial electron donor for the in situ H_2O_2 generation (Scheme 16.41) [93]. Thus, the transformation of ethyl benzene to 1-phenyethanol, or *cis*-β-methylstyrene into epoxides is possible by in situ H_2O_2 generation using the choline oxidase from *A. nicotianae* (*An*ChOx) that converts choline into betaine, and the concomitant generation of 2 equiv of H_2O_2 to drive the corresponding peroxygenase-catalyzed oxyfunctionalization using the recombinant evolved peroxygenase from *Agrocybe aegerita* (r*Aae*UPO). In this manner, the situ hydrogen peroxide formation is leveled in the presence of the unspecific peroxygenase, avoiding oxidative inactive of the biocatalyst. Reactions were carried out in phosphate buffer pH 7.0 employing different (NA) DES sources and concentrations (up to 50%), and seem to be applicable in to other type of transformation such as sulfoxidation reactions as described below.

Finally, examples of sulfoxidation reactions involving DES have been included, since (chiral) sulfoxides are important organic compounds with wide applications in medicinal and organic chemistry [94]. Very recently, Hollmann and coworkers extended the potential of choline-based DES in oxygenation reactions (Scheme 16.41), by studying their participation in the sulfoxidation of methyl phenyl sulfide (35 mM), also known as thioanisole [95]. The best results were found using equal volumes of phosphate buffer and (NA)DES when considering urea as HBD and a temperature of 30°C.

Finally, the benefits of using NADES in the asymmetric sulfoxidation of six sulfides have been reported by de Gonzalo (Scheme 16.42) [96], employing the flavin-containing monooxygenase from *Methylophaga* sp. strain SK1 (mFMO) fused to phosphite dehydrogenase for cofactor regeneration purposes (PTDH-mFMO). Ethyl phenyl sulfide was considered as a benchmark substrate for the optimization of the reaction conditions and analyze the effect of the addition of DES in the reaction medium, which was mostly negative in most of the cases at variable substrate concentrations (10−200 mM). Only the use of ChCl:EG (1:2 mol/mol) or ChCl:Gly (1:2 mol/mol) up to 10% v/v led to comparable reaction results as for the one carried out in pure buffer, and a similar trend was observed with other sulfides, for which the use of NADES has only minimum benefits at high substrate concentrations.

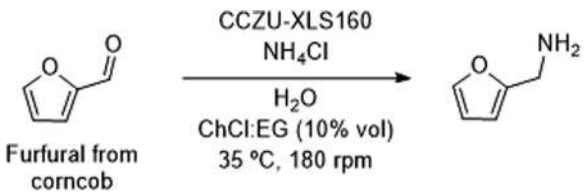
16.4 Lyases and transferases in single transformations using deep eutectic solvents as solvents

Apart from oxidoreductases (EC.1) and hydrolases (EC.3), transferases (EC.2) and lyases (EC.4) provide sustainable solutions for synthetic transformations, although their applications in DES has been scarcely reported in the literature. Inside the transferase class, there is only example dealing with individual transformations developed in DES consisting in the production of furfurylamine from biomass containing furfural (Scheme 16.43) [97]. For that purpose, a recombinant *E. coli* CCZU-XLS160 whole cells harboring ω-transaminase and L-alanine dehydrogenase was constructed and used in combination with inexpensive ammonium chloride as amine source. Thus, the transaminase was able to catalyze the furfural amination in ChCl:EG-water (1:9 v/v) at 35°C, while the L-alanine dehydrogenase was responsible for the transformation of the formed pyruvate into



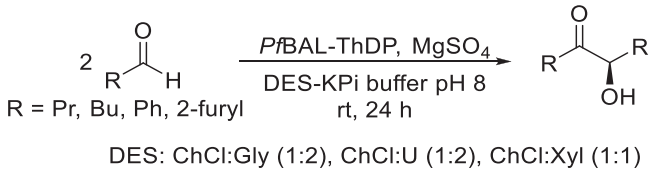
SCHEME 16.42

Enzymatic asymmetric sulfoxidation in buffer and DES-buffer systems.



SCHEME 16.43

Transaminase-catalyzed amination of furfural derived from biomass to furfurylamine.



SCHEME 16.44

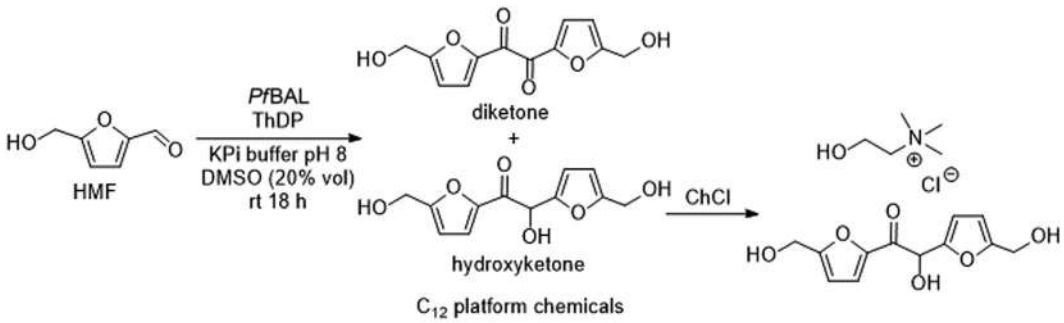
BAL-catalyzed self-condensation of aliphatic or aromatic aldehydes in DES-buffer mixtures.

L-alanine. Overall, 92.3 mM of furfural derived from corncob was entirely transformed to furfurylamine with a productivity of 0.39 g of amine/g xylan in corncob.

Inside the lyase class, the use of benzaldehyde lyases (BALs) and phenolic acid decarboxylases (PADs) has been reported. For instance, Domínguez de María and coworkers reported the use of a thiamine-diphosphate-dependent BAL from *Pseudomonas fluorescens* (*Pf*BAL) in DES-water mixtures for the carboligation of a series of aldehydes (Scheme 16.44) [98]. The reactions were explored in three different ChCl-based DES including Gly (1:2 mol/mol), U (1:2) or Xyl (1:1) as HBDs and variable amount of water (0%−100%), obtaining high to excellent conversions for self-condensation of two aldehydes molecules using ChCl:Gly (1:2)-phosphate buffer (6:4 v/v), and also variable selectivity levels toward the corresponding (*R*)-enantiomers after 24 h at room temperature: butyraldehyde (96% conversion, 52% *ee*), valeraldehyde (98% conversion, 27% *ee*), benzaldehyde (>99% conversion, >99% *ee*), and 2-furaldehyde (75% conversion, 63% *ee*). A decrease of the enzyme activity was found at higher ChCl:Gly percentages, being almost negligible at 90% DES content. However, a full conversion was achieved in the reaction with valeraldehyde when changing to the ChCl:U (1:2 mol/mol) in a 70% ratio.

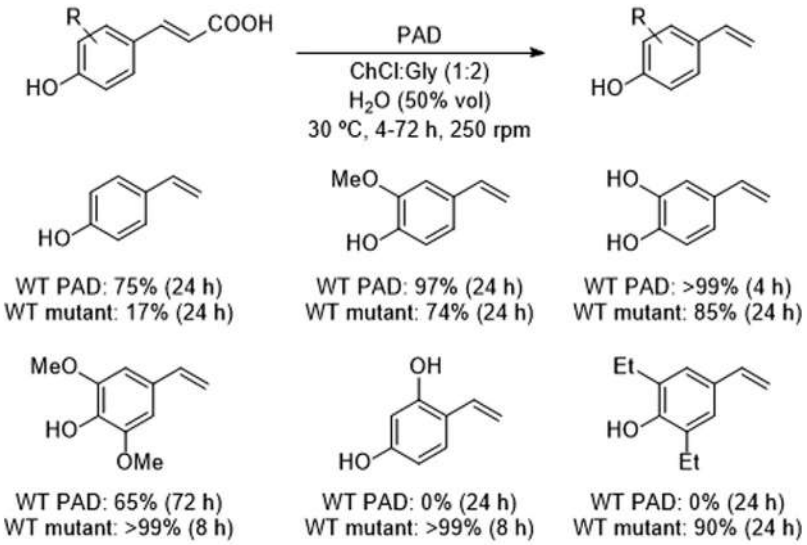
The same research group has reported the use of the *Pf*BAL for the umpolung carboligation of furfural, hydroxymethylfurfural (HMF) and mixtures of them in pure aqueous systems, providing a new C_{10}−C_{12} platform of chemicals (Scheme 16.45) [99]. The so-obtained hydroxyl ketones were later applied as HBDs for the synthesis of novel DES, although their application in enzyme-catalyzed transformations was not tested.

More recently, the use of a phenolic acid decarboxylase from *Bacillus subtilis* (*Bs*PAD) wild-type (PAD WT) and its I85A mutant (PAD mutant) has been explored for the conversion of *p*-hydroxycinnamic acid derivatives to the corresponding *p*-hydroxystyrenes using ChCl-based DES (U, So and Gly as HBDs)-water binary systems (Scheme 16.46) [100]. The DES helps to solubilize



SCHEME 16.45

BAL-catalyzed carboligation of HMF, and later mixing of the hydroxyl ketone with ChCl for the formation of novel DES.



SCHEME 16.46

Conversion values of the *Bs*PAD-catalyzed decarboxylation of *p*-hydroxycinnamic acids performed at 300 mM substrate concentration, except for the 2,6-diethyl-4-vinylphenol formation, that was performed at 10 mM substrate concentration and with addition of DMSO (1%) to favor the solubility of the starting cinnamic acid.

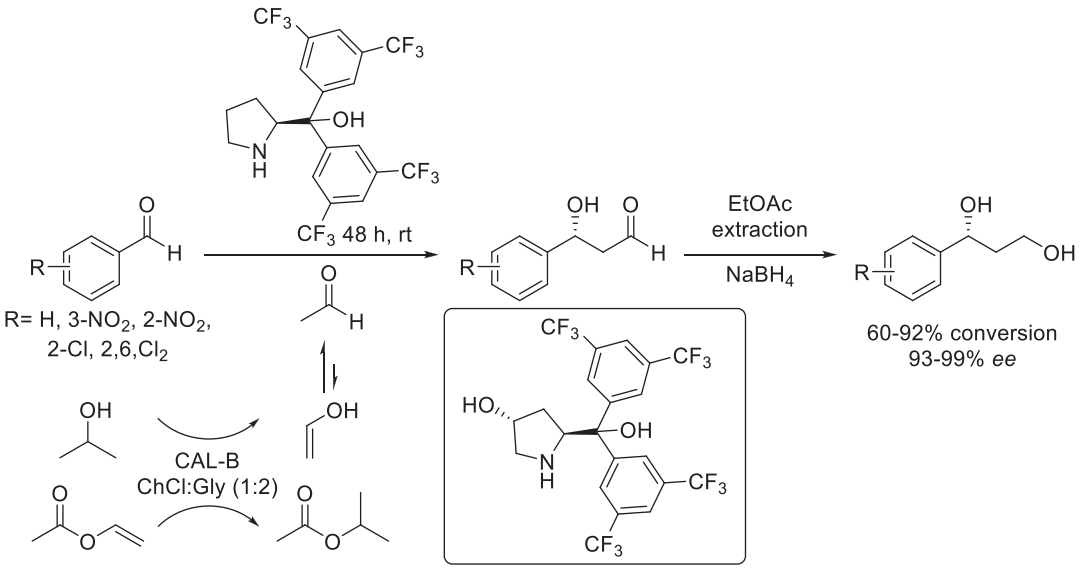
these substrates making possible reactions with substrate concentrations up to 300 mM, studying the influence of water in different percentage (0%−50%). Although, the reactivity of the six tested cinnamic acids was very different, a general methodology was disclosed attaining good to quantitative conversions either with the wild type or with the evolved PAD variant.

16.5 Multicatalytic transformations

The use of single enzyme transformations in DES systems and also in some cases multienzymatic transformations to shift the reaction equilibrium or allowing the cofactor recycling is disclosed, presenting the combination of both enzymes and DES as ideal catalytic systems for practical applications. Next, examples of cascade process developed in a sequential or concurrent manner will be discussed, demonstrating that enzymes can be also used together with organocatalysts and metal species.

16.5.1 Combination of enzymes and organocatalysts

The use of organocatalysts in DES has attracted recent attention mainly for aldol and Michael-type additions [101,102], the combination with enzymes being possible through cascade process, until now when using hydrolases for determined transformations. Domínguez de María and coworkers developed the in situ formation of acetaldehyde through the CAL-B catalyzed transesterification of 2-propanol (3 mmol) with vinyl acetate (3 mmol) in ChCl:Gly (1:2 mol/mol) at room temperature (Scheme 16.47), which subsequently reacted with 4-nitrobenzaldehyde (1 mmol, 1 M) in the presence of a highly substituted proline derivative (0.2 mmol) [103]. Thus, 1-(4-nitrophenyl)propane-1,3-diol was obtained in 92% conversion (70% isolated yield after liquid-liquid extraction with ethyl acetate, EtOAc) and 95% *ee* after reduction of the aldehyde intermediate with sodium borohydride (6 mmol). Recycling experiments of the DES and CAL-B system were performed, after



SCHEME 16.47

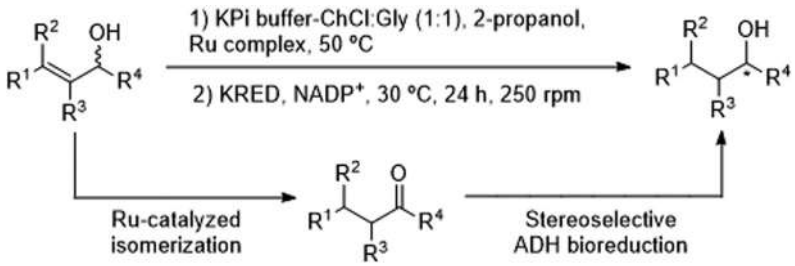
Use of lipase-diaryl prolinol catalytic systems for cross-aldol reactions between aromatic aldehydes and in situ generated acetaldehyde.

recovering the desired hydroxy aldehyde by liquid-liquid extraction with EtOAc, without requiring the addition of the organocatalyst for two cycles. The methodology resulted to be general when applied to other aromatic aldehydes (60%−85% conversion and 93%−99% *ee*), although it failed when attempting an heteroaromatic aldehyde or cinnamaldehydes (<42% conversion). The same research group proposed the use of a structurally similar organocatalyst containing an extra hydroxyl group at the C-3 position of the pyrrolidine ring (boxed in Scheme 16.47) [104], which allows a stronger interaction with the DES, thus improving the conversion and recycling possibilities of the catalytic system for the tandem reaction between 2-propanol, vinyl acetate and 4-nitrobenzaldehyde accelerated by CAL-B in ChCl:Gly (1:2 mol/mol).

16.5.2 Combination of enzymes and metal species

Biocatalysis and metal catalysis are pivotal strategies for the synthesis of organic compounds, usually requiring very different reaction conditions for the correct action of both types of catalysis. In the search for ideal reaction conditions to pair the exquisite selective displayed by biological catalyst with the broad range of applications that offer chemical catalysts, DES has emerged as environmentally friendly reaction medium, or at least part of it in combination with water [105]. Next, a series of cascade metal−enzyme transformations will be discussed including the use of metal complexes derived from palladium or ruthenium, and enzymes such as ADHs, amine transaminases and phenolic acid decarboxylases.

The first application of DES as cosolvents for the combination of biological and metal catalysts consisted of the ruthenium-catalyzed isomerization of racemic allylic alcohols followed by stereoselective bioreduction of the resulting unsaturated ketone intermediates (Scheme 16.48) [106]. Prior to studying the one-pot two-step approach, each of the two steps were studied individually with five ChCl-based DES, finding the best results with the mixture ChCl:Gly (1:2 mol/mol). When applying the multicatalytic approach, the proper selection of ADH allowed the production of both alcohol enantiomers with excellent stereoselection (93%−>99% *ee*) and variable isolated yield depending on the substrate structure (65%−100% conversion) in the one-pot sequential approach. The concurrent strategy was also successfully accomplished when using the commercially available KRED-P2-C11 (68%−98% conversion, >99% *ee*), although failed for the *Lactobacillus kefir* ADH



SCHEME 16.48

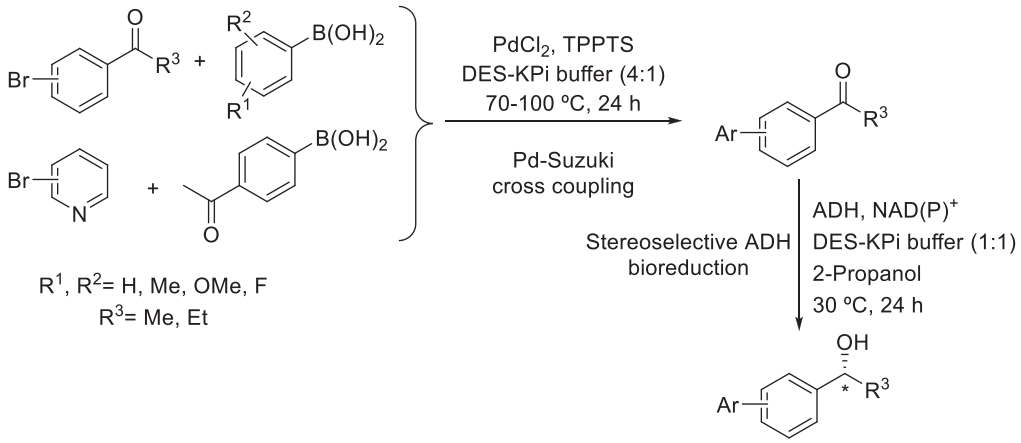
Chemoenzymatic one-pot isomerization-bioreduction sequence developed in a DES-buffer system.

heterologously expressed in *E. coli* since only 21% of the enantiopure (*R*) − 1-phenylpropan-1-ol was formed.

Another metal-catalyzed transformation combined with the stereoselective action of ADHs using DES as part of the solvent is the one consisting in the palladium-catalyzed Suzuki-cross coupling of (het)aryl bromides with arylboronic acids (Scheme 16.49) [107]. The use of DES such as ChCl:Gly (1:2 mol/mol) allowed to work with high substrate concentrations for the overall process (200 mM for the initial Suzuki-cross coupling and then adjusted to 75 mM for the bioreduction step), yielding a series of chiral biaryl compounds in enantiopure form. Apart from the different ideal substrate concentrations for both steps, the approach was developed in a sequential manner because of the high temperatures required for the Suzuki-cross coupling (70°C or 100°C) that are incompatible with the correct ADH action, *L. kefir* to provide access to the (*R*)-alcohols and ADH-A from *R. ruber* for the (*S*)-enantiomers.

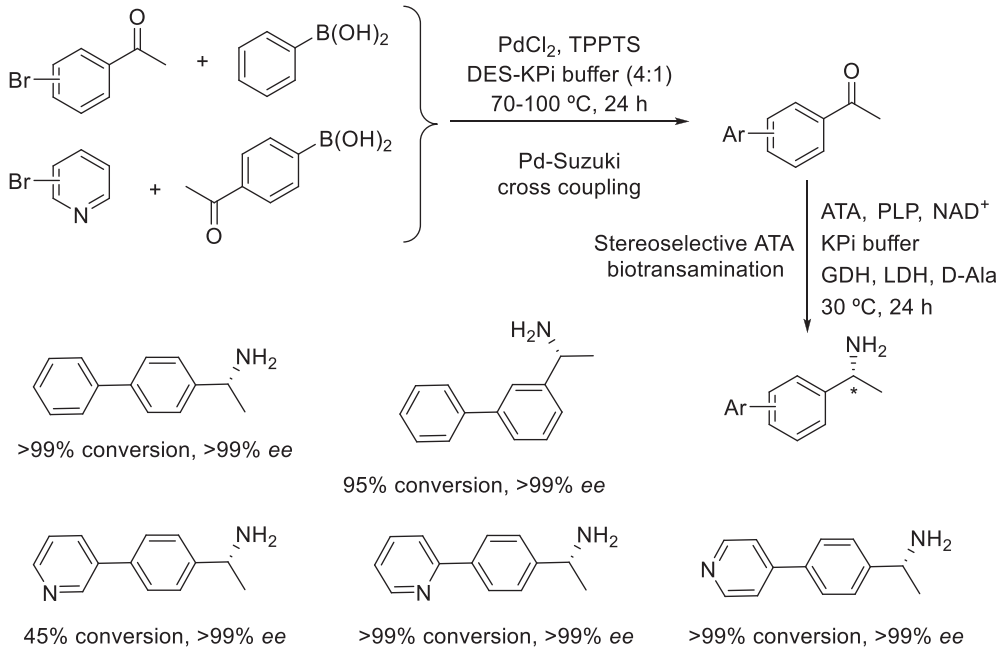
Remarkably and following a similar strategy, the combination of palladium chloride and an amine transaminase was successfully developed by the same authors in a sequential manner for the production of a series of (hetero)aromatic chiral amines, the biotransamination of the biaryl ketone intermediates being possible using D-alanine as amine donor and *Exophiala xenobiotica* wild-type ω-transaminase (EX-ω-TA) or its T273S mutant (EX-STA) as enzyme (Scheme 16.50) [108].

Very recently, a mixture of ChCl-based DES and water has been used first in batch and later in continuous flow for the development of an enzyme-metal sequence to produce the (*E*) − 4-hydroxy-stilbene (Scheme 16.51) [109]. After optimization of the reaction conditions, the continuous mode approach involves the use of an immobilized phenolic acid decarboxylase (PAD) for the decarboxylation of *p*-coumaric acid (30 min for a space-time-yield of 4.8 g/Lh) and subsequent Pd-catalyzed Heck cross coupling with iodobenzene (45 min for a space-time-yield of 0.52 g/Lh). Importantly, the use of DES allowed the development of the tandem enzymatic decarboxylation/Heck coupling in a 20 mM substrate concentration, compared with a maximum 5% achieved in



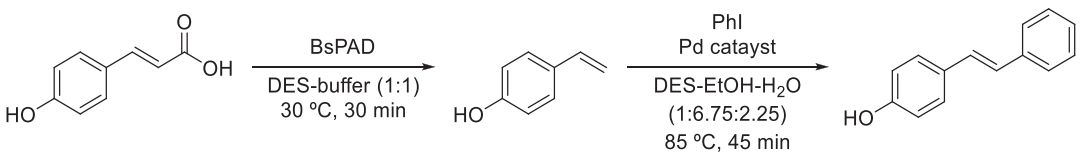
SCHEME 16.49

Chemoenzymatic sequence involving a Suzuki-cross coupling followed by bioreduction of the intermediate biaryl ketones developed in a DES-buffer system.



SCHEME 16.50

Chemoenzymatic sequence involving a Suzuki-cross coupling followed by biotransamination of the intermediate biaryl ketones developed in a DES-buffer system.

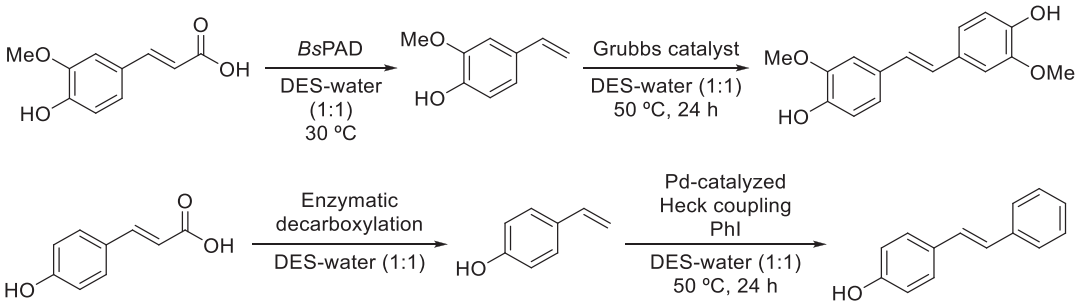


SCHEME 16.51

Chemoenzymatic synthesis of (*E*) − 4-hydroxy-stilbene under flow conditions via PAD-catalyzed decarboxylation of *p*-coumaric acid followed by Pd-catalyzed Heck cross coupling of 4-vinylphenol intermediate with iodobenzene.

water, tackling the incompatibility between both catalysts, and avoiding the isolation of reaction intermediates that are prone to polymerize, thus obtaining a higher yield (20%) than initially expected.

Finally, two independent strategies have been recently explored involving the combination of *Bs*PAD with palladium and ruthenium complexes for the synthesis of biaryl derivatives in DES-water systems (1:1 v/v) [110]. On one hand, the enzymatic decarboxylation of ferulic acid was followed by ruthenium-catalyzed metathesis of the resulting olefin using the Grubbs catalyst,



SCHEME 16.52

Synthesis of stilbenes via enzyme-metal cascades developed in a sequential manner using DES-water systems.

obtaining the corresponding stilbene in only 15% yield (Scheme 16.52 *top*). On the other hand, the aforementioned enzymatic decarboxylation reaction was coupled to a Heck-type C−C coupling using iodobenzene (Scheme 16.52 *bottom*). In this case, the PAD was immobilized onto a solid support and activated with tertiary amine groups (PAD@EC-TEA), giving a lower specific activity than the soluble enzyme but exhibiting and excellent decarboxylation activity in the ChCl:Gly (1:2 mol/mol):water (1:1) system, achieving full conversion after 2 h at a 200 mM substrate concentration. Then after enzyme filtration, the Heck coupling with iodobenzene was developed but no conversion was found possibly due to the partial elution of the *Bs*PAD by the DES, as no problems were observed when water was used as solvent for the first step, leading to a 60% overall yield.

16.6 Conclusions and perspectives

In this chapter, we have focused on showcasing reaction yields or conversions of biocatalytic reactions performed in pure DES and DES-aqueous media mixtures to highlight their applicability in organic synthesis. DES provide advantages as alternatives to organic solvents by enhancing enzyme activity and stability, yield, stereopreference, with higher substrate solubility, while remaining innocuous for the biocatalyst, thus leading to higher yields and a good perspective for synthetic applications. The synergy between biocatalysts and DES contributes to the sustainable production of various industrially relevant compounds with high yields and selectivities, transformations that can be developed in a single transformation or by means of successive steps through chemoenzymatic multicatalytic cascades. Future studies on exploring and exploiting the use of DES as the substrate source itself would be attractive to further implement.

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CHAPTER

Biocatalysis and green solvents: trends, needs, and opportunities 17

Pablo Domínguez de María
Sustainable Momentum, SL., Las Palmas de Gran Canaria, Spain

17.1 On the need of using green solvents to reach truly sustainable processes

Over the last decades solvents have been addressed as one of the largest contributors to the environmental impact of chemical processes [1–3]. This may account for the need of using solvents as the reaction media—to dissolve reagents or products properly—, or for their use as extractive materials during the downstream processing units, to deliver the final (pure) product out of the reactive phase(s). Because of that impact, and fueled by environmental concerns, considerable research is being devoted to generating (and also designing) new solvents that may become useful synthetic solutions, while being environmentally friendly at the same time. Generally speaking, research and development actions are being taken mostly in the production of biogenic solvents—from renewable resources—, as well as in the design of neoteric solvents that can display tuneable properties, to be tailored for specific applications (e.g., deep eutectic solvents, DES, and latest generations of ionic liquids). Also, strategies comprising supercritical fluids as solvents often provide promising figures for being sustainable and effective at the same time (e.g., the case of supercritical CO_2 as media or as extractive for biocatalytic reactions). Likewise, solvent-free processes are gaining a preponderant space in research initiatives, given their huge potential as ecologic and economic systems. Last but not least, studies related to the (eco)toxicology of the new solvents are being conducted as well. Overall, a holistic and critical view of the solvents as an important part of the environmental life-cycle of a given chemical process is gaining space rapidly.

With a broad vision, this book provides a detailed picture of the use of sustainable solvents for enzymatic reactions, covering solvent types, applications, and different synthetic processes. In fact, biocatalysis may become a very relevant discipline to reach green chemistry targets, as biocatalysis *per se* fulfills many of the green chemistry postulates (biodegradability, mild reaction conditions, catalytic systems, etc.) [1–3]. Albeit in many cases the reported enzymatic reactions have not been adequately set for being truly sustainable (e.g., providing very low substrate concentrations, or ineffective long reaction times [2]), the field seems to be developing fast, and an increasing number of biocatalytic applications can be found at industrial level, covering a broad range of chemical conversions [4–6]. Herein, the role of sustainable biocatalysis is gaining importance as well [7,8]. Key-to-success is the combination of using benign solvents—or even better, solvent-free

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processes—, together with high productivity metrics, gathering industrial interests with intensified catalytic systems and sustainable solvents [2]. In particular, the judicious choice of environmentally friendly solvents with biocatalysis and continuous processes may lead to powerful sustainability synergies that can pave the way for future clean(er) chemical strategies [9].

The quest for identifying and use of green solvents in enzymatic reactions has been clearly targeted as an important topic by the scientific community. In that respect, when a literature survey is performed, searching for the combined terms "biocatalysis" and "green solvents," a clear exponential trend in published articles is observed over the years (Fig. 17.1, covering the years 2000−21). Thus, whereas only few hundreds of articles were published yearly two decades ago, in recent times more than four thousand articles have been consistently delivered per year. This gives a clear hint on the importance of the use of green solvents in biocatalysis (although, arguably, it must be assumed that a proportion of these articles may have used the term "green solvents" in a rather generic form only). Nonetheless, and to reinforce the interest, recent (industrial) trends also suggest implementing non-conventional media for biocatalysis (instead of water), to provide intensified processes with high substrate loadings and less water consumption, than can be scaled up properly at commercial level [10].

In the next sections, different aspects related to the use of green solvents in biocatalysis will be discussed. Topics will cover the recent use of solvent-free processes for enzymatic reactions (outside hydrolases), and considerations on the use of some biogenic solvents (derived from biomass), namely 2-methyltetrahydrofuran (2-MeTHF), cyclopentyl-methyl ether (CPME) and Cyrene, with some relevant case studies to be described. Moreover, some recent concepts on the emerging deep

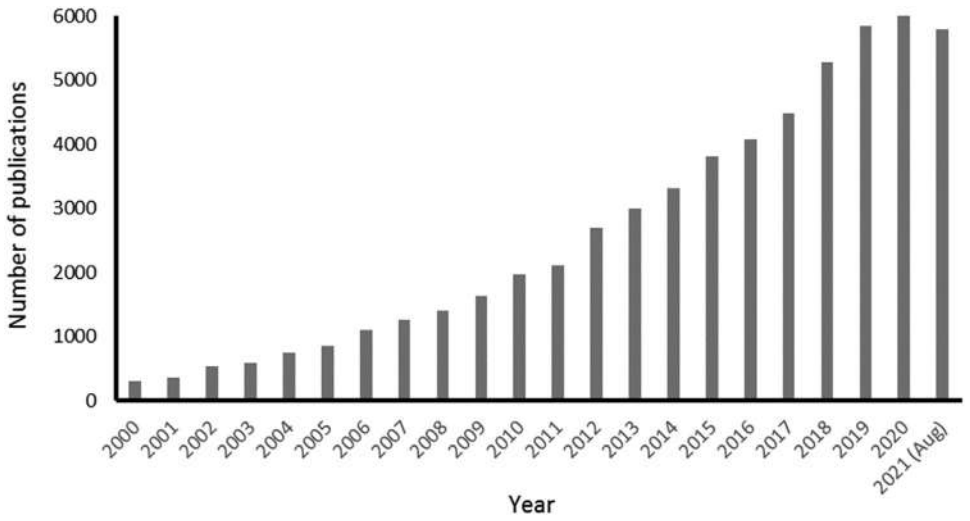


FIGURE 17.1

Number of articles yearly retrieved from the combined search "biocatalysis" and "green solvents" over the last two decades. Search in 2021 is until the end of august.

From Google Scholar (accessed: Beginning of September 2021).

eutectic solvents will be discussed, as prototypical examples of highly versatile promising solutions for sustainable chemistry. When possible, a critical vista on what is (still) needed to further develop the field of biocatalysis and green solvents will be given as well.

17.2 Solvent-free processes in biocatalysis: keeping things as simple as possible, but not simpler!

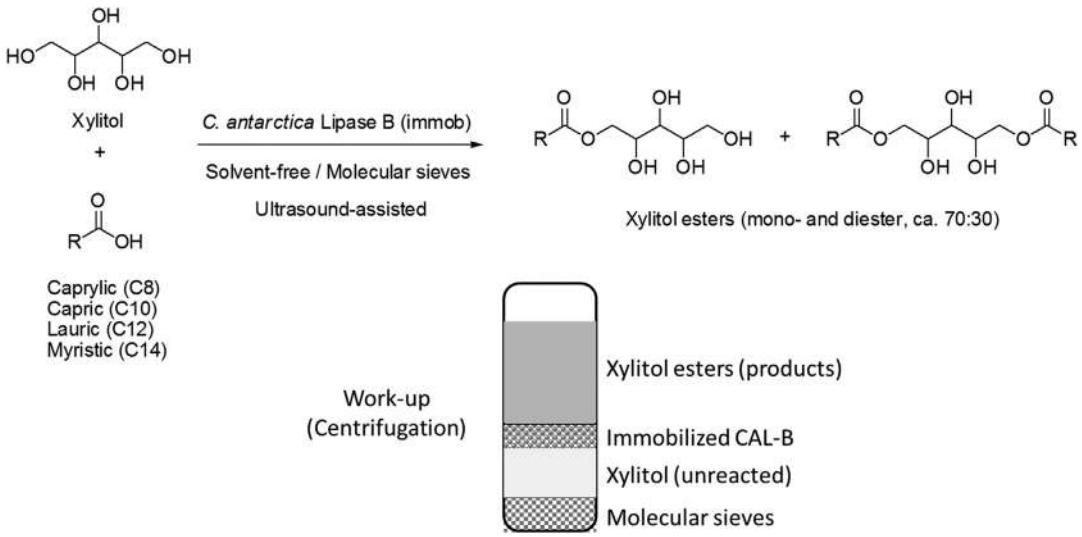
Surely the best approach for a (bio)catalytic reaction is a solvent-free process, in which no solvents are employed at all, and the reaction media is simply composed by the substrates and the suspended (bio)catalyst [1,2,10]. In these cases, substrate loadings are expected to be the highest possible (providing largely improved metrics and productivities), and virtually no wastes associated to solvents or wastewater are generated in the reaction. Furthermore, for these strategies the downstream processing results often straightforward as well (e.g., the product is directly obtained upon filtration of the catalyst). Thus, if every process could be set in solvent-free conditions, there would not be need for considering any other option for the reaction media. However, it must be noted that the set-up of solvent-free systems is not always feasible. For instance, reagents may be solid, or they can display unpaired solubilities among them, generating mass transfer limitations and suboptimal reaction conditions. Ultimately, and for a proper synthetic route, this will force to the use of a solvent. Moreover, not all (bio)catalysts remain stable in such reaction systems, and many enzymes tend to be deactivated in those media, hampering the approach. Thus, despite the potential of solvent-free concepts for biocatalysis, reported examples have been hitherto somewhat scarce, and mostly related to hydrolases, as paradigm of robust enzymes that can perform synthetic reactions in virtually any anhydrous media. Noteworthy, research studies trying to design novel variants with improved performances in solvent-free mixtures are not commonly found in the literature either. Some few academic initiatives have tried to shed light on the reasons of why some enzymes can remain active in solvents (or in solvent-free media), and how these structural motifs could be used as inspiration to translate the catalytic performance to rationally designed variants [11–13]. It appears relevant that more research can be devoted to these aspects, as generating know-how and improved enzymes for solvent-free media would lead to many industrially attractive processes at intensified conditions. The same would apply for the screening of natural samples (e.g., metagenomes, databases, etc.), to spot novel enzymes with outstanding performances in non-conventional media in general, and in solvent-free processes in particular.

Nevertheless, the solvent-free area has experienced increased interest and growth in the last decade, with examples showing that not only hydrolases can perform reactions in nonaqueous solventless media, but also other enzyme types, thus broadening the portfolio and applications. Herein, key-to-success is the protection and/or immobilization of the enzymes, to stabilize them from the somewhat aggressive nonaqueous media. For instance, some oxidoreductases can be used as biocatalysts for the enantioselective reduction of ketones in neat substrates by using *Escherichia coli* whole cells overexpressing the desired enzyme [14–16]. The whole-cell structure serves as protection for the biocatalyst, and reactions can be successfully performed in a neat mixture of the ketone (often the substrate) and an alcohol (as ancillary substrate for cofactor regeneration, e.g., *iso*propanol). Interestingly, the downstream processing is straightforward, and upon filtration of the

suspended whole-cells, followed by the evaporation of the oxidized regeneration substrate (normally acetone), the desired product is achieved with high enantioselectivity (often with *ee* >99%), and with high productivity metrics (over several hundred grams of product per liter per day), aligning industrial applications with environmental approaches [2,10]. Particularly, that kind of strategy is useful when water-sensitive substrates or products are used, as the absence of bulk water enables an efficient synthesis that would be otherwise hardly accessible through biocatalysis [14]. Following these premises, imine reductases have also been successfully applied using whole-cells in neat systems (with few additions of nonbulky water, setting up a micro-aqueous system to keep the enzymatic system active), to afford optically active amines by reducing (water-sensitive) imines as unexpensive substrates [17]. Likewise, biocatalytic solvent-free strategies for oxidoreductases have been demonstrated in continuous mode, leading to highly integrated systems with excellent productivities (>2 kg product/L/day) for the enantioselective reduction of ketones to afford optically active alcohols (e.g., from 2-butanone to (*R*) or (*S*) − 2-butanol) [18]. As stated above, combining biocatalysis with solvent-free systems and continuous reactors may provide superior alternatives for reaching both industrially attractive and environmentally friendly syntheses [9].

Remarkably, the set-up of solvent-free biocatalytic concepts has been also extended to other enzyme types in the last years. As a general pattern, the successful strategies combine (again) the use of whole-cells together with different immobilization procedures to further enhance the stability of the biocatalyst in the media (and enabling its reuse). An outstanding example is the use of aldoxime dehydratases for the synthesis of nitriles from straightforwardly formed aldoximes as substrates, using an aqueous polymeric phase to immobilize the cells (the so-called "superabsorbers"). This system has been successfully described in continuous mode as well [19], providing again sustainable and industrial synergies [9]. As a matter of fact, nitriles are a diverse and important group of chemicals, used in many market segments, from bulk to pharma and fine chemicals applications. As recently shown, aldoxime dehydratases are very promising enzymes for the industrial production of many of these compounds, providing a cyanide-free route for nitriles [20]. In another valuable example, BASF has filed a patent reporting the efficient solvent-free synthesis of different terpene-based nitriles (e.g., citronellyl nitrile fragrance) starting from aldehydes and hydroxyl-amine (to form *in situ* the terpene aldoxime). No solvents were used—the aldoxime was the sole reaction medium—, and high conversions were reported (>90%), yet still at somewhat nonoptimized long reaction times (c. 90 h) [21]. Likewise, peroxygenases have been successfully employed for epoxidations and hydroxylation in neat substrates, using whole-cells immobilized in alginate beads [22,23]. The concept has been also combined with photo-biocatalysts, to *in situ* generate H_2O_2 using LED light and O_2. More recently, another approach for peroxygenases has been reported, combining the double immobilization of whole cells in alginates with cross-linking aggregates (CLEA), to deliver a highly robust biocatalyst with high turnover numbers in solvent-free media [24]. Further recent examples describe other enzymes, such as eugenol oxidase (for the synthesis of vanillin) [25], or the addition of cyclodextrins to stabilize and activate the biocatalyst for ketone reductions in solvent-free media [26]. Overall, the design of a robust catalyst—whole-cell and immobilized—is the key common reason of all examples to enable a successful solvent-free concept for biocatalysis. Given its potential, it may be expected that new options for biocatalytic solvent-free systems will be developed in the coming years. In terms of industrial integration, it appears straightforward to combine a (continuous) solventless enzymatic step with another synthetic units within a production pipeline.

Apart from the efforts related to the use of whole-cells and immobilization procedures to enhance the robustness of the biocatalyst—ultimately enabling their efficient use in solvent-free media—, new promising approaches have been reported for hydrolases as well. As briefly stated above, hydrolases are widely used enzymes that have been known for decades as efficient catalysts for synthetic reactions in solvent-free media [e.g., (trans)esterifications, amidations, epoxide formation, etc.]. However, sometimes it may occur that the unpaired solubility of the substrates (e.g., between the alcohol or the amine and the acyl donor) may hinder an efficient synthetic application in solvent-free. As recently shown by the Lozano group, in several cases, the ultrasound-assistance to lipase-catalyzed esterifications may lead to integrated and efficient synthetic concepts in solventless media [27]. This is exemplified by the lipase-catalyzed esterification of xylitol with different carboxylic fatty acids (from caprylic to myristic) at 40°C. The substrates, xylitol and fatty acids, are not miscible between them, and thus the efficient direct esterification is not technically feasible, due to the mass transfer limitations observed. However, the ultrasound-assistance of the mixture leads to efficient esterification patterns, leading to virtually full conversions, affording mixtures of mono-esters and di-esters of xylitol, depending on the reaction conditions and on the acyl donor (Scheme 17.1). As the catalyst, an immobilized form of *Candida antarctica* lipase B (CALB) was used, and molecular sieves were added to *in situ* remove the formed water in the esterification and to shift the equilibrium to products. High yields (95%) in 90 min reaction time were achieved. Notably, the downstream processing resulted straightforward, and upon centrifugation of the crude reaction mixture and subsequent decantation, a clear separation of the product was achieved as upper phase, also enabling the separation of the immobilized biocatalyst, the unreacted xylitol and



SCHEME 17.1

Ultrasound-assisted solvent-free lipase-catalyzed esterification of xylitol with different fatty acids, using immobilized CAL-B. After centrifugation, the downstream is straightforward as products (mono- and di-ester mixture) remain as upper separated phase [27].

the molecular sieves (Scheme 17.1). Importantly, the biocatalyst could be reused several times without observing an apparent loss of activity, giving hints on the potential and the robustness of the new concept [27].

To summarize this section, it can be stated that, albeit traditionally underexplored by the research community, the set-up of solvent-free processes is gaining interest in biocatalysis. Given the promising figures that the approach may bring—in terms of productivity and sustainability—, it is recommendable that more research initiatives can be taken in this field. Studies may range from delivering new immobilization/stabilization methods for the biocatalysts, to the designing of novel variants (or screening new enzymes) that can perform reactions in these media more efficiently. Furthermore, the fundamental understanding of the structural causes of some enzymes to be stable in these media may also generate knowledge that could enable the development of improved catalysts. Likewise, the combination of solvent-free reactions with other methodologies (e.g., ultrasound) may become very useful for future sustainable processes, especially when continuous systems are envisaged [9].

17.3 Biogenic solvents for biocatalysis: recent examples related to 2-methyltetrahydrofuran, cyclopentyl-methyl ether and cyrene

Another approach for combining green solvents with biocatalysis is the use of biogenic-derived compounds, that can be synthesized from renewable resources (e.g., lignocellulose), and can serve as reaction media or as extractive agents. It must be noted that being produced from bio-based resources does not warrant, as such, that these solvents will be necessarily "green," or "sustainable," and discussions in that respect exist in the scientific literature. Despite in-depth ecotoxicology assessments of these solvents are, in general, still needed, current state-of-the-art suggests that they may exert better biodegradability than other classic solvents. However, some different aspects like the high flammability of many of them, or their inherent volatility, to cite some, have been spotted as problematic. Likewise, their production costs are, in general, still high to consider them as a viable practical option that can replace other more cost-efficient alternatives. Further research and development efforts are needed in that direction as well, to provide a clear picture of the advantages and downsides of the biogenic solvents. Overall, however, they seem to be a promising alternative to ameliorate the ecological impact of chemical production in many cases, by replacing more classic and hazardous solvents (e.g., chloroform, dichloromethane, toluene, etc.). This has stimulated the research and the applications of them in synthetic chemistry in general, and in biocatalysis in particular.

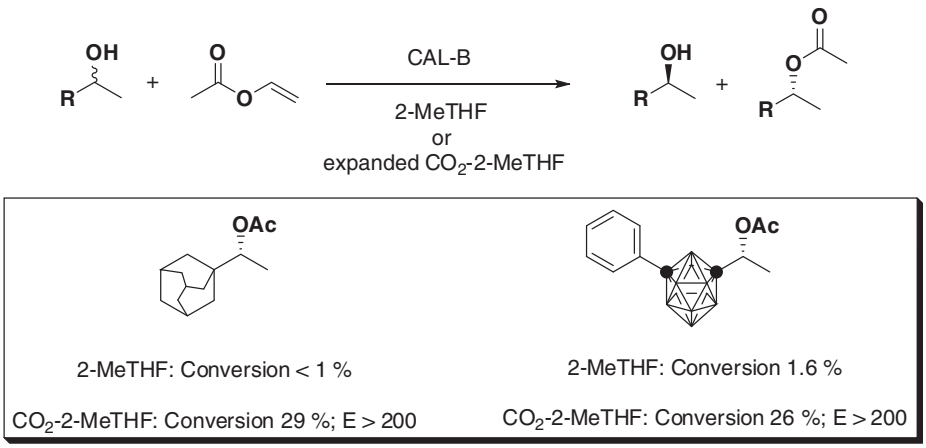
A prototypical example of biogenic solvents is 2-methyltetrahydrofuran (2-MeTHF), which can be produced either from furfural or from levulinic acid—both being lignocellulosic platform chemicals, derived from xylose (hemicellulose) and glucose (cellulose), respectively. 2-MeTHF has been extensively assessed as solvent replacement for many synthetic procedures, in particular as an alternative to THF, due to their analogous structure and properties. Applications also include a broad range of enzyme types and biocatalytic reactions, involving free and immobilized enzymes, as well as whole-cells [3,28,29]. 2-MeTHF exerts a relatively low boiling point (c. 80°C, what facilitates the work-up through distillation) and a good miscibility gap with aqueous media (c. 5% v/v 2-

MeTHF dissolved in water). On that basis, applications in biocatalysis have ranged from using it as solvent for the enzymatic reactions—a non-conventional media—, as cosolvent in aqueous solutions (up to 5% v/v in aqueous media), to assist in substrate dissolution, and as biphasic system with aqueous solutions, to serve as product and substrate reservoirs. A potential drawback for biocatalysis is its relatively low LogP (1.008), although in many cases this has not hampered efficient synthetic processes. Examples of 2-MeTHF in biocatalysis have been recently reviewed, and readers are referred to those contribution for extensive descriptions of the 2-MeTHF use in enzymatic processes [3,29].

Apart from these more "classic" concepts of 2-MeTHF and biocatalysis (solvent, cosolvent and biphasic media) [3,28,29], in recent years 2-MeTHF has been used in combination with CO_2, to trigger the formation of the so-called "CO_2 − 2-MeTHF expanded phases." Broadly speaking, the addition of some CO_2 pressure in 2-MeTHF leads to the dissolution of CO_2 in the solvent, creating the so-called CO_2 − 2-MeTHF expanded phase. The volume of the mixture expands, as the gas dissolves on the solvent. Interestingly, the actual pressures of CO_2 needed to create an expanded phase with 2-MeTHF are relatively low (in the range of 10−50 bar, far from the supercritical region). Notably, many physical-chemical properties of the expanded phase (e.g., solubility capabilities) are different from those of the original 2-MeTHF. Subsequently, when the CO_2 is degassed from the mixture, the original properties of the 2-MeTHF are recovered back, and both solvent and carbon dioxide can be reused. Thus, the strategy enables the straightforward performance of reactions in different solvent media, and depending on the actual CO_2 pressure applied, a *gradient of properties* can be generated, and eventually tailored for specific solutions. The concept has recently been successfully applied for the selective lignin fractionation, using a gradient of CO_2 pressures and triggering the selective precipitation of lignin fragments according to their molecular weight and size (larger polymers precipitate first and selectively at lower CO_2 pressures) [30]. In a broader sense, the concept of expanded phases and biogenic solvents may hold potential for biorefineries, and for many other areas in which polymer distribution needs to be narrowed down to reach different applications.

With respect to biocatalysis, the CO_2 − 2-MeTHF expanded phase has been recently used for the CALB-catalyzed kinetic resolution of racemic bulky alcohols, showing that the expanded phase improves the conversion and enantioselectivity of the lipases significantly [31−33]. Thus, for some bulky substrates the activity of CAL-B in 2-MeTHF results virtually inexistent, while the use of the expanded phase leads to significant catalytic conversions, and excellent enantioselectivities. As hypothesis to explain these findings, it has been postulated that the CO_2 − 2-MeTHF expanded phase would trigger a flexibilization of the enzyme, enabling the acceptance of the substrate (Scheme 17.2) [31−33]. Overall, this research line has been recently disclosed, and it may offer many interesting applications for enzyme catalysis in the future. Besides, fundamental research to understand the different enzymatic performance in expanded phases could bring future innovative ideas to expand the use of enzyme catalysis to other fields.

Another relevant example of a biogenic solvent with promising applications is CPME. Although its production is currently petrochemical (yet with very high atom economy), several biogenic routes have been postulated as well, using furfural derived from xylose as raw material, or adipic acid through fermentation [34,35]. As in the case of 2-MeTHF, CPME exerts low solubility in water, and a manageable boiling point (106°C) for technical purposes. In biocatalysis, CPME has not been used as broadly as 2-MeTHF, but the reported strategies tend to be similar,

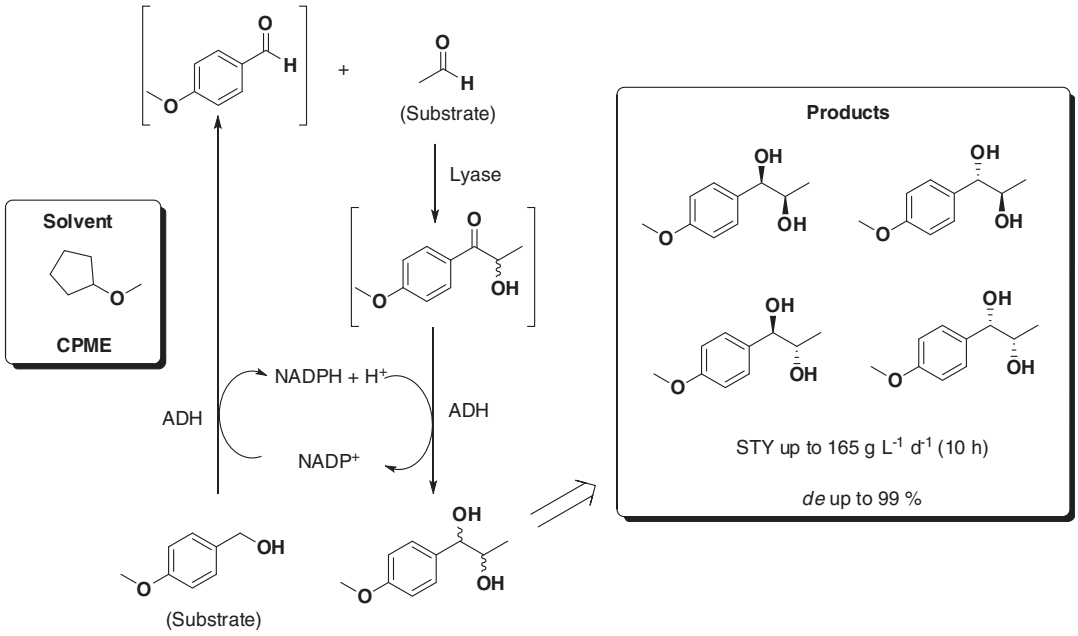


SCHEME 17.2

Improvement in the lipase-catalyzed kinetic resolution of bulky substrate by using expanded CO_2 – 2-MeTHF phases as green solvents, and comparison with the same reaction conducted in 2-MeTHF [31–33].

and thus CPME has been employed as solvent, co-solvent, and in biphasic media [34]. However, the use of CPME in biocatalysis has started to attract an increased interest recently, and new applications are pinpointing the potential that it may have for enzymatic processes. An outstanding recent example illustrates an elegant self-sufficient enzymatic cascade, involving lyases (C-C bond formation) and oxidoreductases (ketone reduction), to afford the four isomers of 4-methoxyphenyl-1,2-propanediol with high yields and high atom economies (Scheme 17.3) [36]. As reaction media, CPME with additions of nonbulk water was set ("micro-aqueous systems"). The substrates for lyases are acetaldehyde and 4-methoxy-benzaldehyde, to perform the *umpolung* C-C bond formation. The latter substrate is in situ formed through ADH-catalyzed oxidation of the correspondent alcohol. Once the optically active hydroxy-ketone is formed through lyase catalysis, the same ADH catalyzes the enantioselective reduction of the formed hydroxy-ketone, affording the diol, and closing the loop in the cofactor-regeneration cycle. Depending on the enantioselectivities of the enzymes employed, different diastereomers can be achieved, in all cases with an outstanding *de* of >99%. Once the cascade systems were optimized, excellent productivities of up to 165 g product/L/d were obtained. Remarkably, products could be crystallized from the reaction media, and thus an environmentally friendly with high atom economy and E factors in the range of 13–45, could be achieved (Scheme 17.3) [36].

Another emerging biogenic dipolar aprotic solvent for biocatalysis is dihydrolevoglucosenone (Cyrene), which can be obtained from cellulose through pyrolysis and hydrogenation steps. Its use in biocatalysis has been scarce so far, with only few applications for hydrolases [37–39] and oxidoreductases [40]. Cyrene has a relatively high boiling point (226°C), and a low LogP (−1.52), properties that may hamper, in principle, many biocatalytic reactions [39]. However, Cyrene displays a rather particular behavior in contact with aqueous media, what may confer some potential for several practical applications (e.g., substrate dissolution). Thus, Cyrene is largely miscible in water, what is supposed to happen through the formation of the

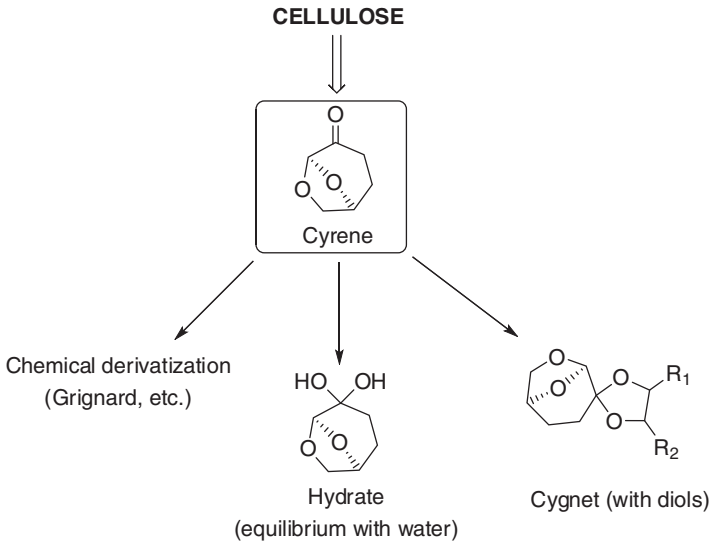


SCHEME 17.3

Self-sufficient enzymatic cascade process involving lyases and oxidoreductases to afford different diastereomeric diols, performed in CPME as solvent [36].

hydrate, forming a ketal, which remains in equilibrium with the ketone. Depending on the amount of water, different proportions of the chemical species may coexist, leading to a gradient of properties in these Cyrene-water mixtures. Moreover, several diols can be used to form other solvent-type structures, called Cygnets, which may change solvent properties again. Furthermore, other derivatizations can be considered (e.g., through Grignard reactions on the ketone group). Overall, starting from Cyrene a broad platform of new solvents can be envisioned, with tuneable polarities and properties. For biocatalysis, this may be promising, due to the straightforward combination of aqueous media with these Cyrene-like derivatives (Scheme 17.4) [39].

To conclude this section, it must be noted that many other biogenic compounds are susceptible to be considered potential solvents for biocatalysis (e.g., terpene-like compounds, limonene, pynene etc.). Important aspects to be considered are the capability of dissolving substrates, their enzyme-compatibility, their potential tuneability to provide an array of options, depending on the applications, and costs associated to them. In this area, it appears necessary that novel enzymes are screened by using these solvents, to try to identify novel catalysts with activity in these media. For instance, oxidoreductases displaying improved tolerance to 2-MeTHF were identified through metagenomic assessments [41]. Understanding the resistance to solvents (in particular biogenic ones) may create new future research avenues with promising options for industrial biocatalysis.



SCHEME 17.4

Derivatization options for cyrene, showing the potential to create a family of biogenic solvents with tailored properties [39].

17.4 Deep eutectic solvents: from proof of concept to continuous biocatalytic processes

Deep Eutectic Solvents (DES) have emerged over the last decade as promising tuneable solvents for many segments in chemistry. By combining different (potentially biogenic) hydrogen-bond acceptors (HBA) with hydrogen-bond donors (HBD), an array of different solvents can be designed, with varied properties. A prototypical example of HBA is choline chloride, an ammonium quaternary salt that is used as feed additive at ton scale worldwide. Albeit largely biodegradable, it must be noted, though, that most of its production is currently petrochemical, but biogenic routes can be envisaged as well. As HBDs different alcohols, carboxylic acids, amines, etc., have been reported, being most of them biogenic and fully biodegradable. The combination of the quaternary ammonium salt with the HBD generates hydrogen interactions which break the crystallinity of the salt and trigger a melting point depression, forming a liquid (the so-called DES). Given the variability of DES, what may lead to a huge number of potential applications, these eutectic mixtures have been coined as the "Solvents of the 21st century" [42]. If fact, the combination of a straightforward preparation—often produced by the gently mixing of the components—, together with their high biodegradability and tuneability, makes DES promising for future sustainable solutions [42].

In biocatalysis, the first example of DES applied as solvent for enzymatic reactions was the seminal work of the Kazlauskas group in 2008, demonstrating that some lipases were able to conduct (trans)esterifications and amidations in different DES media [43]. Interestingly, even DES media containing urea—a well-known as denaturing agent for proteins—, resulted effective for

biocatalytic reactions. Since then, the field has flourished, and many applications have been reported, covering most of the enzyme types, free and immobilized, as well as whole-cells. Improvements in substrate loadings, as well as in the enantioselectivity of the enzymes are often reported as advantageous when using DES as media. The topic has been comprehensively reviewed in several chapters of this book, and elsewhere [44,45].

As stated above, the combination of environmentally friendly media with biocatalysis may become attractive in the quest of sustainable solutions. In the particular case of DES, this can be observed when performing a research literature survey using "biocatalysis" and "deep eutectic solvents" as keywords (covering 2008−2021). As it can be observed, a clear exponential trend is observed (Fig. 17.2), reflecting the interests in DES in biotransformations. Thus, while in years 2008−2013 less than one hundred articles per year were released, the interest increased significantly in latest times, and currently several hundreds of publications appear yearly in the field.

The field of DES and biocatalysis comprise applications of hydrolases, oxidoreductases, lyases, etc., using immobilized enzymes, free biocatalysts and whole-cells [44,45]. In general, DES are used as solvents (non-conventional approach), as co-solvents in aqueous solutions, and as performance additives (low DES content trigger enzyme performance improvements). In particular, DES have attracted interest when substrate(s) dissolution is challenging. Some recent examples in the field show, for instance, the development of improved processes related to immobilized penicillin acylases [46], the set-up of stereoselective biotransformations using plant enzymes (e.g., carrot roots) as inexpensive and readily available biocatalysts [47], or improved processes dealing with β-glucosidases [48], or the establishment of more robust whole-cells processes [49]. Moreover,

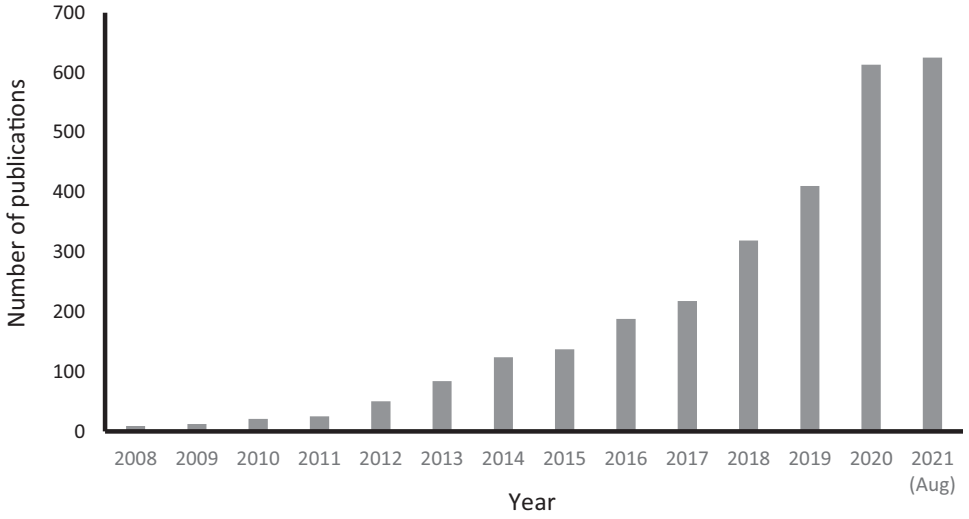


FIGURE 17.2

Number of articles yearly retrieved from the combined search "biocatalysis" and "deep eutectic solvents" over the last years. Search in 2021 is until the end of august.

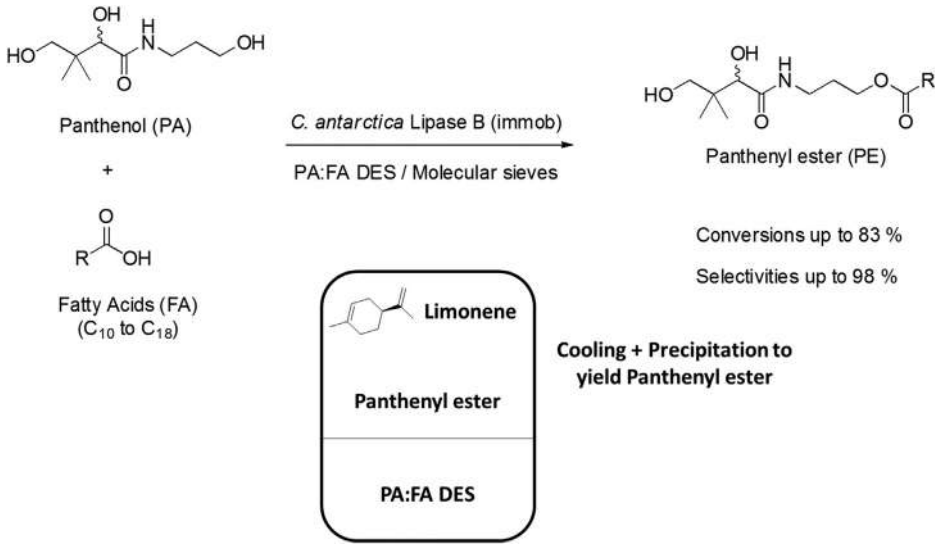
From Google Scholar (accessed: Beginning of September 2021).

research focusing on the understanding of the biocatalyst behavior (and its stability) in DES has been performed, pinpointing the beneficial and deleterious effects that the different DES components may display [50,51]. In general, it appears that DES follow analogous patterns to those observed for other non-conventional solvents in biocatalysis, with direct influence of the water activity of the system in the enzymatic performance [50]. Likewise, the design of novel DES that may be more enzyme-compatible are clear trends in recent works [52]. Overall, the identification of solvents that can dissolve substrates with unpaired solubilities and can be enzyme-compatible and biodegradable at the same time.

A promising line of research when working with DES is using them both as solvent and as substrate. For instance, the efficient lipase-catalyzed esterification of menthol has successfully been reported in a menthol:fatty acid DES used as media and substrate [53]. Also, some sugar-based DES (e.g., choline chloride: glucose) have been employed for the synthesis of glucose esters using fatty acids as acyl donors [54]. Overall, those concepts reduce the waste formation and act in a sort of "solvent-free" media. In this area, an outstanding recent example has been reported by the Lozano group, performing the lipase-catalyzed esterification of panthenol in a DES media composed of panthenol and different fatty acids (from capric to oleic and linoleic acid) [55]. As biocatalyst, immobilized CALB is used, and molecular sieves are used to remove the formed water during the esterification. With that approach, different panthenyl esters were obtained in a clean form, being products that may find commercial use in cosmetics and healthcare. Remarkably, the work-up procedure resulted straightforward and clean, as the formed ester could be selectively extracted by means of a biogenic solvent (limonene). Subsequent cooling triggered the precipitation of the ester with high conversions and selectivities (Scheme 17.5). Moreover, CALB resulted very stable, and could be used several times without apparent loss of activity [55].

One of the drawbacks of many DES used in biocatalysis (in particular choline chloride-containing DES) is their inherent high viscosity, what many hamper many applications due to mass transfer limitations, especially at the temperatures in which biocatalysis is often performed. To circumvent this, water can be added as co-solvent (up to 15%−20% v/v) to the DES, and the resulted mixture DES-water exerts a much lower viscosity than the pure DES. With that strategy, successful esterifications and amidation reactions have been reported by several research groups [44,45]. Importantly, up to additions of 20% v/v of water (or buffer), the DES-water mixture remains as nonconventional media, enabling high substrate dissolution. Moreover, as water is distributed throughout the DES, it is not available for the enzymes, and therefore no hydrolysis reactions are observed, and esterifications can be performed up to full conversions [56]. Notably, the possibility of using less viscous DES-water mixtures has enabled the successful set-up of continuous processes (lipase-catalyzed esterifications), as the reaction media can be efficiently pumped for packed-bed reactors [57]. Under these conditions, higher productivities are achieved, and open in fact many possibilities for using continuous process with DES for truly sustainable synthesis [9]. To further improve the concept, enzymes can be immobilized by means of the CLEA technique, as these derivatives display high stability in DES media, being active in some cases for more than two weeks without apparent loss of activity [58,59]. Moreover, the double immobilization CLEA—Lentikats affords biocatalysts with a more straightforward reuse, displaying high stability and activity [60].

To summarize this section, DES have been shown to be very useful media for enzymatic biotransformations, with many successful cases already reported in the literature. In fact, creating tuneable, cost-effective, biogenic and biodegradable solvents may become highly relevant for the future



SCHEME 17.5

Lipase-catalyzed synthesis of panthenyl esters through esterification of panthenol and different fatty acids, by creating a DES composed of panthenol and the fatty acids. For the work-up, the ester is extracted with the biogenic solvent limonene, and isolated upon cooling and precipitation [55].

sustainable processes. Some creative concepts have been described, such as using DES media as substrate and solvent, or the incorporation of water to design less viscous mixtures, affording continuous processes. It may be expected that more applications, and surely industrial systems will be established using DES and biocatalysis in the future.

17.5 Concluding remarks

Green solvents and biocatalysis must come inevitably together when sustainable synthetic applications are envisioned. This chapter has provided an overview of the increasing importance of green solvents in biotransformations. This can be reflected not only by the exponentially growing number of publications, but also by the many elegant concepts that are being disclosed, combining both the reaction step and the downstream unit, to deliver a pure product. In this area, solvent-free processes are superior to any other system, as high substrate loadings (leading to high productivities) as well as largely reduced waste formation can be envisioned, together with straightforward downstream. While traditionally considered for hydrolases (as robust enzymes), solvent-free concepts have been studied recently for other enzymes, like oxidoreductases, aldoxime dehydratases, or peroxygenases, showing that biocatalysis can benefit from those media in a rather broad sense. For all these reported cases, key-to-success is the use of whole-cells and eventually immobilization of them, to deliver robust biocatalysts that can accept the non-conventional media properly.

Importantly, when the establishment of solvent-free processes is not feasible (e.g solid substrates or unpaired solubilities), biogenic solvents can be considered as reaction media. Virtually, many biogenic compounds can in theory be used as solvents (e.g., esters, alcohols, terpenes, etc.), and in this chapter, several selected examples have been discussed. Solvents are typically used as media, as cosolvent in aqueous solutions, or to set up biphasic systems to act as substrate/product reservoir to avoid enzyme denaturation or inhibitions. Moreover, the combination of CO_2 with biogenic solvents to deliver expanded phases, recently reported, appears as a very promising concept for biocatalysis, since tuneable solvents can be designed, with a gradient of different properties depending on the actual CO_2 pressure applied. Likewise, emerging biogenic solvents with emerging use in biocatalysis can be CPME or Cyrene, from which already some applications have been reported (mostly as solvent). Apart from those cases, many other biogenic solvents can be considered, such as terpenes, alcohols, fragrances, etc. Finally, this chapter has also briefly discussed the importance that DES may have for the future sustainable chemistry. Given their potential, academic applications in biocatalysis have flourished in the last decade. Hence, DES will be widely used in biotransformations in the future.

Overall, green solvents are now an important part of the research devoted to biocatalysis, as this entire book comprehensively shows. Some unexplored research lines would be the need of understanding the factors for enzyme denaturation (or resistance!) to non-conventional media, and how this can be capitalized to deliver more robust biocatalysts, by translating different structural motifs to newly created variants. Moreover, research should be conducted to identify novel enzymes (e.g., through metagenomic screenings) that could be directly assessed in solvent-free, biogenic solvents, etc. Green solvents should be systematically included in these enzyme screenings, to timely find new promising biocatalysts. Likewise, this chapter has discussed some examples in which the creative combination of existing techniques (e.g., ultrasound, microwaves), together with continuous processes, biocatalysis, and green solvents, may provide exciting synergies to shape the sustainable chemistry of the future. Herein, it is key to consider not only the enzymatic process, but also that a realistic and straightforward downstream processing unit can be applied.

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