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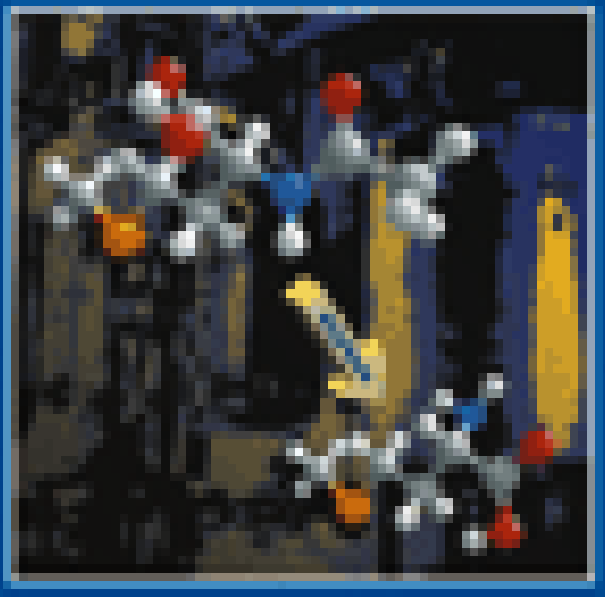
A. Liese, K. Seelbach, C. Wandrey

Industrial Biotransformations



Content

1 Introduction 1

2 History of Industrial Biotransformations – Dreams and Realities 3

2.1 From the "flower of vinegar" to the recombinant *E. coli* – The history of microbial biotransformations 3

2.2 From gastric juice to SweetzymeT – The history of enzymatic biotransformations 11

2.3 Advantages of biotransformations over classical chemistry 25

References 27

3 Enzyme Classification 31

3.1 The Enzyme Nomenclature 31

3.2 The Enzyme Classes 33

EC 1 Oxidoreductases 34

EC 2 Transferases 39

EC 3 Hydrolases 43

EC 4 Lyases 48

EC 5 Isomerases 51

EC 6 Ligases 54

References 56

4 Basics of Bioreaction Engineering 57

4.1 Definitions 58

4.2 Biosynthesis and immobilization of biocatalysts 64

4.3 Characteristics of the different enzyme classes 79

4.4 Kinetics 83

4.5 Basic reactor types and their mode of operation 86

References 90

5 Processes 93

Index

Index of enzyme name 397

Index of strain 400

Index of company 403

Index of starting material 406

Index of product 414

1 Introduction

The main incentive in writing this book was to gather information on one-step biotransformations that are of industrial importance. With this collection, we want to illustrate that more enzyme-catalyzed processes have gained practical significance than their potential users are conscious of. There is still a prejudice that biotransformations are only needed in cases where classical chemical synthesis fails. Even the conviction that the respective biocatalysts are not available and, if so, then too expensive, unstable and only functional in water, still seems to be widespread. We hope that this collection of industrial biotransformations will in future influence decision-making of synthesis development in such a way that it might lead to considering the possible incorporation of a biotransformation step in a scheme of synthesis.

We therefore took great pains in explicitly describing the substrates, the catalyst, the product and as much of the reaction conditions as possible of the processes mentioned. Wherever flow schemes were available for publication or could be generated from the reaction details, this was done. Details of some process parameters are still incomplete, since such information is only sparingly available. We are nevertheless convinced that the details are sufficient to convey a feeling for the process parameters. Finally, the use of the products is described and a few process-relevant references are made.

We would go beyond the scope of this foreword, should we attempt to thank all those who were kind enough to supply us with examples. Of course, we only published openly available results (including the patent literature) or used personally conveyed results with the consent of the respective authors. We are aware of the fact that far more processes exist and that by the time the book is published, many process details will be outdated. Nonetheless, we believe that this compilation with its overview character will serve the above-mentioned purpose. This awareness could be augmented if the reader, using his or her experience, would take the trouble of filling out the printed worksheet at the end of this book with suggestions that could lead to an improvement of a given process or the incorporation of a further industrial process into the collection.

Requesting our industrial partners to make process schemes and parameters more accessible did not please them very much. Even so, we are asking our partners once again to disclose more information than they have done in the past. In many instances, far more knowledge of industrial processes has been gained than is publicly available. Our objective is to be able to make use of these “well known secrets” as well. We would like to express our gratitude to all those who supplied us with information in a progress-conducive manner. Thanks also go to those who did not reject our requests completely and at least supplied us with a photograph in compensation for the actually requested information.

The book begins with a short historical overview of industrial biotransformations. Since the process order of the compilation is in accordance with the enzyme nomenclature system, the latter is described in more detail. We also include a chapter on reaction engineering to enable an easier evaluation of the processes.

The main part of the book, as you would expect, is the compilation of the industrial biotransformations. The comprehensive index will allow a facile search for substrates, enzymes and products.

We sincerely hope that this book will be of assistance in the academic as well as the industrial field, when one wants to get an insight into industrial biotransformations. We would be very thankful to receive any correction suggestions or further comments and contributions. At least we hope to experience a trigger effect that would make it worth while for the readership, the authors and the editors to have a second edition succeeding the first.

We are indebted to several coworkers for screening literature and compiling data, especially to Jürgen Haberland, Doris Hahn, Marianne Hess, Wolfgang Lanters, Monika Lauer, Christian Litterscheid, Nagaraj Rao, Durda Vasic-Racki, Murillo Villela Filho, Philomena Volkmann and Andrea Weckbecker.

We thank especially Uta Seelbach for drawing most of the figures during long nights, as well as Nagaraj Rao and the "enzyme group" (Nils Brinkmann, Lasse Greiner, Jürgen Haberland, Christoph Hoh, David Kihumbu, Stephan Laue, Thomas Stillger and Murillo Villela Filho).

And last but not least we thank our families for their support and tolerance during the time that we invested in our so called 'book project'.

2 History of Industrial Biotransformations – Dreams and Realities

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Throughout the history of mankind, microorganisms have been of tremendous social and economic importance. Without even being aware of their existence, man used them in the production of food and beverages already very early in history. Sumerians and Babylonians practised beer brewing **before 6000 B.C.**, references to wine making can be found in the Book of Genesis, and Egyptians used yeast for baking bread. However, the knowledge of the production of chemicals such as alcohols and organic acids by fermentation is relatively recent and the first reports in the literature appeared only in the **second half of the 19th century**. Lactic acid was probably the first optically active compound to be produced industrially by fermentation. It was accomplished in the USA in **1880** [1]. In **1921**, Chapman reviewed a number of early industrial fermentation processes for organic chemicals [2].

In the course of time, it was discovered that microorganisms could modify certain compounds by simple, chemically well-defined reactions which were further catalyzed by enzymes. Nowadays, these processes are called **"biotransformations".** The essential difference between fermentation and biotransformation is that there are several catalytic steps between substrate and product in fermentation while there are only one or two in biotransformation. The distinction is also in the fact that the chemical structures of the substrate and the product resemble one another in a biotransformation, but not necessarily in a fermentation.

2.1 From the "flower of vinegar" to the recombinant *E. coli* – The history of microbial biotransformations

The story of microbial biotransformations is closely connected with vinegar production which dates back to some **2000 years B.C.**

Vinegar production is perhaps the oldest and best known example of microbial oxidation which may illustrate some of the important developments in the field of biotransformations by living cells (figure 1).

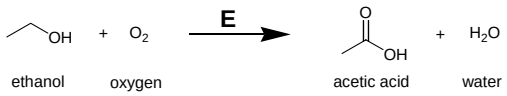


Fig. 1 Vinegar production (**E** = biocatalyst).

A prototype bioreactor with immobilized bacteria has been known in France since the **17th century**. The oldest bioreactor using immobilized living microorganisms, a so-called generator, was developed in **1823** [3,4]. Even today, acetic acid is still known as "vinegar" if it is obtained by oxidative fermentation of ethanol-containing solutions by acetic acid bacteria [5].

In **1858**, Pasteur [6] was the first to demonstrate the microbial resolution of tartaric acid. He performed fermentation of the ammonium salt of racemic tartaric acid, mediated by the mold *Penicillium glaucum.* The fermentation yielded (–)-tartaric acid (figure 2).

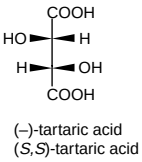


Fig. 2 Pasteur's product of the first resolution reaction.

This was also the first time that a method in which microorganisms degrade one enantiomer of the racemate while leaving the other untouched was used.

In **1862**, Pasteur [7] investigated the conversion of alcohol to vinegar and concluded that the pellicle, which he called "the flower of vinegar", "serves as a transport of air oxygen to a multitude of organic substances".

In **1886**, Brown confirmed Pasteur's findings and named the causative agent in vinegar production as *Bacterium xylinum*. He also found that it could oxidize propanol to propionic acid and mannitol to fructose (figure 3) [8].

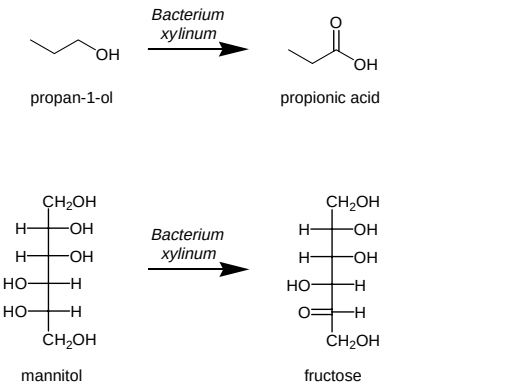
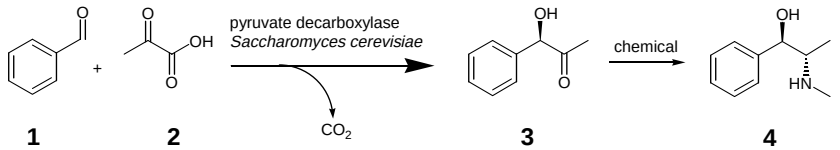


Fig. 3 Reactions catalyzed by *Bacterium xylinum*, the vinegar biocatalyst.

In **1897**, Buchner [9] reported that cell-free extracts prepared by grinding yeast cells with sand could carry out alcoholic fermentation reactions in the absence of living cells. This initiated the usage of resting cells for biotransformations.

Neuberg and Hirsch [10] discovered in **1921** that the condensation of benzaldehyde with acetaldehyde in the presence of yeast forms optically active 1-hydroxy-1-phenyl-2-propanone (figure 4).



1 = benzaldehyde
2 = 2-oxo-propionic acid
3 = 1-hydroxy-1-phenylpropan-2-one
4 = 2-methylamino-1-phenylpropan-1-ol

Fig. 4 L-Ephedrine production.

The obtained compound was further chemically converted into D-(–)-ephedrine by Knoll AG, Ludwigshafen, Germany in **1930** (figure 5) [11].

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Knoll A.-G. Chemische Fabriken in Ludwigshafen a. Rh., Dr. Gustav Hildebrandt und Dr. Wilfrid Klavehn in Mannheim

Verfahren zur Herstellung von l-1-Phenyl-2-methylaminopropan-1-ol

Patentiert im Deutschen Reiche vom 9. April 1930 ab

Racemisches 1-Phenyl-2-methylaminopropan-1-ol kann bereits nach verschiedenen Verfahren synthetisch hergestellt werden (vgl. z.B. Nagai u. Kanao, Annalen 470 [1929], S. 157; Patentschrift 469 783; Skita u. Keil, Ber. 62 [1929], S. 1142 ff.; Patentschrift 524 806).

Das racemische 1-Phenyl-2-methylaminopropan-1-ol läßt sich nach bekannten Verfahren (vgl. Nagai u. Kanao, Annalen 470 [1929], S. 157; britische Patentschrift 297 385) in seine optischen Isomeren spalten. Das bei der Spaltung entstehende l-1-Phenyl-2-methylaminopropan-1-ol ist identisch mit dem natürlichen Ephedrin und wird neuerdings therapeutisch erfolgreich verwendet.

Bisher ist jedoch kein Verfahren bekannt geworden, nach welchem l-1-Phenyl-2-methylaminopropan-1-ol auf unmittelbarem Wege dargestellt werden kann.

Es wurde nun gefunden, daß man mit guten Ausbeuten unmittelbar zum l-1-Phenyl-2-methylaminopropan-1-ol gelangt, wenn man links drehendes Phenylpropanolon (Neuberg, Biochem. Zeitschrift 115 [1921], S. 282 ff., und 128 [1922], S. 610 ff.) in Gegenwart von Methylamin der Reduktion unterwirft.

Die Bildung von l-1-Phenyl-2-methylaminopropan-1-ol war keineswegs vorauszusehen, da bekanntlich Abwandlungen von optisch aktiven Verbindungen nicht notwendig zu Verbindungen von optisch gleicher Drehungsrichtung führen müssen, sondern ebensogut zu solchen der entgegengesetzten Drehungsrichtung führen können (so entsteht z. B. l-Mandelsäure aus d-Benzaldehydcyanhydrin bei der Verseifung).

Ferner ist hervorzuheben, daß in der vorliegenden Erfindung eine asymmetrische Synthese vorliegt, bei welcher wiederum nicht vorauszusehen war, welche von den möglichen Konfigurationen entstehen würde. Es stand zu erwarten, daß sowohl d- oder l- oder dl-1-Phenyl-2-methylaminopropan-1-ol als auch d- oder l- oder dl-Pseudo-1-Phenyl-2-methylaminopropan-1-ol oder endlich ein Gemisch von mehreren dieser Komponenten entstehen würde. Daß bei der Reduktionskondensation des l-Phenylpropanolons mit Methylamin fast ausschließlich l-1-Phenyl-2-methylaminopropan-1-ol entsteht, war daher durchaus überraschend.

Die Erhaltung der optischen Aktivität war nach derwegen überraschend, weil auf Grund der Neubergschen Beobachtung (Biochem. Zeitschrift 128 [1922], S. 613) l-Phenylpropanolon sich bereits in verdünnt alkalischer Lösung in kurzer Zeit racemisiert. Da bei dem Verfahren der vorliegenden Erfindung die Reduktion in alkalischer Lösung stattfindet, war vorwiegend mit der Bildung von racemischen Basen zu rechnen.

548 459

Das Verfahren stellt eine neue Methode dar, um das links drehende Phenylpropanolon in Form von l-1-Phenyl-2-methylaminopropan-1-ol zutreffend zu verwerten.

Diese unmittelbare Synthese des l-1-Phenyl-2-methylaminopropan-1-ols hat ferner den Vorzug, daß kein therapeutisch wertloses d-1-Phenyl-2-methylaminopropan-1-ol ausfällt, wie es bei den bekannten Spaltungsverfahren des Racemkörpers der Fall ist.

Beispiel 1

120 g des durch Ätherauszug gewonnenen phenylpropanololhaltigen Gärungsproduktes (vgl. Biochem. Zeitschrift 115 [1921], S. 282 ff.) läßt man ohne weitere Reinigung in eine Lösung von 10 g Methylamin in 500 ccm Äther in Gegenwart von 20 g aktiviertem Aluminium unter Rühren im Verlaufe von 2 Stunden eintropfen. Gleichzeitig läßt man 20 bis 30 g Wasser tropfenweise zufließen. Die sofort heftig einsetzende Umsetzung wird zeitweilig durch Kühlung gemäßigt. Nach beendigter Reduktion wird der filtrierten ätherischen Lösung die entstandene optisch aktive Base mit verdünnter Säure entzogen. Die Aufarbeitung erfolgt in bekannter Weise.

Man erhält das Hydrochlorid des l-1-Phenyl-2-methylaminopropan-1-ols vom F. 214°, welches die aus der Literatur bekannte Linksdrehung zeigt. Die Ausbeute beträgt je nach Art des verwendeten Ausgangsstoffes 25 bis 45 g Hydrochlorid.

Beispiel 2

360 g des in Beispiel 1 verwendeten phenylpropanololhaltigen Ätherauszuges werden unter verminderten Druck destilliert. 300 g der bei 100 bis 150° unter 14 mm Druck übergehenden Fraktion werden in Gegenwart von kolloidalem Platin (70 ccm 1%iger Lösung) und 85 g 33%iger Methylaminlösung der katalytischen Reduktion unterworfen. Es ist vorteilhaft, etwas Äther zuzusetzen. Nach Beendigung der Wasserstoffaufnahme wird die ätherische Lösung mit Salzsäure ausgeschüttelt und das l-1-Phenyl-2-methylaminopropan-1-ol in bekannter Weise abgetrennt.

Das Hydrochlorid schmilzt bei 214° und zeigt die aus der Literatur bekannte Linksdrehung. Die Ausbeute an Hydrochlorid beträgt 210 g.

Beispiel 3

100 g nach Neuberg (Biochem. Zeitschrift 128 [1922], S. 611) abgetrenntes l-1-Phenylpropan-1-ol-2-on werden in 200 ccm Äther gelöst, mit 75 g 33%iger Methylaminlösung versetzt und etwa eine halbe Stunde lang geschüttelt. Hierbei findet unter Wärmeentwicklung Kondensation statt. Anschließend wird in Gegenwart von 70 ccm 1%iger kolloidaler Platinlösung mit Wasserstoff reduziert.

Die Aufarbeitung geschieht nach Beispiel 2.

Das Hydrochlorid des l-1-Phenyl-2-methylaminopropan-1-ols kristallisiert aus Alkohol in derben Prismen vom F. 214 bis 215°. Der F. der freien Base liegt bei 40°.

Patentansprüche:

1. Verfahren zur Darstellung von l-1-Phenyl-2-methylaminopropan-1-ol, dadurch gekennzeichnet, daß man links drehendes l-Phenylpropan-1-ol-2-on mit Methylamin kondensiert und das Kondensationsprodukt gleichzeitig oder nachträglich mit Reduktionsmitteln, wie aktiviertem Aluminium in Gegenwart von Wasser oder Wasserstoff in Gegenwart eines Platinkatalysators, behandelt.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß man — unter Umgehung der Reindarstellung des l-Phenylpropanolons — Destillate oder Auszüge aus l-Phenylpropanolon enthaltenden Gemischen verwendet, wie sie z. B. bei der Vergärung von Zuckern oder von zuckerhaltigen Produkten in Gegenwart von Benzaldehyd entstehen.

Fig. 5 Knoll's patent of **1930**.

The bacterium *Acetobacter suboxydans* was isolated in **1923** [12]. Its ability to carry out limited oxidation was used in a highly efficient preparation of L-sorbose from D-sorbitol (figure 6).

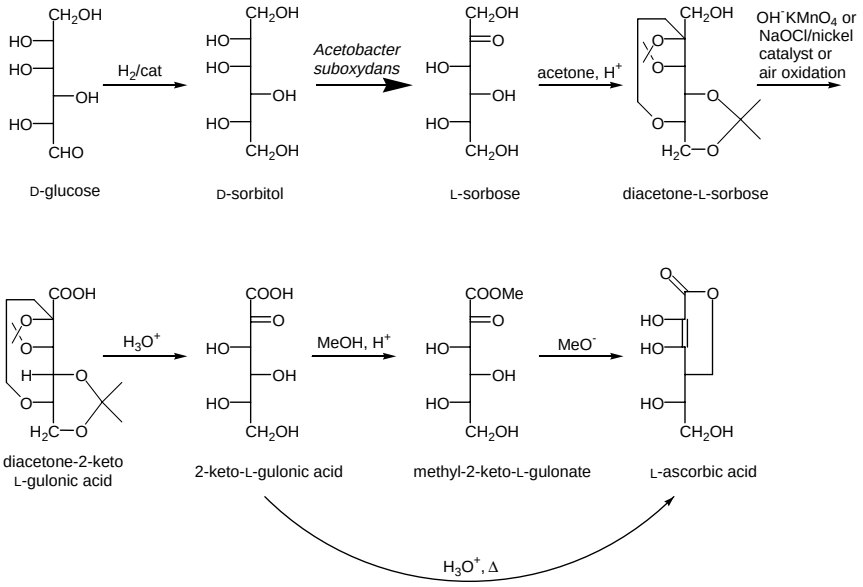

Fig. 6 Reichstein-Grüssner synthesis of vitamin C (L-ascorbic acid).

L-Sorbose became important in the **mid-1930's** as an intermediate in the Reichstein-Grüssner synthesis of L-ascorbic acid [13].

In **1953**, Peterson at al. [14] reported that *Rhizopus arrhius* converted progesterone to 11α-hydroxyprogesterone (figure 7), which was used as an intermediate in the synthesis of cortisone.

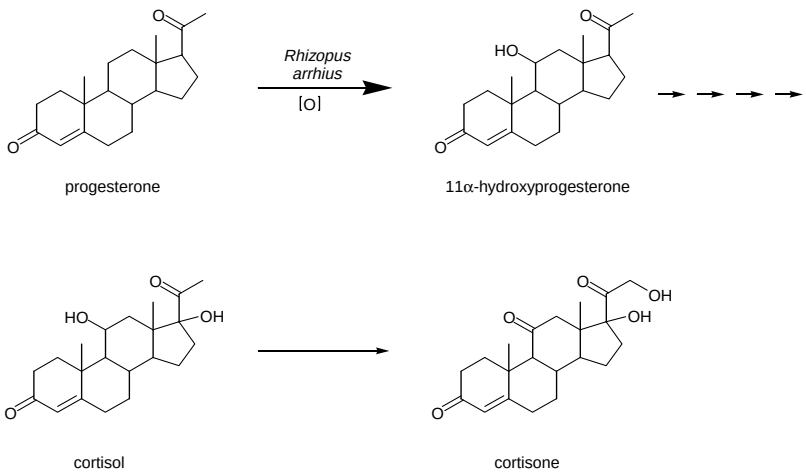

Fig. 7 Microbial 11α-hydroxylation of progesterone.

This microbial hydroxylation simplified and considerably improved the efficiency of the multi-step chemical synthesis of corticosteroid hormones and their derivatives. Although the chemical synthesis [15] (figure 8) from deoxycholic acid that was developed at Merck, Germany, was workable, it was recognized that it was complicated and uneconomical: 31 steps were necessary to obtain 1 kg of cortisone acetate from 615 kg of deoxycholic acid. The microbial 11α-hydroxylation of progesterone quickly reduced the price of cortisone from $200 to $ 6 per gram. Further improvements have led to a current price of less than $1 per gram [16].

In the **1950's**, the double helix structure and the chemical nature of RNA and DNA – the genetic code of heredity – were discovered. This discovery can be regarded as one of the milestones among this century's main scientific achievements. It led to the synthesis of recombinant DNA and gave a fillip to genetic engineering in the seventies'. Such developments quickly made the rDNA technology a part of industrial microbial transformations. Application of this technology for the production of small molecules began in **1983**. Ensley et al. [17] reported on the construction of a strain of *E.coli* that excreted indigo, one of the oldest known dyes. They found that the entire pathway for conversion of naphthalene to salicylic acid is encoded by genes of *Pseudomonas putida.* These genes can be expressed in *E.coli*. Their results led to the unexpected finding that a subset of these genes was also responsible for the microbial production of indigo. Moreover, they showed that indigo formation was a property of the dioxygenase enzyme system that forms *cis*-dihydrodiols from aromatic hydrocarbons. Finally, they proposed a pathway for indigo biosynthesis in a recombinant strain of *E. coli* (figure 9).

Genencor International is developing a commercially competitive biosynthetic route to indigo using recombinant *E.coli* that can directly synthesize indigo from glucose [18]. Anderson et al. in **1985** [19] reported the construction of a metabolically engineered bacterial strain that was able to synthesize 2-keto-L-gulonic acid (figure 10), a key intermediate in the production of L-ascorbic acid (vitamin C).

BASF, Merck and Cerestar are building a 2-keto-L-ketogulonic acid plant in Krefeld, Germany. The start up of operation is scheduled for **1999**. They developed a new fermentation route from sorbitol directly to the ketogulonic acid [20]. This method is probably similar to the method described in **1966** [21].

The Cetus Corporation (Berkeley, California, USA) bioprocess for converting alkenes to alkene oxides emerged in **1980** [22]. This bioprocess appeared to be very interesting, thanks to the possibility of replacing energy-consuming petrochemical processes.

There were high hopes that the development of recombinant DNA technology would speed up technological advances. Unfortunately, there is still a lot left to be done about the development and application of bioprocesses before the commercial production of low-value chemicals becomes feasible [23]. However, today even the traditional chemical companies like Dow Chemical, DuPont, Degussa-Hüls AG etc., pressurized by investors and technological advances, are trying to use microbial or enzymatic transformations in production. They are doing this to see whether natural feedstocks can bring more advantages than crude oil. One only needs to compare the cost of a barrel of oil with that of corn starch to see that the latter is quite cheaper [20].

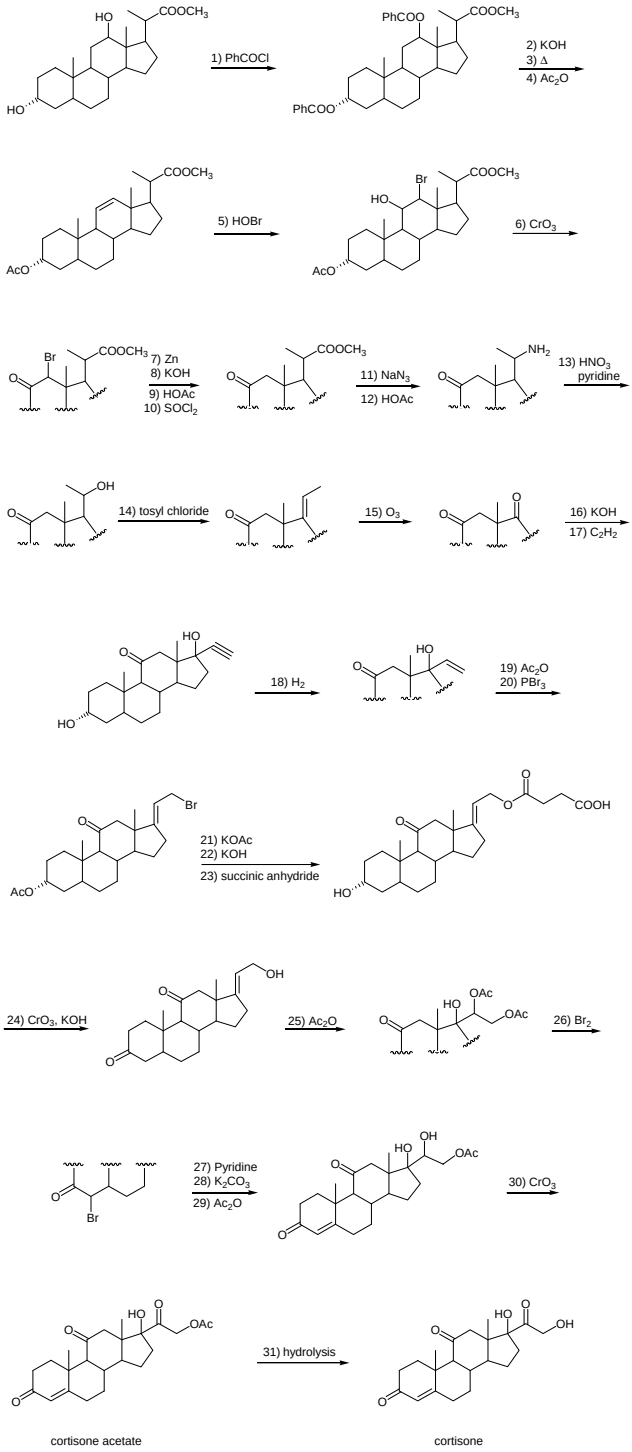

Fig. 8 Chemical synthesis of cortisone.

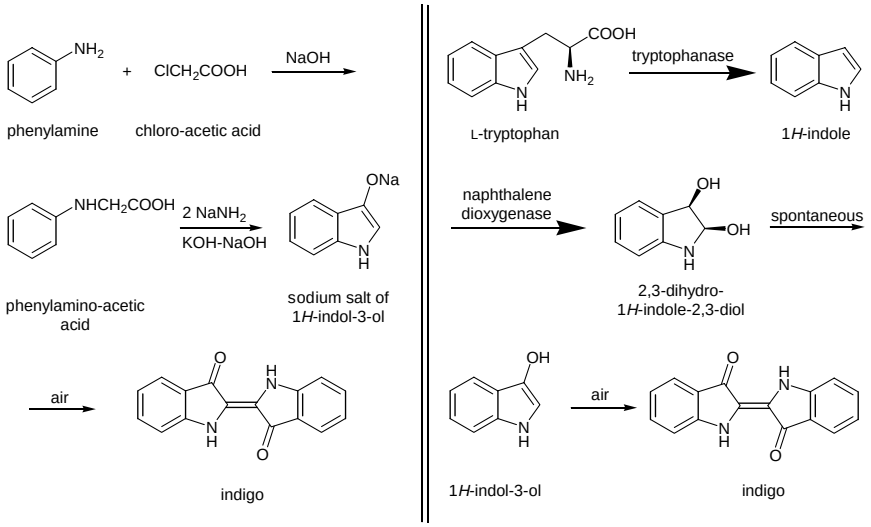

Fig. 9 Comparison of chemical and biological routes to indigo.

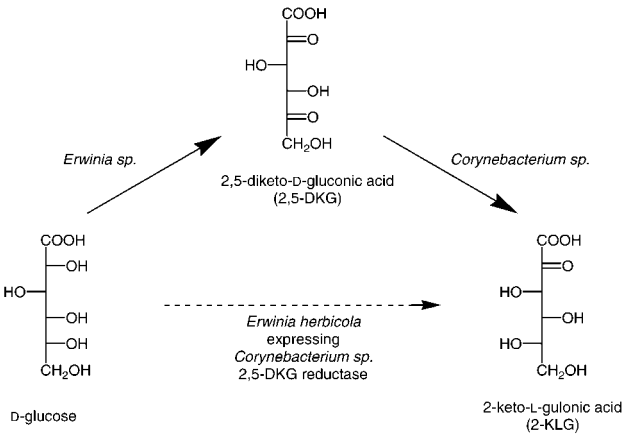

Fig. 10 Biosynthesis of 2-keto-L-gulonic acid.

Acrylamide is one of the most important commodity chemicals. Its global consumption is about 200,000 tonnes per year. It is used in the production of various polymers for use as flocculants, additives or for petroleum recovery. In conventional synthesis, copper salts are used as catalysts in the hydration of nitriles. However, this is rather disadvantageous as the preparation of the catalysts is quite complex. Additionally, it is difficult to regenerate the used catalyst and separate and purify the formed acrylamide. Furthermore, since acrylamides are readily polymerized, their production under moderate conditions is highly desirable. In contrast to the conventional chemical process, there is no need to recover unreacted acrylonitrile in the enzymatic process, because the conversion and yield of the enzymatic hydration pro-

cess are almost 100 %. The removal of the copper ions from the product is no longer necessary. Overall, the enzymatic process – being carried out below 10 °C under mild reaction conditions and requiring no special energy source – proves to be simpler and more economical. The immobilized cells are used repeatedly and a very pure product is obtained. The enzymatic process, which was first implemented in **1985**, is already producing about 6000 tons of acrylamide per year for Nitto [24,25]. The use of biocatalyst for the production of acrylamide may not be the first case in which biotransformation as a part of biotechnology was used in the petrochemical industry. However, it is the first successful example of the introduction of an industrial biotransformation process for the manufacture of a commodity chemical (figure 11).

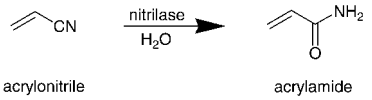

Fig. 11 Acrylamide synthesis.

Some representative industrial microbial transformations are listed in Table I.

Table I: Some representative industrial biotransformations catalyzed by whole cells.

Product	**Biocatalyst**	**Operating since**	**Company**
vinegar	bacteria	1823	various
L-2-methylamino-1-phenylpropan-1-ol	yeast	1930	Knoll AG, Germany
L-sorbose	*Acetobacter suboxydans*	1934	various
prednisolone	*Arthrobacter simplex*	1955	Schering AG, Germany
L-aspartic acid	*Escherichia coli*	1958	Tanabe Seiyaku Co., Japan
7-ADCA	*Bacillus megaterium*	1970	Asahi Chemical Industry, Japan
L-malic acid	*Brevibacterium ammoniagenes*	1974	Tanabe Seiyaku Co., Japan
D-*p*-hydroxyphenylglycine	*Pseudomonas striata*	1983	Kanegafuchi, Chemical Co., Japan
acrylamide	*Rhodococcus sp.*	1985	Nitto Chemical Ltd, Japan
D-aspartic acid and L-alanine	*Pseudomonas dacunhae*	1988	Tanabe Seiyaku Co., Japan
L-carnitine	*Agrobacterium sp.*	1993	Lonza, Czech.Rep.
2-keto-L-gulonic acid	*Acetobacter sp.*	1999	BASF, Merck, Cerestar, Germany

2.2 From gastric juice to SweetzymeT – The history of enzymatic biotransformations

Enzymes were in use for thousands of years before their nature was gradually understood. No one really knows when the calf stomach was used as a catalyst for the first time in the manufacture of cheese.

As early as **1783**, Spallanzani showed that gastric juice secreted by cells could digest meat *in vitro*. In **1836**, Schwan called the active substance pepsin [26]. In **1876**, Kühne (figure 12) presented a paper to the Heidelberger Natur-Historischen und Medizinischen Verein, suggesting that such non-organized ferments should be called **e n z y m e s** [27]. At that time two terms were used: "organized ferment" such as cell-free yeast extract from Büchner, and "unorganized ferment" such as gastric juice secreted by cells. Today the terms "intracellular" and "extracellular" are used. Kühne also presented some interesting results from his experiments with trypsin. The word "enzyme" comes from Greek for "in yeast" or "leavened" [28].

Microorganisms synthesize numerous enzymes, each having its own function. **Intracellular** enzymes operate inside the cell in a protected and highly structured environment, while **extracellular** enzymes are secreted from the cell, thus working in the medium surrounding the microorganism.

The commercial usage of extracellular microbial enzymes started in the West around **1890**, thanks to the Japanese entrepreneur Takamine. He settled down in the United States and started an enzyme factory based on Japanese technology. The principal product was called takadiastase. This was a mixture of amylolytic and proteolytic enzymes prepared by cultivation of *Aspergillus oryzae*. In France, Boidin and Effront developed bacterial enzymes in **1913**. They found that the hay bacillus, *Bacillus subtilis,* produces an extremely heat-stable α-amylase when grown in still cultures on a liquid medium prepared by extraction of malt or grain [29].

In **1894**, Emil Fischer [30,31] observed in his studies of sugars that the enzyme called emulsin catalyzes the hydrolysis of β-methyl-D-glucoside, while the enzyme called maltase is active towards the α-methyl-D-glucoside as substrate (figure 13).

This led Fischer to suggest his famous "lock–and-key" theory of enzyme specificity, which he would describe in his own words as follows: "To use a picture, I would say that enzyme and the glucoside must fit into each other like a lock and key, in order to effect a chemical reaction on each other" [1].

In **1913**, Michaelis and Menten published a theoretical consideration of enzymatic catalysis. This consideration envisaged the formation of a specific enzyme-substrate complex which further decomposed and yielded the product with the release of the enzyme. This led to the development of the famous Michaelis-Menten equation to describe the typical saturation kinetics observed with purified enzymes and single substrate reactions [32].

By **1920**, about a dozen enzymes were known, none of which had been isolated [33]. Then, in **1926**, Sumner [34] crystallized urease from jack bean, *Canavalia ensiformis,* and announced that it was a simple protein.

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Ueber das Verhalten verschiedener organisirter und sog. ungeformter Fermente.

Ueber das Trypsin (Enzym des Pankreas).

Von **W. Kühne.**

1876

Ueber das Verhalten verschiedener organisirter und sog. ungeformter Fermente.

Sitzung am 4. Februar 1876.

Hr. W. Kühne berichtet über das Verhalten verschiedener organisirter und sog. ungeformter Fermente. Um Missverständnissen vorzubeugen und lästige Umschreibungen zu vermeiden schlägt Vortragender vor, die ungeformten oder nicht organisirten Fermente, deren Wirkung ohne Anwesenheit von Organismen und ausserhalb derselben erfolgen kann, als *Enzyme* zu bezeichnen. — Genauer untersucht wurde besonders das Eiweiss verdauende Enzym des Pankreas, für welches, da es zugleich Spaltung der Albuminkörper veranlasst, der Name *Trypsin* gewählt wurde. Das Trypsin vom Vortr. zuerst dargestellt und zwar frei von durch dasselbe noch verdaulichen und zersetzbaren Eiweissstoffen, verdaut nur in alkalischer, neutraler, oder sehr schwach sauer reagirender Lösung. Dasselbe wird durch nicht zu kleine Mengen Salicylsäure, welche das Enzym in bedeutenden Quantitäten löst, bei 40° C. gefällt, ohne dabei seine specifische Wirksamkeit zu verlieren. Wird die Fällung in Sodalösung von 1 pCt. gelöst, so verdaut sie höchst energisch unter Bildung von Pepton, Leucin, Tyrosin u. s. w. Nur übermässiger Zusatz von Salicylsäure bis zur Bildung eines dicken Krystallbreies vernichtet die enzymotischen Eigenschaften. Dies Verhalten war kaum zu erwarten, seit Kolbe und J. Müller die hemmende, selbst vernichtende Wirkung kleiner Mengen Salicylsäure auf einige Enzyme hervorgehoben hatten. Die Beobachtungen des Vortr., der ausser dem Trypsin noch das Pepsin eingehender untersuchte, stehen jedoch mit den Angaben von J. Müller, nach welchen Salicylsäure bei einem Gehalte der

Fig. 12 W. F. Kühne [27].

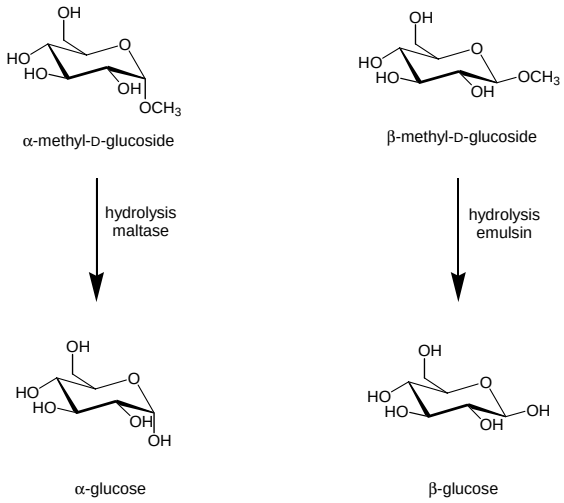

Fig. 13 Emil Fischer' s substrates.

Northrop and his colleagues [26] soon supported Sumner's claim that an enzyme could be a simple protein. They isolated many proteolytic enzymes beginning with pepsin in **1930** by applying classical crystallization experiments. By the late **1940s** many enzymes were available in pure form and in sufficient quantity for investigation of their chemical structure. Currently, more than 3,000 enzymes have been catalogued [35]. The ENZYME data bank contains information related to the nomenclature of enzymes [36]. The current version contains 3,705 entries. It is available through the ExPASy WWW server (http://www.expasy.ch/). Several hundreds of enzymes can be obtained commercially [37].

In **1950**, there was still no evidence that a given protein had a unique amino acid sequence. Lysosyme was the first enzyme whose tertiary-structure (figure 14) was defined in **1966** with the help of X-ray crystallography [38].

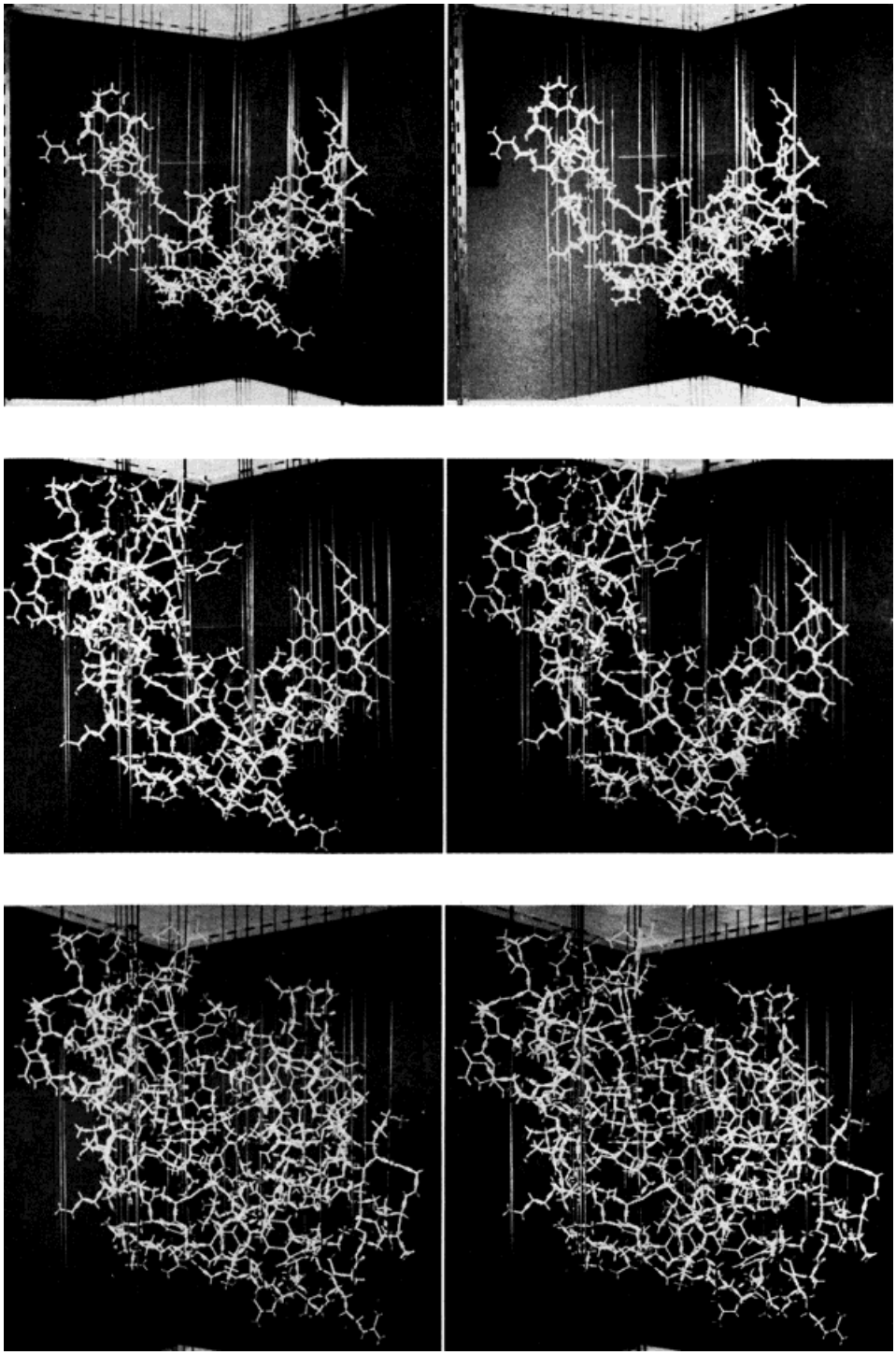

Fig. 14 Stereo photographs of models of part of the lysozyme molecule [38].

Further, ribonuclease A was one of the first enzymes made on a laboratory scale by organic chemistry methods. In **1969**, Gutte and Merrifield synthesized its whole sequence in 11,931 steps [39].

By **1970**, the complete molecular structures of several enzymes had been established and plausible reaction mechanisms could be discussed [26].

Hill (**1897**) was the first to show that the biocatalysis of hydrolytic enzymes is reversible [40].

Pottevin (**1906**) went further and demonstrated that crude pancreatic lipase could synthesize methyl oleate from methanol and oleic acid in a largely organic reaction mixture [41].

While the first benefit for the industry from the microbiological development had come early, the investigations with isolated enzymes hardly influenced the industry at that time. Consequently, industrial enzymatic biotransformations have a much shorter history than microbial biotransformations in the production of fine chemicals.

Invertase was probably the first immobilized enzyme to be used commercially for the production of Golden Syrup by Tate & Lyle during World War II, because sulfuric acid as the preferred reagent was unavailable at that time (figure 15) (42).

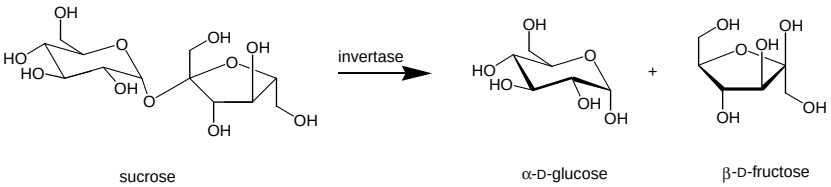

Fig. 15 Inversion of sucrose by invertase.

Yeast cells were autolysed and the autolysate clarified by adjustment to pH 4.7, followed by filtration through a calcium sulphate bed and adsorption into bone char. A bone char layer containing invertase was incorporated into the bone char bed, which was already used for syrup decolorisation. The scale of operation was large, the bed of invertase-char being 60 cm deep in a 610 cm deep bed of char. The preparation was very stable since the limiting factor was microbial contamination or loss of decolorising power rather than the loss of enzymatic activity. The process was cost-effective but the product did not have the flavor quality of the acid-hydrolysed material. This is the reason why the immobilized enzyme was abandoned once the acid became available again [42].

Industrial processes for L-amino acid production based on the batch use of soluble aminoacylase were already in use in **1954**. However, like many batch processes with soluble enzymes, they had their disadvantages such as higher labor costs, complicated product separation, low yields, high enzyme costs and non-reusability of enzyme. During the **mid-1960s** the Tanabe Seiyaku Co. of Japan was trying to overcome these problems by using immobilized aminoacylases. In **1969**, they started the industrial production of L-methionine by aminoacylase immobilized on DEAE-Sephadex in a packed bed reactor (figure 16). This was the first full scale industrial use of an immobilized enzyme. The most important advantages are the relative simplicity and ease of control [44].

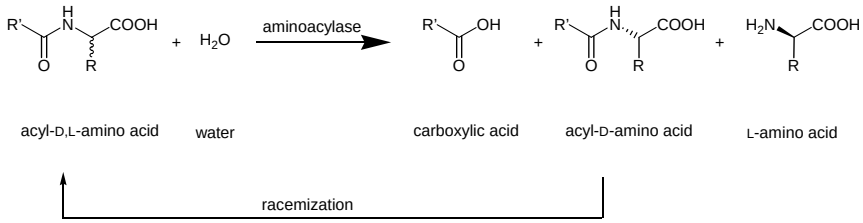

Fig. 16 L-Amino acid production catalyzed by aminoacylase.

In a membrane reactor system developed at Degussa-Hüls AG in Germany in **1980** [45], native enzymes, either pure or of technical grade, are used in homogeneous solution for the large scale production of enantiomerically pure L-amino acids (figure 17).

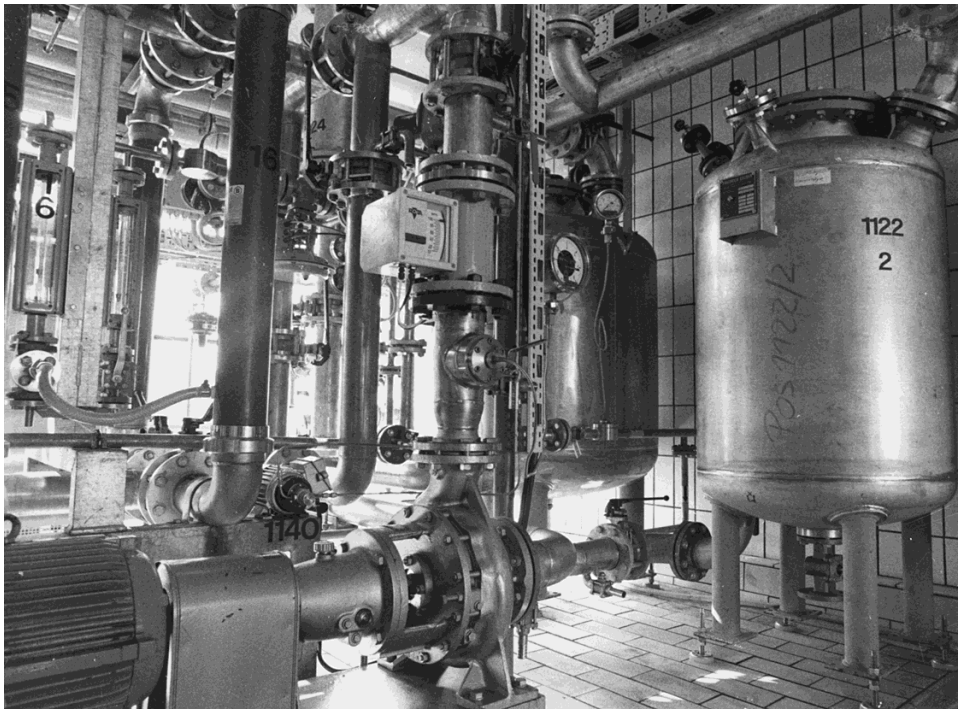

Fig. 17 Enzyme membrane reactor (Degussa-Hüls AG, Germany).

A membrane reactor is particularly well suited for cofactor-dependent enzyme reactions, especially if the cofactor is regenerated by another enzyme reaction and retained by the membrane in modified form [46]. There are several advantages of carrying out biocatalysis in membrane reactors over heterogeneous enzymatic catalysis: there are no mass transfer limitations, enzyme deactivation can be compensated for by adding soluble enzyme and the reactors can be kept sterile more easily than immobilized enzyme systems. The product is mostly pyrogen free (major advantage for the production of pharmaceuticals), because the prod-

uct stream passes through an ultrafiltration membrane. Scale-up of membrane reactors is simple because large units with increased surface area can be created by combining several modules.

The enzymatic isomerization of glucose to fructose (figure 18) represents the largest use of an immobilized enzyme in the manufacture of fine chemicals.

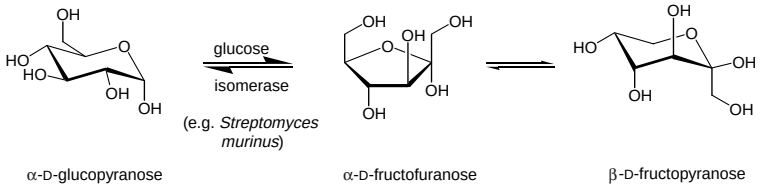

Fig. 18 Isomerization of glucose to fructose.

High-fructose corn syrup HFCS has grown to become a large-volume biotransformation product [47]. While sucrose is sweet, fructose is approximately 1.5 times sweeter and consequently high quality invert syrups (i.e. hydrolyzed sucrose) may be produced. Invert syrups contain glucose and fructose in a 1:1 ratio. However, the food industry needed a long time to become acquainted with the glucose isomerase potential to produce high quality fructose syrups from glucose. Again, the Japanese were the first to employ soluble glucose isomerase to produce high quality fructose syrups in **1966**. At the beginning of **1967**, Clinton Corn Processing Company, Iowa, USA, was the first company to manufacture enzymatically produced fructose corn syrup [47].The glucose-isomerase catalyzed reversible reaction gave a product containing about 42 % of fructose, 50 % of glucose and 8 % of other sugars. Due to various reasons, economic viability being the more important among them, the first commercial production of fructose syrups using glucose isomerase immobilized on a cellulose ion-exchange polymer in a packed bed reactor plant started only in **1974**. It was initiated by Clinton Corn Processing [44]. In **1976**, Kato was the first company in Japan to manufacture HFCS in a continuous process as opposed to a batch process. In **1984**, it became the first company to isolate crystalline fructose produced in this process by using an aqueous separation technique.

The glucose isomerase Sweetzyme T, produced by Novo, Denmark is used in the starch processing industry in the production of high fructose syrup. The key to its long life is immobilization. The enzyme is chemically bound to a carrier, making the particles too large to run out through the sieve at the bottom of the isomerization columns. Sweetzyme T is packed into columns where it is used to convert glucose into fructose. The record for the longest lifetime of a column is 687 days, held by a Japanese company called Kato Kagaku in Kohwa near Nagoya. The reaction conditions are pH 7.5 and T = 55 °C. Though enzyme activity is reduced at this temperature, its stability and productivity are considerably improved [48].

The engineers from Kato used to say: “The better the substrate you put in, the better the results you get out”. Each column at Kato contains 1,800 kg of Sweetzyme T. The column needs to be changed when the flow rate decreases to about 10 % of the initial value. Sweetzyme T displays a linear decay curve under steady state operating conditions. With regard to productivity, the yield from the record-

breaking column was 12,000 kg of fructose syrup (containing 42 % fructose) (dry substance)/ kg of Sweetzyme T. The normal column productivity was 8,000–10,000 kg / kg enzyme. The 687 days' record for Sweetzyme T is also a world record in the starch industry [48] (figure 19).

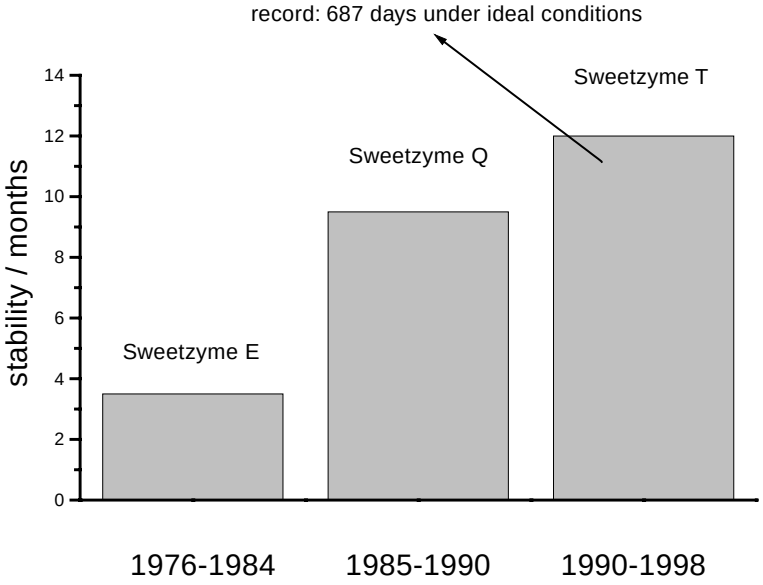

Fig. 19 Improved biocatalyst stability by biocatalyst engineering at Novo.

"Central del Latte" of Milan, Italy, was the first company which commercially hydrolyzed milk lactose with immobilized lactase using SNAMprogetti technology [49]. An industrial plant with a capacity of 10 tons per day is situated in Milan. The entrapped enzyme is lactase obtained from yeast and the reaction is performed batchwise at low temperature. Lactase hydrolyses lactose, a sugar with poor solubility properties and a relatively low degree of sweetness, to glucose and galactose (figure 20).

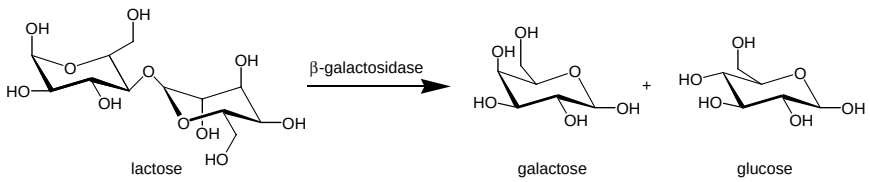

Fig. 20 β-Galactosidase catalyzed hydrolysis of lactose to galactose and glucose.

After the processed milk reaches the desired degree of hydrolysis of lactose, it is separated from the enzyme fibers, sterilized, and sent for packing and distribution. SNAMprogetti's process enables the manufacture of a high-quality dietary milk at low cost. This milk has a remarkable digestive tolerance, pleasant sweetness, unaltered organoleptic properties, and good shelf-life. It does not contain foreign matter. The industrial plant is shown in figure 21.

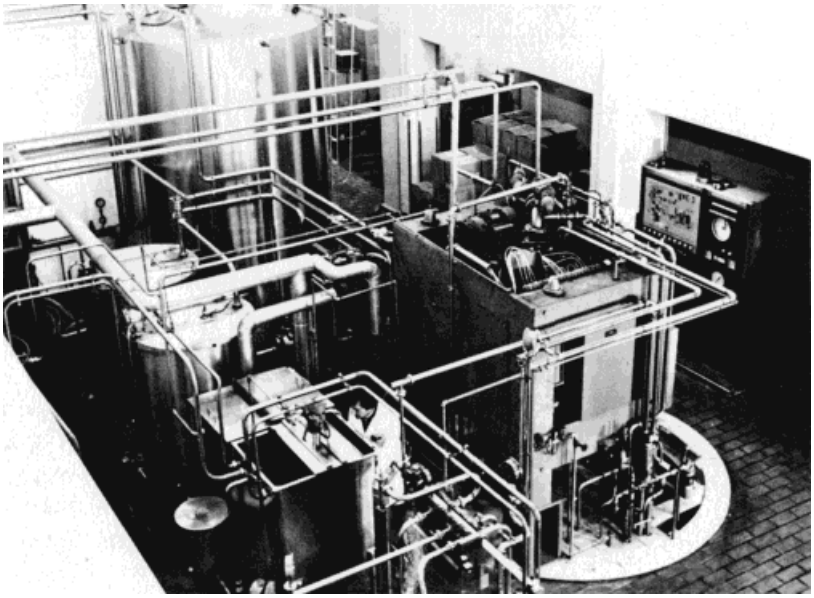

Fig. 21 Industrial plant for processing low – lactose milk [49].

Penicillin G, present in *Penicillum notatum* and discovered by Fleming in **1929**, revolutionized chemotherapy against pathogenic microorganisms. Today, β-lactam antibiotics such as penicillins and cephalosporins are very widely used. Thousands of semisynthetic β-lactam antibiotics are being synthesized to find more effective compounds. Most of these compounds are prepared from 6-aminopenicillanic acid (6-APA), 7-aminocephalosporanic acid (7-ACA) and 7-amino-desacetoxycephalosporanic acid (7-ADCA).

At present, 6-APA is mainly produced either by chemical deacylation or by enzymatic deacylation using penicillin amidase from penicillin G or V. This process, which exemplifies the best known usage of an immobilized enzyme in the pharmaceutical industry, is being used since around **1973** (figure 22). Several chemical steps are replaced by a single enzymatic reaction. Organic solvents, the use of low temperature (-40 °C) and the need for absolutely anhydrous conditions, which made the process difficult and expensive, were no longer necessary in the enzymatic process [50].

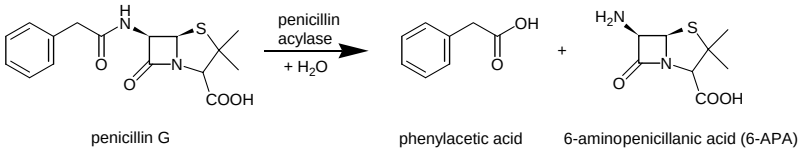

Fig. 22 Enzymatic synthesis of 6-aminopenicillanic acid (6-APA).

For many years enzymatic 7-ACA production was nothing but a dream. This changed in **1979**, when Toyo Jozo, Japan, in collaboration with Asahi Chemical Industry, also Japan, developed and succeeded in the industrial production of 7-ACA by a chemoenzymatic two-step process starting from cephalosporin C (figure 23):

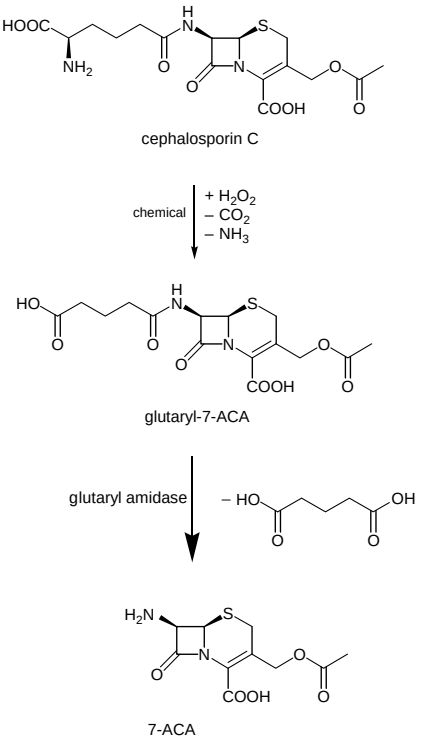

Fig. 23 Two-step process of 7-ACA production from cephalosporin C.

The chemical process requires highly purified cephalosporin C as raw material. A number of complicated reaction steps are carried out at –40 °C to –60 °C, and the reaction time is long. Furthermore, hazardous reagents, such as phosphorous pentachloride, nitrosyl chloride and pyridine are used in this process. The removal of such reagents causes significant problems. Therefore, the development of an enzymatic process was a dream for a long time. In the enzymatic process, liberated glutaric acid reduces the pH and inhibits the glutaryl-7-ACA amidase, the enzyme that catalyzes the deacylation of cephalosporin C. Because of this change in pH the reaction rate is decreased, requiring strict pH control during the reaction process. For these reasons, a recirculation bioreactor with immobilized glutaryl-7-ACA amidase and an automatic pH controller were designed for the 7-ACA production. The bioreactor for industrial 7-ACA production is shown in figures 24 and 25. The process has been in operation at Asahi Chemical Industry since **1973**. It is reported that about 90 tons of 7-ACA are thus produced annually [51].

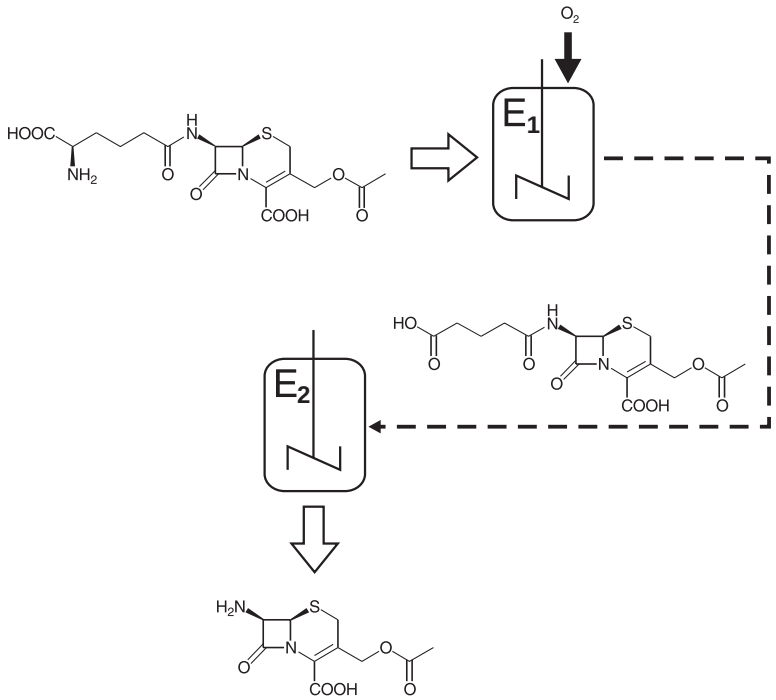

Fig. 24 Flow scheme for the production of 7-ACA. Production carried out at Asahi Chemical Industry. (**E_1** = D-aminoacid oxidase; **E_2** = glutaryl amidase).

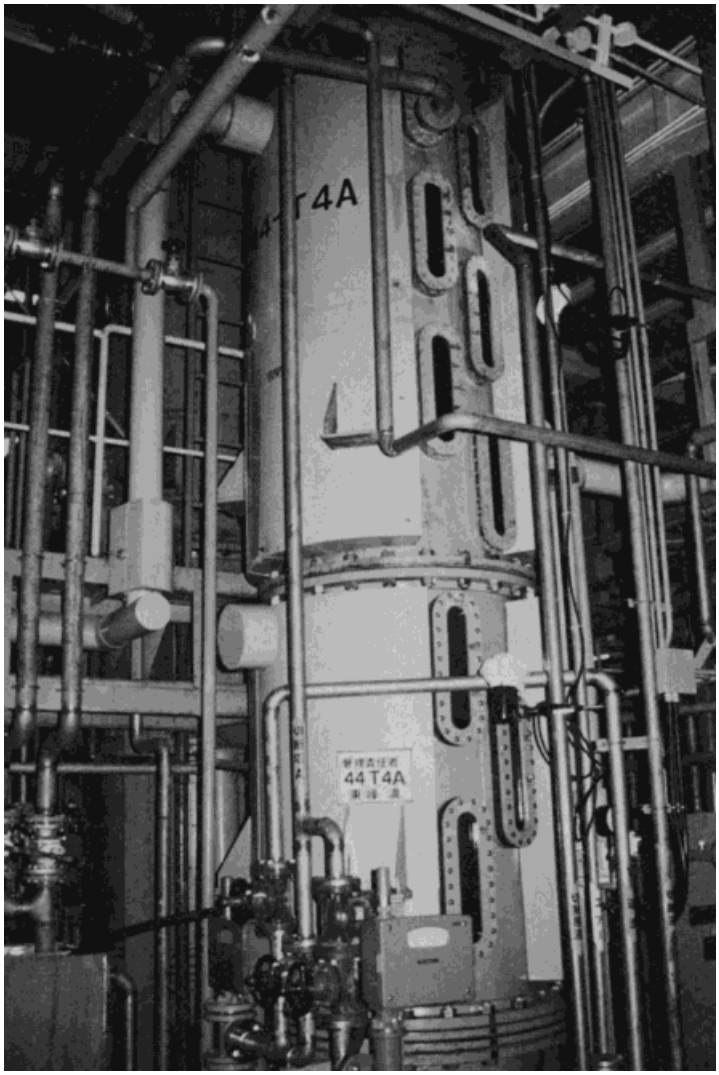

Fig. 25 The bioreactor plant for 7-ACA production carried out at Asahi Chemical Industry (Reprinted from Ref. [51], p. 83 by courtesy of Marcel Dekker Inc.).

Four technological advances, having major impact on enzymatic biotransformations, were required for the acceptance of enzymes as 'alternative catalysts' in industry [52].

The first technological advance was the development of large-scale techniques for the release of enzymes from the interior of microorganisms [53]. Although the majority of industrial purification procedures are based on the same principles as those employed at laboratory scale, the factors under consideration while devising industrial scale purification regimes are somewhat different. When isolating enzymes on an industrial scale for commercial purposes, a prime consideration has to be the cost of production in relation to the value of the end prod-

uct. Therefore, techniques used on a laboratory scale are not always suitable for large scale work [54]. Production and isolation of an intracellular microbial enzyme are quite expensive. The costs of the usage of water-soluble protein as catalyst for biotransformations can be justified only by its repeated use [55].

The second technological advance was the development of techniques for large-scale immobilization of enzymes. As mentioned earlier, the first enzyme immobilized in the laboratory was invertase, adsorbed onto charcoal in the year **1916** [56]. However, only after the development of immobilization techniques on a large scale occured in the **1960s**, many different industrial processes using immobilized biocatalysts have been established. The historical invertase column operating since **1968** on a laboratory scale is shown in figure 26.

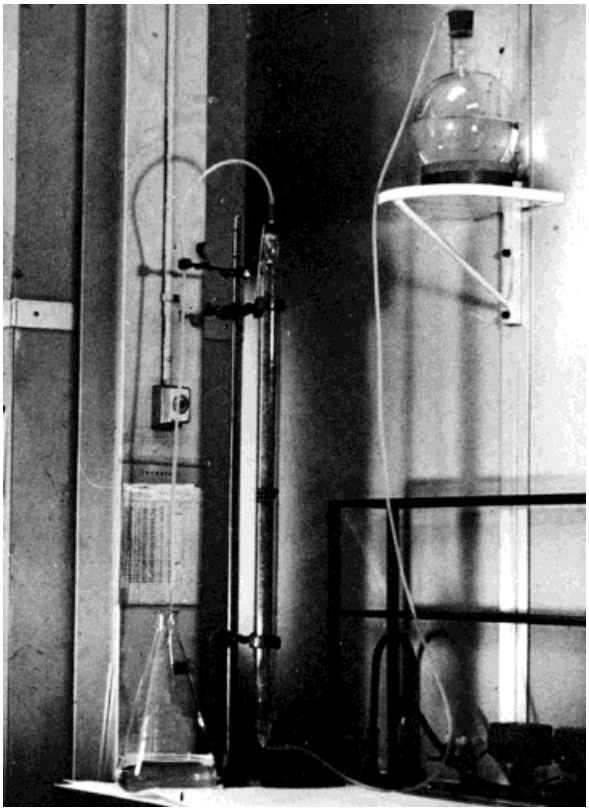

Fig. 26 Historical invertase column [49].

It was shown that by increasing the concentration of sucrose, the efficiency of the fiber-entrapped invertase (which hydrolyses sucrose) can be increased. This occurred because the substrate, which is an inhibitor of the enzyme, could not reach high concentration levels inside the microcavities of the fibers owing to diffusion limitations [49].

Table II lists some industrial biotransformations performed by isolated enzymes.

Table II: Selected historical, industrial applications of isolated enzymes.

Product	Biocatalyst	Operating since	Company
L-amino acid	aminoacylase	1954, 1969	Tanabe Seiyaku Co. Ltd., Japan
6-aminopenicillanic acid	penicillin acylase	1973	SNAMProgetti and others*
low lactose milk	lactase	1977	Central del Latte, Milan, Italy (SNAMProgetti technology)
7-amino-cephalosporanic acid	D-amino acid oxidase	1979	Toyo Jozo and Asahi Chemical Industry, Japan

* Beecham, Squibb, Astra Lakenedal, Bayer, Gist-Brocades, Pfizer, Bristol Myers, Boehringer Mannheim, Biochemie, Novo, Hindustan Antibiotics

The first Enzyme Engineering Conference was held at Hennicker, New Hampshire, in **1971**. The term "immobilized enzymes" describing "enzymes physically confined at or localized in a certain region or space with retention of their catalytic activity and which can be used repeatedly and continuously" was adopted at this conference [57].

The third technological advance was the development of techniques for biocatalysis in organic media. The usage of very high proportions of organic solvents for increasing the solubility of reactants was examined in **1975** in the reaction with isolated cholesterol oxidase to produce cholestenone [58]. The enzymatic synthesis was believed to be incompatible with most organic syntheses carried in nonaqueous media. This changed after Klibanov [59] recognized in **1986** that most enzymes could function quite well in organic solvents. Since that time different processes involving an organic phase have been established in industry (Table III).

Table III: Industrial biotransformations involving poorly water-soluble reactants.

Process	Biocatalyst	Operating since	Company
fat interesterification	lipase	1979, 1983	Fuji Oil, Unilever
ester hydrolysis	lipase	1988	Sumitomo
transesterification	lipase	1990	Unilever
aspartame synthesis	thermolysin	1992	DSM
acylation	lipase	1996	BASF

The fourth and most recent technological advance is recombinant DNA technology. This technology is only now being widely used for biotransformations.

Generally, microorganisms isolated from nature produce the desired enzyme at levels which are too low to offer a cost-effective production process. Consequently, the modification of the organism would be highly desirable for process development. Currently, there are three principal approaches available for strain improvement. The first one, *direct evolution* [60], i.e. improvement by mutation and selection, has been successfully used in many industrial microbiological fields for many years. In **1978**, Clarke showed that evolution processes can be performed on a laboratory scale. Microevolution occuring in bacterial cultures grown in the chemostat gives rise to altered enzyme specificity, enabling microorganisms to degrade some unusual synthetic organic compounds. Successive muta-

tional steps could be responsible for the evolution of new enzymatic specificities. The rate of production of existing enzymes and the expression of previously dormant genes are also typically affected by this event [61]. The second method is *hybridization*. It involves modification of the cellular genetic information by transference of DNA from another strain. The third method is *recombinant DNA technology*, whereby genetic information from one strain can be manipulated *in vitro* and then inserted into the same or another strain.

Recombinant DNA technology has dramatically changed enzyme production, because enzymes are synthesized in cells by the normal protein synthesis methods [62,63]. A 5–10 year period required for classical enzyme development can be reduced to 1–2 years. Protein engineering, in combination with recombinant expression systems, allows to plug in a new enzyme variant and to be very quick at manufacturing levels [64]. Novel microbial catalysts, together with recent advances in molecular biology, offer scientists an opportunity to rapidly evolve selected genes and considerably improve bacterial biocatalysts [65]. For example, a method for the rapid generation of thermostable enzyme variants has been developed [66,67]. This is done by introducing the gene coding for a given enzyme from a mesophilic organism into a thermophile. Variants that retain the enzymatic activity at higher growth temperatures of the thermophile are selected. This can be accomplished by constructing the artificial environment in which only the evolutional adaptation of the enzyme can permit cell growth. This strategy can be readily extended to the general method of screening mutant enzymes. Another example is random mutation, developed as a method for highly efficient generation of mutant enzymes. The cloned gene coding for a given enzyme can be mutated either chemically or enzymatically *in vitro*. The mutant enzymes can be readily screened because mutant genes can be separated from intact genes. Various mutant enzymes have a change in properties, such as substrate specificity, thermal stability and coenzyme selectivity, have been isolated by this technique. These methods do not require predictive strategies, unlike, for example, site-directed mutagenesis. It is hoped that in course of time they will make enzymes excellent catalysts fulfilling all the requirements for industrial use. This research field may be referred to as **biocatalyst engineering** [23].

2.3 Advantages of biotransformations over classical chemistry

Enzymes are proteins, things of beauty and a joy forever [26]

Biocatalysis is a relatively green technology. Enzyme reactions can be carried out in water at ambient temperature and neutral pH, without the need for high pressure and extreme conditions, thereby saving process energy. Biocatalysis has proven to be a useful supplementary technology for the chemical industry, allowing, in some cases, reactions which are not easily conducted by classical organic chemistry or, in other cases, allowing reactions which can replace several chemic-

al steps. Today, highly chemo-, regio- and stereoselective biotransformations can simplify manufacturing processes and make them even more economically attractive and environmentally acceptable [68].

Both new discoveries and incrementalism describe how the industrial enzyme business changed during **1996**. Enzymes have competed well with chemical methods for resolution but not with synthesis. Ibuprofen, phenylethylamine and acrylamide are commonly cited as compounds using enzyme-based chiral processes. There is also an unconfirmed suspicion that the fat substitute Olestra, because of some of its structural features, may require enzymatic steps for synthesis. The outlook for industrial enzymes is positive. The suppliers have extensive portfolios of promising new enzymes in their product pipelines. The range of customers considering the utilization of enzymes, as a replacement to conventional chemical methods, appears to be growing. New niche applications continue to be discovered in otherwise mature segments [69]. It appears that enzyme-based processes are gradually replacing conventional chemical-based methods. Finally, the latest literature on enzymology suggests that other biocatalysts will add to future sales, both in established and new markets. The enzyme "nitrogenase", converting dinitrogen to ammonia, a basic chemical compound, has been discovered recently [70]. Dream reactions of organic chemists might become true in the future, with biocatalysts where functional or chiral groups are introduced into molecules by utilizing H_2, O_2 or CO_2. Recently Aresta reported of a carboxylase enzyme that utilizes CO_2 in the synthesis of 4-hydroxybenzoic acid starting from phenyl-phosphate [71].

Although the production of D-amino acids is currently of great interest, there has been no known industrial manufacture of D-amino acids except for D-*p*-hydroxyphenylglycine and D-phenylglycine. Chemical methods are not suitable for large scale production of D-amino acids at the moment due to low yield and high cost. Most L-amino acids are efficiently manufactured by fermentation, but D-amino acids are hardly produced by fermentation, apart from a few exceptions, because it is difficult to obtain high optical purity and productivity. Enzymatic methods are most plausible for the industrial manufacture of D-amino acids with respect to optical purity and productivity. D-Amino acids such as D-*p*-hydroxy phenylglycine and D-phenylglycine are produced from D,L-hydantoins. From an industrial point of view, availability of cheap starting materials and the development of suitable biocatalysts are most important. The number of substrates that are available on an industrial scale is limited. Based on these criteria, synthetic intermediates of D,L-amino acids and L-amino acids produced by biotransformations would be the most important starting materials for the production of D-amino acids. The enzymatic production of D-amino acids is classified into three categories based on the starting materials [72]:

1. D,L-Amino acids (D-amino acylase)
2. Synthetic intermediates (D,L-hydantoin:D-hydantoinhydrolase; D,L-amino acid amides:D-amidase)
3. Prochiral substrates (α-keto acids, L-amino acids; D-transaminase and amino acid racemase)

The fed batch process [73] used in the production of L-DOPA, having a final product concentration of 110 $g \cdot L^{-1}$, has many advantages over the classical chemical process, such as: a single reaction step, water as the only reaction by-

product, no need for optical separation, shorter production cycle of three days, simple down-stream processing and process sustainability. L-DOPA is a metabolic precursor of dopamine, a very important drug in the treatment of Parkinsonism.

It is difficult to directly assess the true commercial value of biocatalysis, because the real value of the products made using the biocatalysts must be taken into account. Of course, its major advantage lies in stereoselective reactions. A good example of its technological power and commercial potential is the aforementioned stereoselective hydroxylation of steroids.

In comparison to fermentation processes fewer side-products are formed in enzymatic biotransformations, complex and expensive fermenters are not required, aeration, agitation and sterility need not necessarily be maintained and the substrate is not diverted into the formation of *de novo* cellular biomass [50]. Isolated biocatalysts are especially useful if the reaction they catalyze is about to be completed, if they are resistant to product inhibition, and if they are active in the presence of low concentrations of substrate (such as in detoxification reactions where pollutants are present in the waste stream). "One-pot" multi-enzyme reactions are much more feasible than a combined use of several chemical catalysts or reagents, especially as the latter often have to be used in reactors made of special resistant materials to tolerate extreme conditions, such as the use of concentrated acids under elevated temperatures and pressures [50].

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3 Enzyme Classification

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3.1 The Enzyme Nomenclature

In early times of biochemistry there were no guidelines for naming enzymes. The denomination of newly discovered enzymes was given arbitrarily by individual workers. This practice had proved to be inadequate. Occasionally two different enzymes had the same name while in other cases two different names were given to the same enzyme. Furthermore, there emerged denominations which provided no clue about the catalyzed reaction (e.g. catalases, or pH 5 enzyme).

With the great progress experienced by biochemistry in the 1950's, a large number of enzymes could be isolated and characterized. By this time it became evident that it was necessary to regulate the enzyme nomenclature. So, the International Union of Biochemistry and Molecular Biology (IUBMB), formerly International Union of Biochemistry (IUB), set up in consultation with the International Union for Pure and Applied Chemistry (IUPAC), an Enzyme Commission in charge of guiding the naming and establishing a systematic classification for enzymes. In 1961, the report of the commission was published. The proposed classification was used to name 712 enzymes. This work has been widely used as a guideline for enzyme nomenclature in scientific journals and textbooks ever since. It has been periodically updated, new entries have been included or old ones have been deleted, while some other enzymes have been reclassified. The sixth complete edition of the Enzyme Nomenclature (1992) contains 3196 enzymes [1]. Five supplements to the Enzyme Nomenclature with various additions and corrections have been published until today [2,3,4,5,6] signaling the constantly growing number of new enzyme entries. An update documentation of the classified enzymes is available on the ENZYME data bank server [7,8].

The Enzyme Nomenclature suggests two names for each enzyme, a **recommended name** convenient for every day use and a **systematic name** used to minimize ambiguity. Both names are based on the nature of the catalyzed reaction. The recommended name is often the former trivial name, sometimes after little change to prevent misinterpretation. The systematic name also includes the involved substrates. This taxonomy leads to the classification of enzymes into six main classes (Table 1).

Table 1: The main enzyme classes

Enzyme class	Catalyzed reaction
1. Oxidoreductases	oxidation-reduction reactions
2. Transferases	transfer of functional groups
3. Hydrolases	hydrolysis reactions
4. Lyases	group elimination (forming double bonds)
5. Isomerases	isomerization reactions
6. Ligases	bond formation coupled with a triphospate cleavage

As the systematic name may be very extensive and uncomfortable to use, the Enzyme Commission (EC) has also developed a numeric system based on the same criteria, which can be used together with the recommended name to specify the mentioned enzyme. According to this system, each enzyme is assigned a four-digit EC number (Table 2). The first digit denotes the main class that specifies the catalyzed reaction type. These are divided into subclasses, according to the nature of the substrate, the type of the transferred functional group or the nature of the specific bond involved in the catalyzed reaction. These subclasses are designated by the second digit. The third digit reflects a further division of the subclasses according to the substrate or co-substrate, giving origin to sub-sub-classes. In the fourth digit a serial number is used to complete the enzyme identification.

Table 2: Constitution of the four-digit EC number

EC number EC (i).(ii).(iii).(iv)	
(i)	the main class, denotes the type of catalyzed reaction
(ii)	sub-class, indicates the substrate type, the type of transferred functional group or the nature of one specific bond involved in the catalyzed reaction
(iii)	sub-subclass, expresses the nature of substrate or co-substrate
(iv)	an arbitrary serial number

As an example, aminoacylase (*N*-acyl-L-amino-acid amidohydrolase, according to the systematic nomenclature), an enzyme used in the industrial production of L-methionine, has the classification number EC 3.5.1.14 (see process on page 300). The first number (i = 3) indicates that this enzyme belongs to the class of hydrolases. The second number (ii = 5) expresses that a carbon-nitrogen bond is hydrolyzed and the third number (iii = 1) denotes that the substrate is a linear amide. The serial number (iv = 14) is needed for full classification of the enzyme.

As the biological source of an enzyme is not included in its classification, it is important to mention this together with the enzyme number for full identification. So the enzyme used in the production of "acrylamide" should be mentioned as "nitrilase (EC 4.2.1.84) from "*Rhodococcus rhodochrous*" (see process on page 362).

An important aspect concerning the application of the enzyme nomenclature is the direction how a catalyzed reaction is written for purposes of classification. To make the classification more transparent the direction should be the same for all enzymes of a given class, even if this direction has not been demonstrated for all enzymes of this class. Many examples for the use of this convention can be found in the class of oxidoreductases.

A further implication of this system is the impossibility of full classification of an enzyme if the catalyzed reaction is not clear. Complete classification of the enzymes only depends on the natural substrates. Non-natural substrates are not considered for the classification of the biocatalyst.

Finally, it is important to emphasize that the advantageous influence of the enzyme classification is not limited to biochemistry's enzyme nomenclature. It is also very beneficial for organic preparative chemists because it facilitates the choice of enzymes for synthetic applications. Since the classification of the enzymes is based on the catalyzed reactions it helps chemists to find an appropriate biocatalyst for a given synthetic task. An analogous nomenclature for chemical catalysts has not been set up until today.

The number of existing enzymes in nature is estimated to reach the 25,000 mark [9]. It is one essential part of biochemistry and related sciences to try to find and identify them. The scientist isolating and characterizing a new enzyme is free to report the discovery of that "new" biocatalyst to the Nomenclature Committee of the IUBMB and may form a new systematic name for this enzyme. An appropriate form to draw the attention of the editor of the Enzyme Nomenclature to enzymes and other catalytic entities missing from this list is available online [10].

3.2 The Enzyme Classes

The following part of this chapter aims at giving a compact overview of the six main enzyme classes and their subclasses. Since the industrial bioprocesses and biotransformations illustrated in the following chapters of the book are divided according to the involved enzymes and their classes, this short survey should provide the reader with the most important information on the enzyme classes.

The six main enzyme classes are resumed separately by giving a general reaction equation for every enzyme subclass according to the Enzyme Nomenclature. The reaction equations are picturized in a very general manner pointing out just the most important attributes of the catalyzed reactions. The authors would like to emphasize that no attempt has been made to provide a complete summary of the reactions catalyzed by the enzymes listed in the Enzyme Nomenclature. The reaction schemes have been elaborated to give reaction equations being as general and clear as possible and as detailed as necessary.

An important point that needs to be considered in this context concerns the enzymes classified as EC (i).99 or EC (i).(ii).99. These enzymes are either very substrate specific and therefore cannot be classified in already existing enzyme subclasses (or sub-subclasses) or a substrate of these enzymes has not been completely identified yet.

For instance, in the enzyme main class EC 5.(ii).(iii).(iv) (isomerases), the EC number 5.99 only describes "other isomerases" that cannot be classified within the other existing subclasses EC 5.1 to EC 5.5. It is important to point out that the enzymes classified with a 99-digit have not been considered in the reaction equations unless stated explicitly. The catalyzed reactions of these enzymes differ exceedingly from those of the other enzymes in the same main division.

The following short remarks on the generalized reaction schemes should help the reader to understand the illustrated enzyme catalyzed reactions:

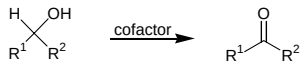
1. Each main enzyme class is introduced by a short paragraph giving a general idea of the respective enzymes.
2. By generalizing nearly all catalyzed reactions of one enzyme subclass to only one or a few reaction equations, some details of the single reactions had to be neglected, e.g. specification of the cofactor, reaction conditions (pH, temperature), electric charge or stoichiometry. Correct protonation of the substrates and products depending on the pH value of each reaction mixture has not been taken into consideration as well. Also, the enzyme itself does not appear in the reaction schemes of this chapter.
3. If the catalyzed reaction leads to a defined equilibrium, only one direction of this reaction is considered according to its direction in the Enzyme Nomenclature. In consequence, no equilibrium arrows are used in any reaction scheme of this chapter.
4. Enzymes of a given subclass may show some frequently appearing common properties or some very worthwhile uniqueness. These qualities are taken into account by additional comments below the reaction schemes.

EC 1 Oxidoreductases

The enzymes of this first main division catalyze oxidoreduction reactions, which means that all these enzymes act on substrates through the transfer of electrons. In the majority of the cases the substrate that is oxidized is regarded as hydrogen donor. Various cofactors or coenzymes serve as acceptor molecules. The systematic name is based on *donor:acceptor oxidoreductase*.

Whenever possible the nomination as a *dehydrogenase* is recommended. Alternatively, the term *reductase* can be used. If molecular oxygen (O_2) is the acceptor, the enzymes may be named as *oxidases*.

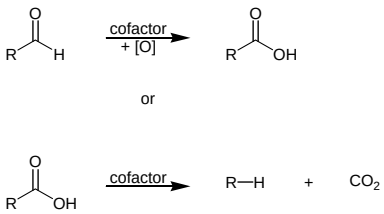
EC 1.1 Acting on CH-OH group of donors



R^1 = hydrogen, organic residue
R^2 = hydrogen, organic residue, alcoxy residue

The sub-subclasses are defined by the type of cofactor.

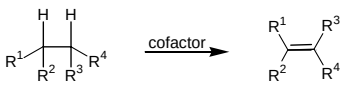
EC 1.2 Acting on aldehyde or oxo group of donors



R = hydrogen, organic residue

Analogous with the first depicted reaction, the aldehyde can be oxidized to the respective thioester with coenzyme A (CoA). In the case of oxidation of carboxylic acids, the organic product is not necessarily bound to hydrogen as suggested in the figure. It can also be bound to the cofactor. The sub-subclasses are classified according to the cofactor.

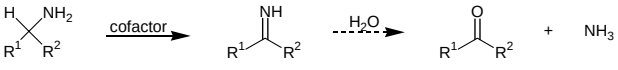
EC 1.3 Acting on the CH-CH group of donors



$R^{1,2,3,4}$ = hydrogen, organic residue

In some cases the residues can also contain heteroatoms, e.g. dehydrogenation of *trans*-1,2-dihydroxycyclohexa-3,5-diene to 1,2-dihydroxybenzene (catechol). Further classification is based on the cofactor.

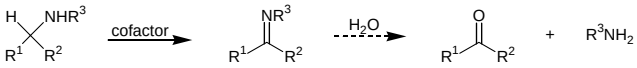
EC 1.4 Acting on the CH-NH_2 group of donors



$R^{1,2}$ = hydrogen, organic residue

In most cases the imine formed is hydrolyzed to give an oxo-group and ammonia (deaminating). The division into sub-subclasses depends on the cofactor.

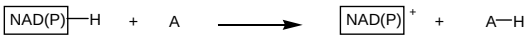
EC 1.5 Acting on the CH-NH group of donors



$R^{1,2}$ = hydrogen, organic residue
R^3 = organic residue

In some cases the primary product of the enzymatic reaction may be hydrolyzed. Further classification is based on the cofactors.

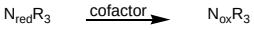
EC 1.6 Acting on NAD(P)H



A = acceptor

Generally enzymes that use NAD(P)H as reducing agent are classified according to the substrate of the reverse reaction. Only enzymes which need some other redox carrier as acceptors to oxidize NAD(P)H are classified in this subclass. Further division depends on the redox carrier used.

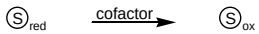
EC 1.7 Acting on other nitrogen compounds as donors



R = hydrogen, organic residue, oxygen

The enzymes that catalyze the oxidation of ammonia to nitrite and the oxidation of nitrite to nitrate belong to this subclass. The subdivision is based on the cofactor.

EC 1.8 Acting on sulfur group of donors

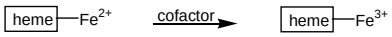


S_{red} = sulfide, sulfite, thiosulfate, thiol, etc.

S_{ox} = sulfite, sulfate, tetrathionate, disulfite, etc.

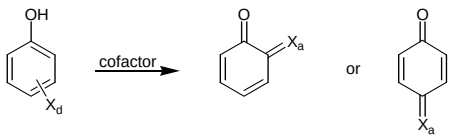
The substrates may be either organic or inorganic sulfur compounds. The nature of the cofactor defines the further classification.

EC 1.9 Acting on a heme group of donors



The sub-subclasses depend again on the cofactor.

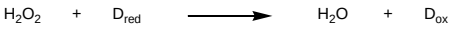
EC 1.10 Acting on diphenols and related substances as donors



X_d = OH, NH_2
X_a = O, NH

The aromatic ring may be substituted; ascorbates are also substrates for this subclass. The primary product may undergo further reaction. The subdivision in four sub-subclasses depends on the cofactor.

EC 1.11 Acting on a peroxide as acceptor

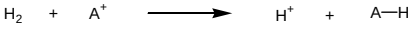


$H_2O_2 + D_{red} \longrightarrow H_2O + D_{ox}$

D = donor

The single sub-subclass contains the peroxidases.

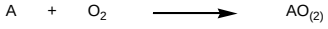
EC 1.12 Acting on hydrogen as donor



$H_2 + A^+ \longrightarrow H^+ + A{-}H$

Sub-subclass 1.12.1 contains enzymes using NAD^+ and $NADP^+$ as cofactors. Other hydrogenases are classified under 1.12.99. Enzymes using iron-sulfur compounds as cofactor are listed under 1.18.

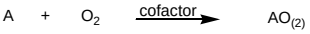
EC 1.13 Acting on single donors with incorporation of molecular oxygen



$A + O_2 \longrightarrow AO_{(2)}$

If two oxygen atoms are incorporated, the enzyme belongs to the sub-subclass 1.13.11 and if only one atom of oxygen is used the enzyme is classified as 1.13.12. All other cases are classified under 1.13.99.

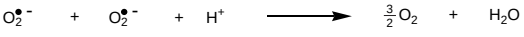
EC 1.14 Acting on paired donors with incorporation of molecular oxygen



$A + O_2 \xrightarrow{\text{cofactor}} AO_{(2)}$

The classification into sub-subclasses depends on whether both oxygen atoms or just one is bonded to the substrate. The difference to subclass 1.13 is the requirement of a cofactor.

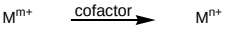
EC 1.15 Acting on superoxide radicals as acceptor



$O_2^{\bullet -} + O_2^{\bullet -} + H^+ \longrightarrow \frac{3}{2}O_2 + H_2O$

The only enzyme classified under this subclass is superoxide dismutase.

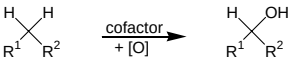
EC 1.16 Oxidizing metal ions



$m \geq 0$
$n > m$

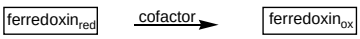
The two sub-subclasses are divided according to the cofactor.

EC 1.17 Acting on CH_2 groups

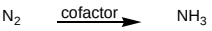


The origin of the oxidizing oxygen is either molecular oxygen or water.

EC 1.18 Acting on reduced ferredoxin as donor



EC 1.19 With dinitrogen as acceptor



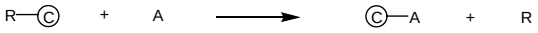
The only enzyme classified under this subclass is nitrogenase.

EC 2 Transferases

The transferases are enzymes that transfer a chemical group from one compound (generally regarded as the donor) to another compound (generally regarded as the acceptor). Of all biological reactions, this class of biocatalysts is one of the most common [11]. To avoid any confusion, the following reaction schemes of the subclasses all show the same pattern: the donor molecule is always the first one among the substrates, the acceptor is the second one. If possible, some detailed information is given on the acceptor, but also a general denomination as A = acceptor has been chosen in three cases.

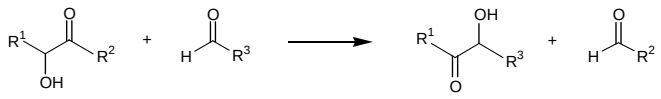
In general, the systematic names of these biocatalysts are formed according to the scheme *donor:acceptor* group*transferase*. In many cases, the donor is a cofactor (coenzyme) carrying the often activated chemical group to be transferred.

EC 2.1 Transferring one-carbon groups



A = acceptor
R = organic residue
Ⓒ = methyl-, hydroxymethyl-, formyl-, carboxyl-, carbamoyl- and amidino-groups

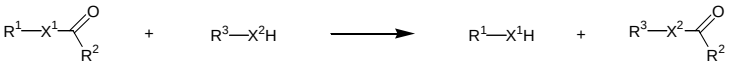
EC 2.2 Transferring aldehyde or ketone residues



R^1 = hydrogen or methyl residue
R^2 = methyl residue or polyol chain
R^3 = hydrogen or polyol chain

Three of the only four enzymes in this class depend on thiamin-diphosphate as a cofactor. The catalyzed reactions may be regarded as an aldol addition. Some enzymes also accept hydroxypyruvate as a donor to form CO_2 and the resulting addition product.

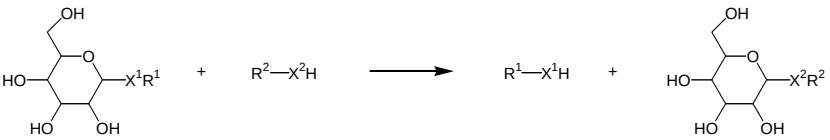
EC 2.3 Acyltransferases



X^1 = S, O, NH
X^2 = S, O, NH, CH_2
R^1 = hydrogen, alkyl-, aryl- or monophosphate residue
R^2 = hydrogen, alkyl- or aryl-residue
R^3 = alkyl-, aryl-, acyl- or monophosphate residue, aryl-NH

Transferred acyl-groups are often activated as coenzyme A (CoA) conjugates.

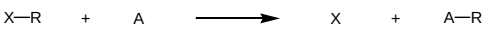
EC 2.4 Glycosyltransferases



X^1 = O, PO_4^{3-}
X^2 = O, NH
R^1 = hydrogen, hexosyl, pentosyl, oligosaccharide, monophosphate
R^2 = hexosyl, pentosyl, oligosacharide, monophosphate, organic residue with OH- or NH_2-groups
X^1R^1 = nucleoside di- or monophosphates (e.g. UDP, ADP, GDP or CMP), purine

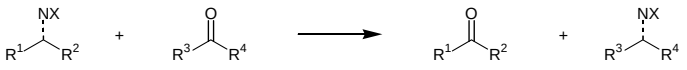
This enzyme class is subdivided into the hexosyl- (sub-subclass 2.4.1) and pentosyltransferases (sub-subclass 2.4.2). Although illustrating a hexosyl transfer in the figure, this general scheme is meant to describe both the enzyme sub-subclasses.

EC 2.5 Transferring alkyl or aryl groups, other than methyl groups



A = acceptor
X = OH, NH, SR, SO_4^-, mono-, di- or triphosphate
R = organic residue other than a methyl group

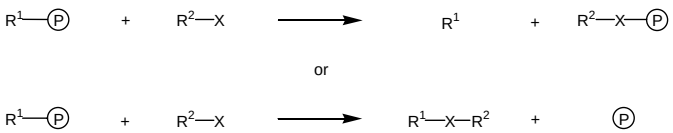
EC 2.6 Transferring nitrogenous groups



If NX = NH_2, then --- is a single bond.
If NX = NOH, then --- is a double bond.
R^1 = hydrogen, carboxy or methyl residue
R^2 = organic residue
R^3 = hydrogen, carboxy or hydroxymethyl residue
R^4 = organic residue

Pyridoxal-phosphate is the most frequently-appearing cofactor for these enzymes. For NX = NH_2 the substrates are often α-amino acids and 2-oxo acids.

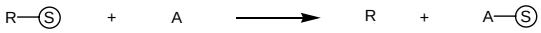
EC 2.7 Transferring phosphorous-containing groups



X = OH, COOH, NH_2, PO_4^{2-}
R^1 = hydrogen, NDP, NMP, adenosine, monosaccharide residue, acyl residue, polyphosphate, histidine, *syn*-glycerol, organic residues carrying more functional groups
R^2 = hydrogen, monosaccharide residue, nucleosides, nucleotides, organic residues carrying more functional groups, proteins, polyphosphate
Ⓟ = mono- or diphosphates

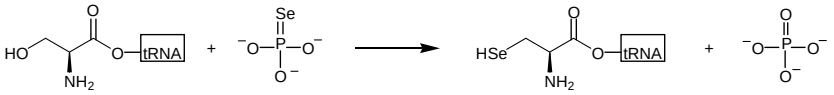
The enzymes transferring a phosphate residue from an ATP molecule to an acceptor are called kinases. The enzyme EC 2.7.2.2 (carbamate kinase) transfers a phosphate residue from an ATP molecule on CO_2 and NH_3 to form carbamoyl phosphate.

EC 2.8 Transferring sulfur-containing groups



A = acceptor, e.g. cyanide, phenols, alcohols, carboxylic acids, amino acids, amines, saccharides
R = sulfur atom, (phosphorous-) organic residue
Ⓢ = sulfur atom, SO_3^{2-}, SH, CoA

EC 2.9 Transferring selenium-containing groups



The only enzyme classified under this subclass is L-seryl-tRNA (Sec) selenium transferase.

EC 3 Hydrolases

This third main class of enzymes plays the most important role in today's enzymatic industrial processes. It is estimated that approximately 80 % of all industrial enzymes are members of this enzyme class [12]. Hydrolases catalyze the hydrolytic cleavage of C-O, C-N, C-C and some other bonds, including P-O bonds in phosphates. The applications of these enzymes are very diverse: the most well-known examples are the hydrolysis of polysaccharides (see process on page 231), nitriles (see process on page 361 and 362), proteins or the esterification of fatty acids (see process on page 217). Most of these industrial enzymes are used in processing-type reactions to degrade proteins, carbohydrates and lipids in detergent formulations and in the food industry.

Interestingly, all hydrolytic enzymes could also be classified as transferases, since every hydrolysis reaction can be regarded as the transfer of a specific chemical group to a water molecule. But, because of the ubiquity and importance of water in natural processes, these biocatalysts are classified as hydrolases rather than as transferases.

The term *hydrolase* is included in every systematic name. The recommendation for the naming of these enzymes is the formation of a name which includes the name of the substrate and the suffix *–ase*. It is understood that the name of the substrate with this suffix means a hydrolytic enzyme.

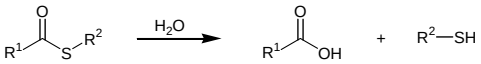
EC 3.1 Acting on ester bonds

The nature of the substrate may differ largely, as shown in the three examples.

EC 3.1.1 Carboxylic ester hydrolase

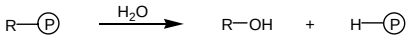
R^1 = hydrogen, organic residue
R^2 = organic residue

EC 3.1.2 Thiolester hydrolase



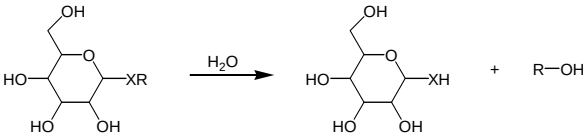
R^1 = hydrogen, organic residue
R^2 = organic residue

EC 3.1.3 Phosphohydrolase ("phosphatase")



Ⓟ = monophosphate
R = organic residue

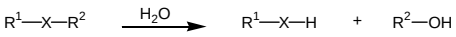
EC 3.2 Glycosidases



X = O, N or S
R = organic residue

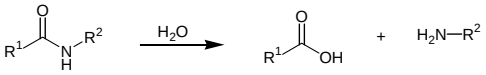
The illustration shows the hydrolysis of a hexose derivative although pentose derivatives are also accepted as substrates.

EC 3.3 Acting on ether bonds



X = O or S
$R^{1,2}$ = organic residue

EC 3.4 Acting on peptide bonds



$R^{1,2}$ = part of amino acids or proteins

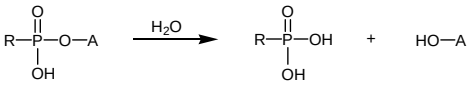
EC 3.5 Acting on carbon-nitrogen bonds other than peptide bonds

R = organic residue

For some nitriles a similar reaction takes place. The enzyme involved is called nitrilase (EC 3.5.5).

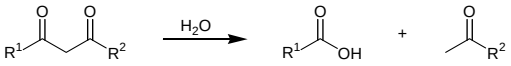
R = aromatic, heterocyclic and certain unsaturated aliphatic residues

EC 3.6 Acting on acid anhydrides



A = phosphate, organic phosphate, sulfate
R = organic residue, hydroxy group

EC 3.7 Acting on carbon-carbon bonds



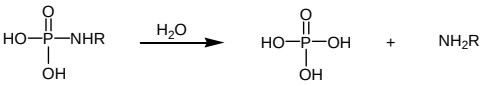
$R^{1,2}$ = organic residue, hydroxy group

There is only one sub-subclass.

EC 3.8 Acting on halide bonds

X = halogen
R = hydrogen, organic residue, hydroxy group

EC 3.9 Acting on phosphorous-nitrogen bonds



R = organic residue

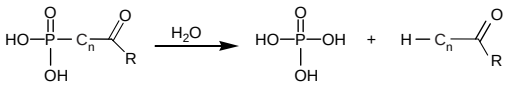
The only enzyme classified under this subclass is phosphoamidase.

EC 3.10 Acting on sulfur-nitrogen bonds

Ⓢ = sulfon group
R = organic residue

There is only one subdivision of this subclass.

EC 3.11 Acting on carbon-phosphorous bonds



R = CH_3, OH
n = 0, 1

If n = 0, the product is an aldehyde.

EC 3.12 Acting on sulfur-sulfur bonds

S1 = sulfate
S2 = thiosulfate

The only enzyme classified under this subclass is trithionate hydrolase.

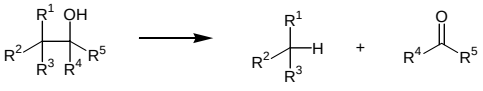
EC 4 Lyases

From the commercial perspective, these enzymes are an attractive group of catalysts as demonstrated by their use in many industrial processes (see chapter 5). The reactions catalyzed are the cleavage of C-C, C-O, C-N and some other bonds. It is important to mention that this bond cleavage is different from hydrolysis, often leaving unsaturated products with double bonds that may be subject to further reactions. In industrial processes these enzymes are most commonly used in the synthetic mode, meaning that the reverse reaction – addition of a molecule to an unsaturated substrate – is of interest. To shift equilibrium these reactions are conducted at very high substrate concentrations which results in very high conversions to the desired products. For instance, a specific type of lyase, the *phenylalanine ammonia lyase* (EC 4.1.99.2), catalyzes the formation of an asymmetric C-N bond yielding the L-amino acid dihydroxy-L-phenylalanine (L-DOPA). This amino acid is produced on a ton scale and with very high optical purities (see process on page 342).

Systematic denomination of these enzymes should follow the pattern *substrate* group-*lyase*. The hyphen should not be omitted to avoid any confusion, e.g. the term *hydro-lyase* should be used instead of *hydrolyase*, which looks quite the same as a *hydrolase*.

In the recommended names, terms like *decarboxylase*, *aldolase* or *dehydratase* (describing the elimination of CO_2, an aldehyde or water) are used. If the reverse reaction is much more important, or the only one known, the term *synthase* may be used.

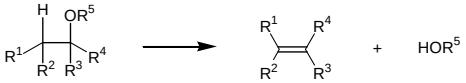
EC 4.1 Carbon-carbon lyases



$R^{1,2,3,4,5}$ = hydrogen, organic residue

If the substrate is a carboxylic acid, one of the products will be carbon dioxide. If the substrate is an aldehyde, carbon monoxide may be a product.

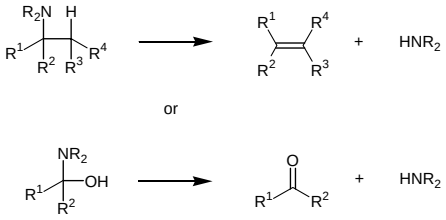
EC 4.2 Carbon-oxygen lyases



$R^{1,2,3,4,5}$ = hydrogen, organic residue

A further addition of water to the product may lead to an oxo acid. This is the case for some amino acids, where ammonia is then eliminated.

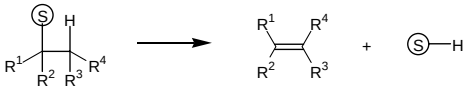
EC 4.3 Carbon-nitrogen lyases



R = organic residue

The resulting double bond may change its position in order to deliver a more stable product, for instance in the case of keto-enol tautomerism. The product may also undergo a further reaction.

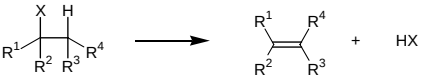
EC 4.4 Carbon-sulfur lyases



Ⓢ = SH, (di)substituted sulfide, sulfur-oxide, SeH
R = organic residue

According to the Enzyme Nomenclature the carbon-selenium lyase also belongs to this subclass. Similar to other lyases, further reactions may occur on the product. In the case of disubstituted sulfides, there is no hydrogen bonded to the sulfur in the product.

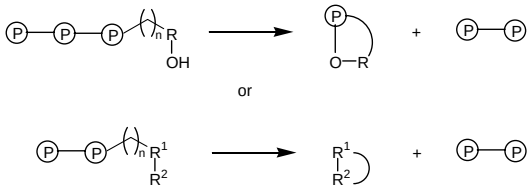
EC 4.5 Carbon-halide lyases



X = halogen
R = organic residue

The primary product may also undergo further reaction. In the case of dihalo-substituted methane the sequential reaction will lead to the aldehyde. Amino compounds may react under elimination of ammonia to oxo compounds. If thioglycolate is a cofactor, a sulfur-carbon bond will replace the halogen-carbon one.

EC 4.6 Phosphorous-oxygen lyase



Ⓟ = monophosphate
R = organic residue

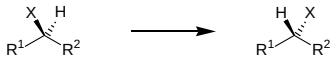
With the exception of EC 4.6.1.4 all enzymes of this subclass lead to cyclic products.

EC 5 Isomerases

This enzyme class only represents a small number of enzymes, but nevertheless one of them plays a major role in todays industry. This enzyme, known as *glucose isomerase* (EC 5.3.1.5), catalyzes the conversion of D-glucose to D-fructose which is necessary in the production of high-fructose corn syrup (HFCS) (see process on page 387). This syrup is a substitute for sucrose and is used by the food and beverage industries as a natural sweetener.

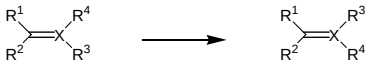
In general, the isomerases catalyze geometric or structural changes within one single molecule. Depending on the type of isomerism, these enzymes may be called as *epimerases*, *racemases*, cis-trans-*isomerases*, *tautomerases* or *mutases.*

EC 5.1 Racemases and epimerases



X = NH_2, NHR, NR_2, OH, CH_3, COOH
$R^{1,2}$ = organic residue

EC 5.2 *cis-trans*-Isomerases

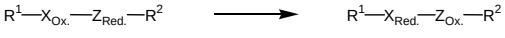


X = C or N
$R^{1,2,3,4}$ = organic residue

If X = N the substrate is an oxime. In this case R^4 represents the single electron pair.

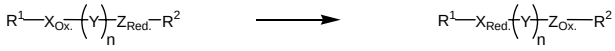
EC 5.3 Intramolecular oxidoreductases

General scheme for the subclasses 5.3.1–5.3.4



$R^{1,2}$ = organic residue

General scheme for the subclass 5.3.99



n = 0,1,2
$R^{1,2}$ = organic residue

For these enzymes the centers of oxidation and reduction in the substrate need not to be adjacent.

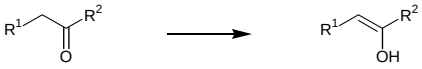
To avoid misunderstandings the sub-subclasses 5.3.1–5.3.4 are presented separately.

EC 5.3.1 Interconverting aldoses and ketoses

$R^{1,2}$ = hydrogen, organic residue

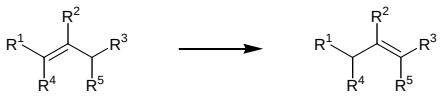
If R^1 is a hydrogen atom, then R^2 is any organic residue and *vice versa.*

EC 5.3.2 Interconverting keto-enol-groups



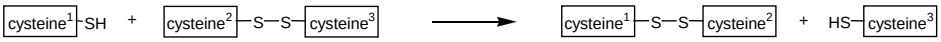
$R^{1,2}$ = hydrogen, organic residue

EC 5.3.3 Transposing C=C bonds



$R^{1,2,3,4,5}$ = organic residue

EC 5.3.4 Transposing S-S bonds

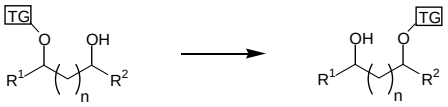


The cysteine residues are parts of proteins.

EC 5.4 Intramolecular transferases (mutases)

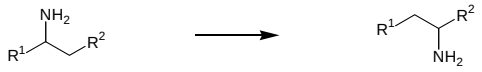
This enzyme sub-class can be divided into two groups.

The enzymes belonging to 5.4.1 and 5.4.2 catalyze the transfer of a functional group from one oxygen atom to another oxygen atom of the same molecule.



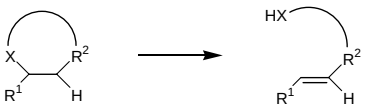
TG = **t**ransferred **g**roups are acyl or orthophosphate groups
$R^{1,2}$ = organic residue
n = 0 or 4

The enzymes classified under 5.4.3 catalyze the transfer of a whole amino group from one carbon atom of a molecule to a neighboring atom of the same molecule.



$R^{1,2}$ = organic residue

EC 5.5 Intramolecular lyases



X = O, CH_2
$R^{1,2}$ = organic residue

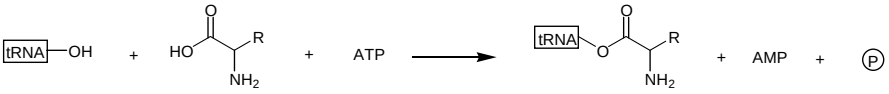
EC 6 Ligases

In contrast to all other five enzyme classes this last main division in the Enzyme Nomenclature is the only one where no member is used for the production of any fine chemical in an industrial process. Nevertheless these biocatalysts play a major role in genetic engineering and genetic diagnostics, since specific enzymes in this class called DNA ligases catalyze the formation of C-O bonds in DNA synthesis. This reaction is essential in genetic engineering sciences, allowing connection of two DNA strings into a single one.

To generalize, ligases are enzymes catalyzing a bond formation between two molecules. This reaction is always coupled with the hydrolysis of a pyrophosphate bond in ATP or a similar triphosphate. The bonds formed are, e.g., C-O, C-S, and C-N bonds.

The systematic names should be formed on the system *X:Y ligase.*

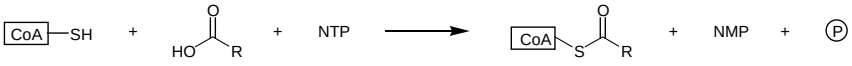
EC 6.1 Forming carbon-oxygen bonds



Ⓟ = diphosphate
R = organic residue

The tRNA-hydroxy group is the 2′- or 3′-hydroxy group of the 3′-terminal nucleoside.

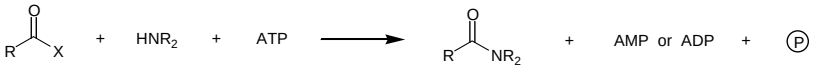
EC 6.2 Forming carbon-sulfur bonds



Ⓟ = diphosphate
R = organic residue
NTP = **n**ucleotide **trip**hosphate (ATP, GTP)
NMP = **n**ucleotide **m**ono**p**hosphate (AMP, GMP)

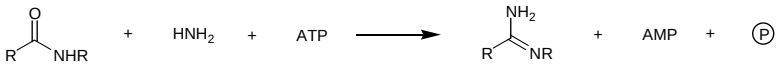
The thiol group is the terminal group of the coenzyme A molecule.

EC 6.3 Forming carbon-nitrogen bonds



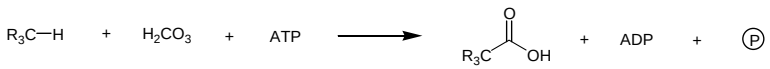
Ⓟ = monophosphate, diphosphate
X = OH, H, COOH
R = hydrogen, organic residue

CO_2 is the substrate for the enzyme EC 6.3.3.3. There are exceptions to this reaction pattern, like ligase EC 6.3.4.1, that catalyzes the following reaction:



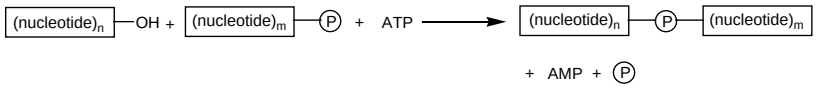
Ⓟ = diphosphate

EC 6.4 Forming carbon-carbon bonds



Ⓟ = monophosphate
R = hydrogen, organic residue

EC 6.5 Forming phosphoric ester bonds



Ⓟ = monophosphate
R = hydrogen, organic rest

These enzymes are repair enzymes for DNA. The enzyme EC 6.5.1.2 uses NAD^+ as cofactor.

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4 Basics of Bioreaction Engineering

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The prerequisite for a process development is rational design. The starting point is the availability of the reactants as well as of the catalyst (figure 1). This is a very important point that has not only a practical impact but also an economical one. The next step should be the characterization of the reaction system by the

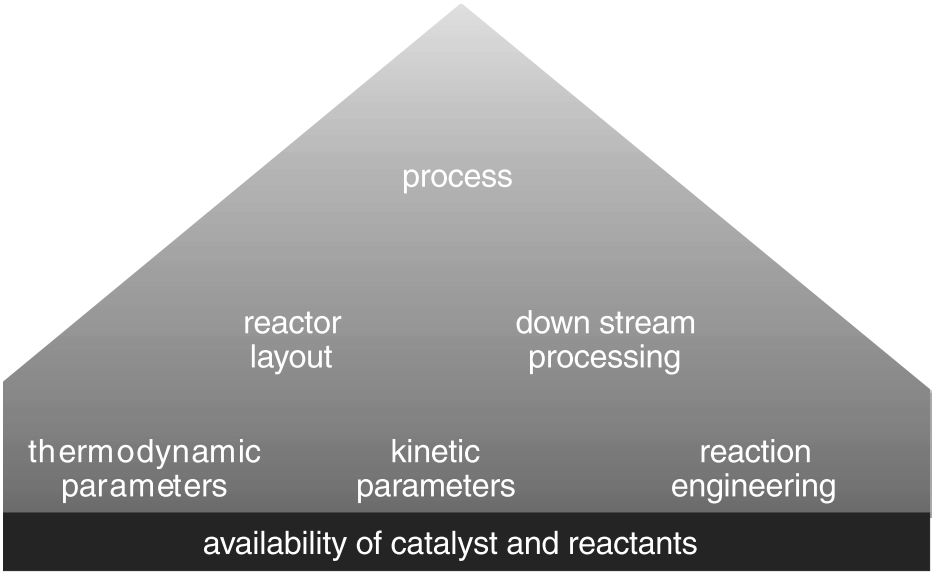


Fig. 1 Objective – rational process design.

kinetic, thermodynamic and reaction engineering parameters. These together then determine the type of reactor to be chosen and how the down stream processing looks like, thereby forming the total process.

In this chapter some fundamental aspects of reaction engineering, kinetics and enzymatic synthesis are described that are needed for the understanding of the data given in chapter 5.

We will start with definitions of key reaction engineering terms that are used throughout the book. These are followed by an introduction to enzyme biosynthesis and a brief overview of general characteristics of the different enzyme classes. Further topics discussed are the fundamental types of reactors and their mode of operation.

4.1 Definitions

4.1.1 Conversion

The conversion is the number of converted molecules per number of starting molecules:

$$X_s = \frac{n_{s0} - n_s}{n_{s0}} \tag{1}$$

X_s conversion of substrate s

n_{s0} amount of substrate s at the start of the reaction (mol)

n_s amount of substrate s at the end of the reaction (mol)

The conversion has to be maximized firstly to avoid recycling of unconverted reaction solution and secondly to minimize reactor volumes. On the other hand, high conversions can result in long reaction times or high amounts of catalyst being employed. Unwanted subsequent reactions of the product will then result in the formation of by-products.

4.1.2 Yield

The yield is the number of synthesized molecules per number of starting molecules:

$$\eta_p = \frac{n_p - n_{p0}}{n_{s0}} \cdot \frac{|\nu_s|}{|\nu_p|} \tag{2}$$

η_p yield of product p

n_{p0} amount of product p at the start of the reaction (mol)

n_p amount of product p at the end of the reaction (mol)

ν_s stoichiometric factor for substrate s

ν_p stoichiometric factor for product p

In combination with the conversion or the selectivity it describes how many product molecules are synthesized in relation to the starting amount of substrate molecules. The described yield is the analytical one. Often the isolated yield is given instead, which describes the synthesized amount of product after down stream processing. The latter does not help in understanding single reaction steps and developing correct kinetic models. If an entire process is considered, the overall yield can be calculated by multiplication of all single yields.

4.1.3 Selectivity

The selectivity is the number of synthesized product molecules per number of converted molecules:

$$\sigma_p = \frac{n_p - n_{p0}}{n_{s0} - n_s} \cdot \frac{|\nu_s|}{|\nu_p|} \tag{3}$$

σ_p selectivity to component p

n_{s0} amount of substrate s at the start of the reaction (mol)

n_s amount of substrate s at the end of the reaction (mol)

n_{p0} amount of product p at the start of the reaction (mol)

n_p amount of product p at the end of the reaction (mol)

ν_s stoichiometric factor for substrate s

ν_p stoichiometric factor for product p

The selectivity describes the synthesized product molecules in relation to the substrate molecules converted. Selectivity has to be as close to '1' as possible to avoid waste of educt. It belongs to the most important economical factors.

If only a very short reaction course is looked at, the selectivity leads to the differential form. This is interesting for gaining information on the synthesis of by-products at every step of conversion. It is decisive for estimating whether a premature stop of the reaction is efficient with regard to the overall yield of the reaction.

The combination of conversion, yield and selectivity leads to the equation:

$$\eta = \sigma \cdot X \tag{4}$$

4.1.4 Enantiomeric excess

The enantiomeric excess (*ee*) is the difference in the number of both enantiomers per sum of the enantiomers:

$$ee_R = \frac{n_R - n_S}{n_R + n_S} \tag{5}$$

ee_R enantiomeric excess of (*R*)-enantiomer

n_R amount of (*R*)-enantiomer (mol)

n_S amount of (*S*)-enantiomer (mol)

The enantiomeric excess describes the enantiomeric purity of an optically active molecule. Small differences in the constellation sequence of the binding partners of one central atom lead to big differences in chemical behavior, in biological pathways and recognition. Since not only different organoleptic properties for both the enantiomers can be found, but also contrary pharmacological effects, it is most important to find syntheses with clear enantiomeric selectivities. Since biological catalysts (enzymes) have improved performance as a result of evolution, they often fulfill this task in the best way. Many pharmaceuticals as well as herbicides and fungicides used to be sold as racemic mixtures, if the unwanted enantiomer did not have a deleterious effect on the organism. But in the last decades, attempt has often been made to switch over to one enantiomer. These products are called racemic switches. Less of the unwanted enantiomer is produced and plant capacity is increased. Organisms are exposed to lesser quantities of chemicals. The environmental benefits are also quite significant.

4.1.5 Turnover number

The turnover number (*tn*) is the number of synthesized molecules per number of used catalyst molecules:

$$tn = \frac{n_p}{n_{cat}} \cdot \frac{1}{|\nu_p|} \tag{6}$$

tn turnover number

n_p amount of product *p* at the end of the reaction (mol)

ν_p stoichiometric factor for the product *p*

n_{cat} amount of catalyst (mol)

The turnover number is a measure of the efficiency of a catalyst. Especially when using expensive catalysts, the *tn* should be as high as possible to reduce the cost of the product. It is very important to name defined reaction parameters in combination with the *tn* to make this value comparable. Instead of the *tn*, the deactivation rate or half life may be given.

The turnover number can also be given for cofactors / coenzymes.

4.1.6 Turnover frequency

The turnover frequency (*tof*) is defined as the number of converted molecules per unit of time:

$$tof = \frac{\partial n_s}{\partial t \cdot n_{catalyst}} \tag{7}$$

tof turnover frequency (s^{-1})

∂n_s differential amount of converted substrate (mol, μmol)

∂t differential time for conversion (s)

$n_{catalyst}$ mole of catalyst (mol, μmol)

The turnover frequency expresses the enzyme activity. It is noteworthy that chemical catalysts are very slow in comparison to enzymes. An example is the epoxidation catalyst Mn-Salen with a *tof* of 3 h^{-1} and the enzymatic counterpart chloroperoxidase (CPO) with a *tof* of 4,500 h^{-1}. But usually the great advantage in activity is lost when educt/product solubility, stability and molecular mass of the enzyme are also considered (here molecular masses are: 635 $g \cdot mol^{-1}$ and 42,000 $g \cdot mol^{-1}$, respectively) [1, 2].

4.1.7 Enzyme activity

The enzyme activity is defined as the reaction rate per unit weight of catalyst (protein):

$$V = \frac{\partial n_s}{\partial t \cdot m_{catalyst}} \tag{8}$$

V maximum activity of enzyme at defined conditions (katal $\cdot kg^{-1}$, U $\cdot mg^{-1}$)

∂n_s differential amount of converted substrate (mol, μmol)

∂t differential time for conversion (s, min)

$m_{catalyst}$ mass of catalyst (kg, mg)

The SI-unit for the enzyme activity is katal (kat = mol $\cdot s^{-1}$, 1 kat = $6 \cdot 10^7$ U), which results in very low values so that often μ-, n- or pkat are used. More practical is the Unit (1 U = 1 μmol $\cdot min^{-1}$). It is very important that the activity be named with the used substrate and with all necessary reaction conditions, like temperature, buffer salts, pH-value etc. The concentration of a protein solution can be determined using indirect photometric tests (e. g. Bradford [3]) or analytical methods (e. g. electrophoresis). Only when the enzyme contains a photometrically active component, e. g. a heme protein, the concentration can be easily determined by direct photometric absorption. The activity can also be given per reaction volume (U $\cdot mL^{-1}$), if the enzyme concentration cannot be determined due to missing molecular mass or the inability to analyze the mass of solubilized enzyme.

4.1.8 Deactivation rate

The deactivation rate is defined as the loss of catalyst activity per unit of time:

$$V_1 = V_0 \cdot e^{-k_{deact} \cdot (t_1 - t_0)} \tag{9}$$

k_{deact} deactivation rate (min^{-1}, h^{-1}, d^{-1})

V_0 enzyme activity at the start of the measurement ($U \cdot mg^{-1}$)

V_1 enzyme activity at the end of the measurement ($U \cdot mg^{-1}$)

t_0 start time of the measurement (min, h, d)

t_1 end time of the measurement (min, h, d)

The deactivation rate expresses the stability of a catalyst.

4.1.9 Half life

The half life is defined as the time in which the activity is halved:

$$V_1 = V_0 \cdot e^{-k_{deact} \cdot (t_1 - t_0)} \tag{10}$$

$$V_2 = V_0 \cdot e^{-k_{deact} \cdot (t_2 - t_0)} \tag{11}$$

$$V_1 = \frac{1}{2} \cdot V_2 \tag{12}$$

$$\Rightarrow \quad t_{1/2} = \frac{ln(2)}{k_{deact}} \tag{13}$$

$t_{1/2}$ half life of catalyst (min, h, d)

V_x enzyme activity at time t_x ($U \cdot mg^{-1}$)

t_x time of measurement (min, h, d)

k_{deact} deactivation rate (min^{-1}, h^{-1}, d^{-1})

The half life expresses the stability of a catalyst. The activity usually shows a typical exponential decay. Therefore the half life can be calculated and it gives an extent of the catalyst deactivation independent of considered time differences.

4.1.10 Catalyst consumption

The biocatalyst consumption (*bc*) is defined as the mass or activity of catalyst consumed per mass of synthesized product:

$$bc = \frac{m_{catalyst}}{m_{product}} \tag{14}$$

bc biocatalyst consumption ($g \cdot kg^{-1}$ or $U \cdot kg^{-1}$)

$m_{catalyst}$ mass or activity of catalyst used for synthesized mass of product (g or U)

$m_{product}$ mass of synthesized product (g)

If an expensive catalyst is used, the biocatalyst consumption should be as low as possible to decrease the biocatalyst consumption cost in production. Often, pharmaceutical products are valued so high that in discontinuous reactions the catalyst can be discarded without recycling. Since the catalyst stability can change with conversion due to deactivating by-products, it is interesting to look at the differential catalyst consumption to find the optimal conversion for the end of the reaction and separating the reaction solution from the catalyst.

4.1.11 Residence time

The residence time (τ) is defined as the quotient of reactor volume and feed rate:

$$\tau = \frac{V_R}{F} \tag{15}$$

τ residence time or reaction time (h)

V_R reactor volume (L)

F feed rate ($L \cdot h^{-1}$)

The residence time describes the average time of a molecule in the reactor. Since the residence times of different molecules are not the same, usually the average residence time is used. Diffusion effects and non-ideal stirring in a continuously operated stirred tank reactor (CSTR) or back mixing in plug flow reactors results in a broad distribution of single residence times. For a detailed simulation of the process this distribution has to be taken into account. For example, one educt molecule could leave the reactor directly after it was fed into the reactor or it could stay in the reaction system forever. Therefore, the selectivity can be strongly influenced by a broad distribution.

4.1.12 Space-time yield

The space-time yield (STY) is the mass of product synthesized per reactor volume and time. It is also named as the **volumetric productivity**:

$$STY = \frac{m_p}{\tau \cdot V_R} \tag{16}$$

STY space-time yield ($g \cdot L^{-1} \cdot d^{-1}$)

m_p mass of synthesized product (g)

τ residence time or reaction time (d)

V_R reactor volume (L)

The space-time yield expresses the productivity of a reactor system. The *STY* should be as high as possible to decrease investment costs of a plant. A low *STY* means low product concentrations or bad reaction rates. Low concentrations lead to more complex down stream processing, while low reaction rates necessitate larger reactor volumes. The reactor volume for heterogeneously catalyzed reactions is usually the volume of the catalyst itself.

4.2 Biosynthesis and immobilization of biocatalysts

This chapter tries to give a brief introduction to the biosynthesis of enzymes, which are the biological catalysts. For a more detailed introduction the reader is referred to textbooks [4–6]. Additionally the immobilization of biocatalysts is discussed that is often used on an industrial scale to reduce the catalyst costs and to increase the stability.

4.2.1 Types of biocatalysts

The biocatalyst is always described as a whole cell or an enzyme. In the first case we face a mini-reactor with all necessary cofactors and sequences of enzymes concentrated in one cell. In the second case the main catalytic unit is isolated and purified. In both cases optimization is possible. Furthermore, multistep biosynthetic pathways can be changed to prevent degradation of the desired product or produce precursors normally not prioritized in the usual pathway. All these changes for the whole cell lead to an optimized mini-plant. The optimization of the main catalyst is comparable with catalyst development inside a reaction system. In this chapter the composition and biological synthesis of enzymes as well as the different genetic terms (e.g. mutation, cloning, etc.) that are used in chapter 5 are briefly explained.

Whole cells can be bacteria, fungi, plant cells or animal cells. They are subdivided into the two groups of prokaryotic cells and eukaryotic cells.

4.2.1.1 Prokaryotic cells

Prokaryotic cells are the “lowest microorganisms” and do not possess a true nucleus. The nuclear material is contained in the cytoplasm of the cell. They reproduce by cell division. They are relatively small in size (0.2 to 10 μm) and exist as single cells or as mycelia. When designing bioreactors, an adequate supply of nutrients as well as oxygen into the bioreactor must be assured, since the cells, e.g. bacteria, grow rapidly. Parameters such as pH, oxygen feed rate and temperature in the bioreactor must be optimized. Perhaps the most widely used prokaryotic microorganism in industrial biotransformations is *Escherichia coli,* which is a native to the human intestinal flora.

4.2.1.2 Eukaryotic cells

Eukaryotic cells are higher microorganisms and have a true nucleus bounded by a nuclear membrane. They reproduce by an indirect cell division method called mitosis, in which the two daughter nuclei normally receive identical compliments of the number of chromosomes characteristic of the somatic cells of the species. They are larger in size (5–30 μm) and have a complex structure. When eukaryotic cells are used as biocatalysts, high or low mechanical stress must be avoided by using large stirrers at slow speed and by eliminating dead zones in the fermenter. *Saccharomyces cerevisiae* and *Zymomonas mobilis* represent the most important eukaryotic cells used in industrial biotransformations.

4.2.2 Enzyme structure and biosynthesis

An enzyme is an accumulation of one or more polypeptide chains in the form of a protein. It is unique in being capable of accelerating or producing by catalytic action a transformation in a substrate for which it is often specific.

The three-dimensional structure of an enzyme is determined at different levels [7]:

- Primary structure: sequence of connected amino acids of a protein chain.
- Secondary structure: hydrogen bonds from the type of R–N-H – O=C-R are responsible for the formation of the secondary structure, the α-helix or the β-sheet, of one protein chain.
- Tertiary structure: hydrogen and disulfide bonds, as well as ionic and hydrophobic forces lead to the tertiary structure, the folded protein chain.
- Quaternary structure: If several protein chains are combined in the form of subunits, the quaternary structure is formed. Not covalent bonds, but molecular interactions occuring in the secondary, tertiary and quaternary structures, are responsible for the formation of the well-functioning catalytic system.

The amino acid sequence of a protein is determined by the nucleic acids, which are the non-protein constituents of nucleoproteins present in the cell nucleus. The nucleic acids are complex organic acids of high molecular weight consisting of chains of alternate units of phosphate and a pentose sugar with a purine and a pyrimidine base attached to the sugar. The DNA (desoxyribonucleic acid) consists of four bases, namely adenine, thymine, cytosine and guanine, whereas the RNA (ribonucleic acid) contains uracil instead of thymine (figure 2). In RNA, the sugar is ribose instead of 2-desoxyribose. Figure 3 shows the matching bases in a short section of a DNA strand.

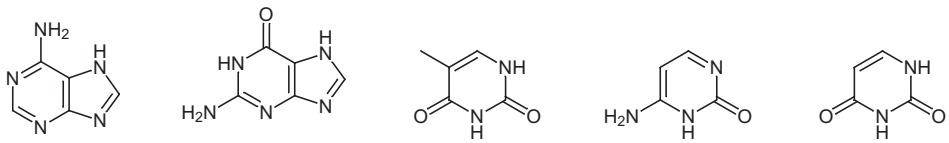


Fig. 2 Bases of nucleic acids.

Two chains are combined to form a helical structure, but only thymine–adenine (T-A) and cytosine-guanine (C-G) can be coupled. The phosphoric ester can be bound on the 3′- or 5′-hydroxy group of the sugar component (figure 3):

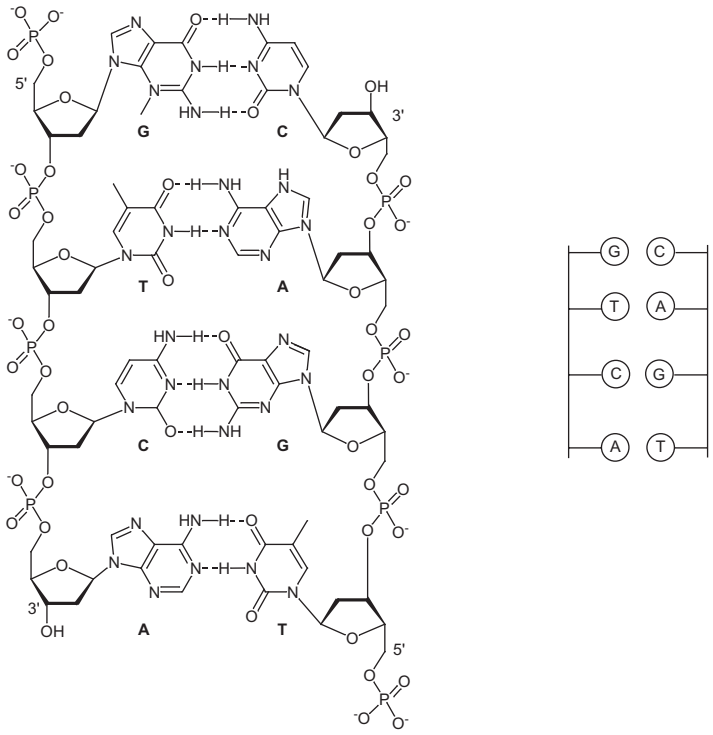


Fig. 3 Matching bases in a short section of a DNA strand.

The process of transcribing DNA, transferring mRNA out of the nucleus and reading mRNA to build a protein, e.g. an enzyme, is called **gene expression**. The expression of a gene that is encoding a protein can be divided into the two main steps of **transcription** and **translation**. In prokaryotic cells that do not contain a nucleus both steps of transcription and translation take place at the same time and at the same place, in the cytosol.

Transcription

In transcription, a double-stranded DNA serves as a template to synthesize a messenger RNA (mRNA) with the help of a RNA-polymerase. Starting from the linear genetic determinants on a DNA strand, the flow of cellular information descends to specify the structure of the proteins through the transcription into mRNA molecules having complimentary base sequences to the parent DNA strands. In short, the information of a gene is transferred to a mRNA by a RNA-

polymerase and can thus leave the nucleus. The enzyme polymerase needs a start sequence, the promoter, to begin with the building of the mRNA. The transcription ends when a termination sequence is found (figure 4).

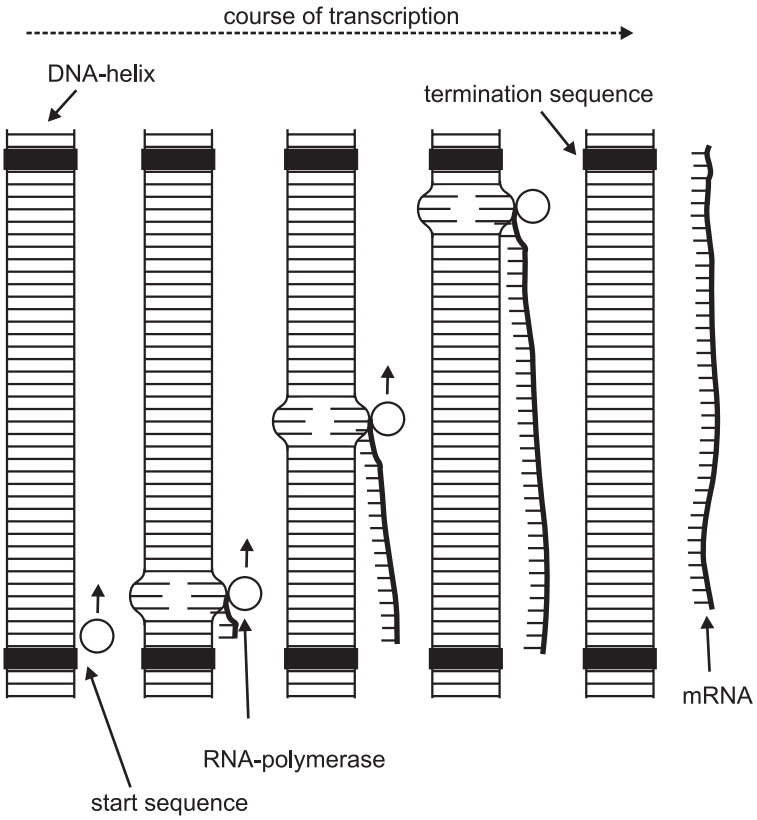


Fig. 4 Transcription of DNA information on mRNA.

Translation

In translation, the mRNA serves as a template to form the protein molecule. The information that is contained in the mRNA can be translated into a protein building sequence by the ribosomes. The ribosome needs a binding sequence where it binds to the mRNA. It moves along the mRNA until it finds a start sequence (AUG). Here the protein biosynthesis begins. Each amino acid incorporated into the protein is defined by the combination of three nucleotides known as the codon. Of the 64 possible codons, 61 are used to code 20 amino acids and the remaining three are used as stop signals for translation. The translation table of the codons depends on the organism. A transfer-RNA (tRNA), which looks like a clover leaf due to intramolecular bonds, is used as a carrier for one special amino acid at one end. The tRNA binds to the mRNA and the corre-

sponding amino acid is connected to the end of the existing protein chain. This procedure is repeated until a stop sequence (stop codon) is found (UAA, UAG or UGA). Figure 5 shows the translation of the mRNA to a protein:

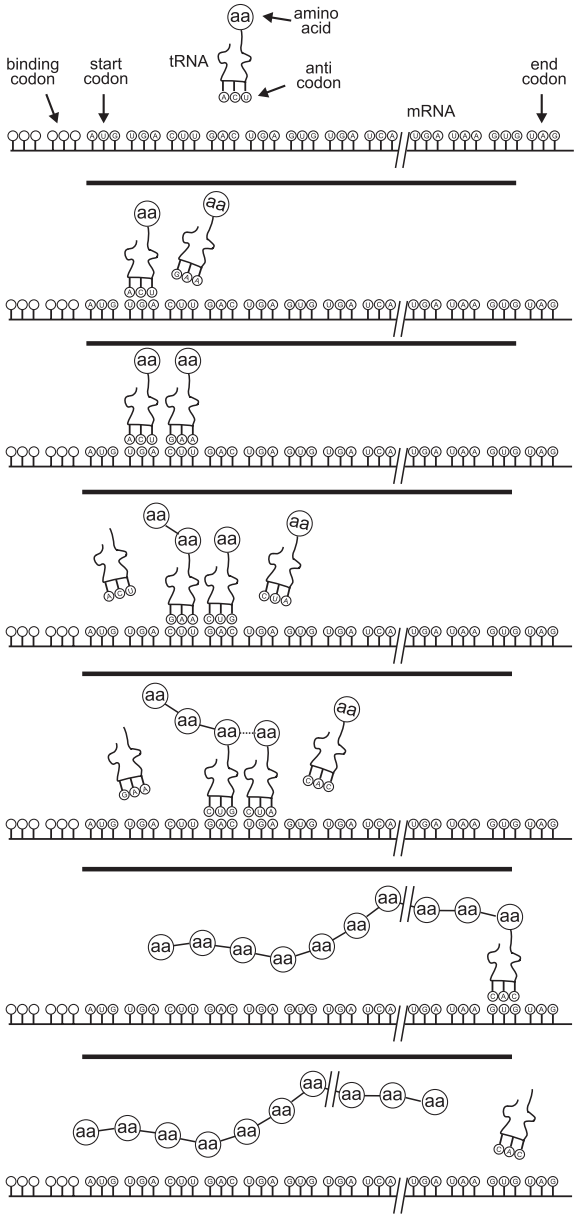


Fig. 5 Synthesis of protein by translation of mRNA.

In some cases the protein is modified post-translationally (e.g. for insulin production).

4.2.3 Cloning of biocatalysts

The aim of cloning is to improve the expression of a specific gene, which is responsible for the synthesis of the desired enzyme, a protein. In principle, it should be sufficient to cut out the gene fragment and transfer it into a cell. In this cell the protein will be synthesized but the aim is to produce a high quantity of protein, which is not possible with only one cell. Therefore the cell has to be divided and multiplied. But during this process only the cell typical DNA will be multiplied for each cell during cell division. This replication procedure is catalyzed by the DNA-polymerase which needs a special start sequence, the promoter (see above). This sequence usually does not exist for DNA-fragments. Therefore a cloning vector has to be found which contains the interesting gene and an origin of replication. Vectors are DNA molecules which serve as a recipient or carrier for foreign DNA. They carry an origin of DNA replication and genetic markers which allow them to be detected in host cells. The most commonly used vectors are the plasmids, which are self-replicating rings of DNA and are not contained in the main set of chromosomes of a cell. Figure 6 shows the difference in cell replication using the single DNA-fragment and the modified plasmid:

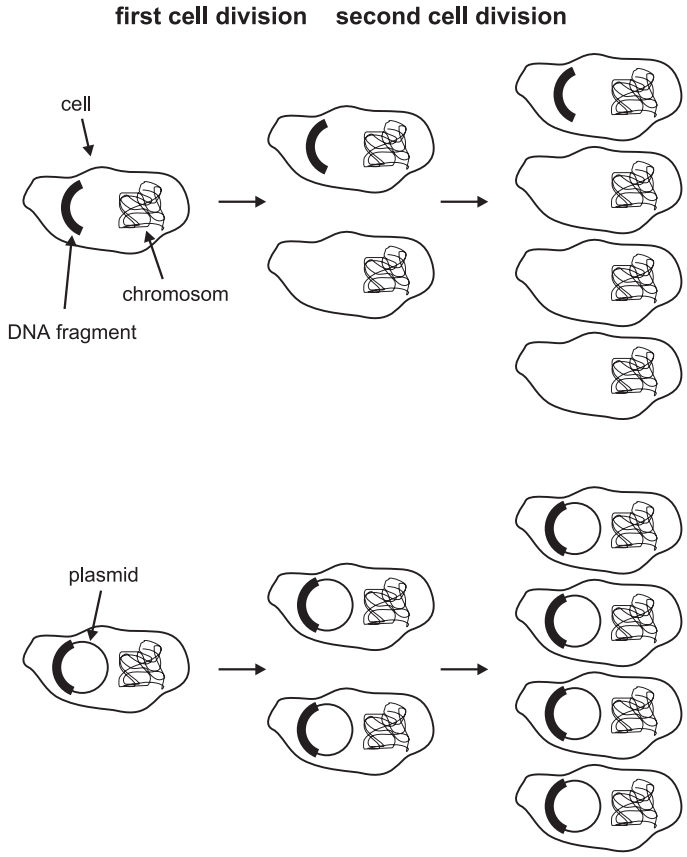


Fig. 6 The use of plasmids for the replication of the interesting DNA-fragment.

The desired fragment can be inserted into the plasmid ring by use of restriction enzymes (restriction endonucleases) which cut a nucleotide sequence to sticky or blunt ends [8]. Thus one chain is longer than the other or both are of the same length (figure 7). Blunt ends have to be converted to sticky ends in order to insert them into a plasmid ring by the use of a ligase:

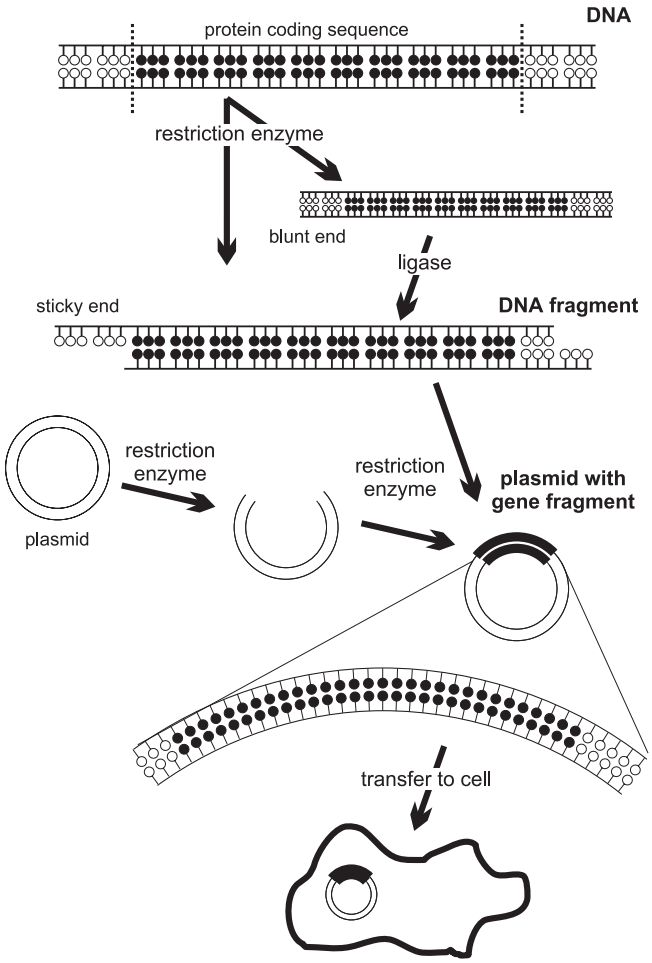


Fig. 7 The insertion of a DNA-fragment into a plasmid vector.

For genes from bacteria and viruses (prokaryotic organisms) this procedure works in the above-described manner. But, if a more complex gene from an eukaryotic organism is to be expressed, the protein cannot be translated since some codons do not contain any coding informations (**introns**), as opposed to the informative **exons**. These introns have to be removed prior to translation (**splicing**) (figure 8):

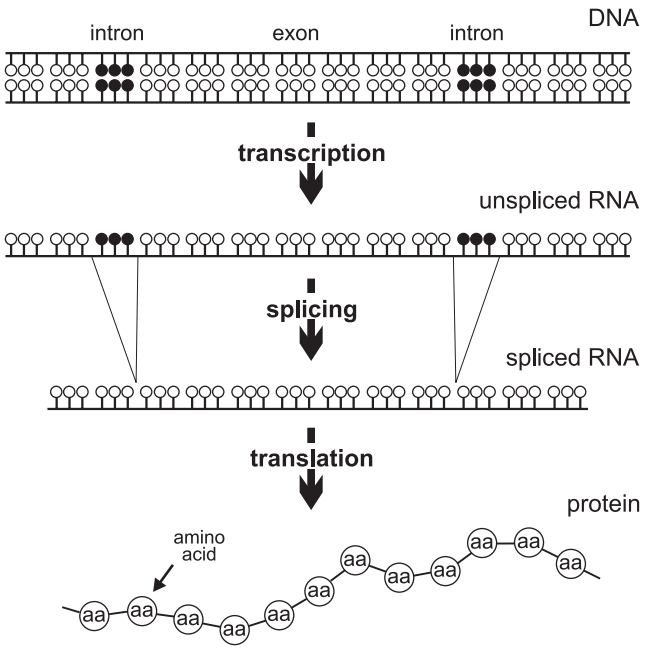


Fig. 8 Splicing of RNA before translation is possible.

Only few organisms employed for cloning can be used for splicing. For example, yeast can be cloned, but cloning is very inefficient and is therefore seldom used. A better method can be the isolation of the mRNA from the protein-producing organism. The mRNA can be converted to the DNA segment named cDNA by use of reverse transcriptase. cDNA (complimentary DNA) is a double-stranded DNA copy of the mRNA and serves as a template for protein biosynthesis. The blunt ends can be converted into sticky ends by a ligase and inserted to the plasmid by the restriction enzymes. A further method, which can only be applied if the amino acid sequence and thus the nucleic base sequence is known, is to synthesize the RNA step by step by chemical methods. Figure 9 summarizes the different approaches:

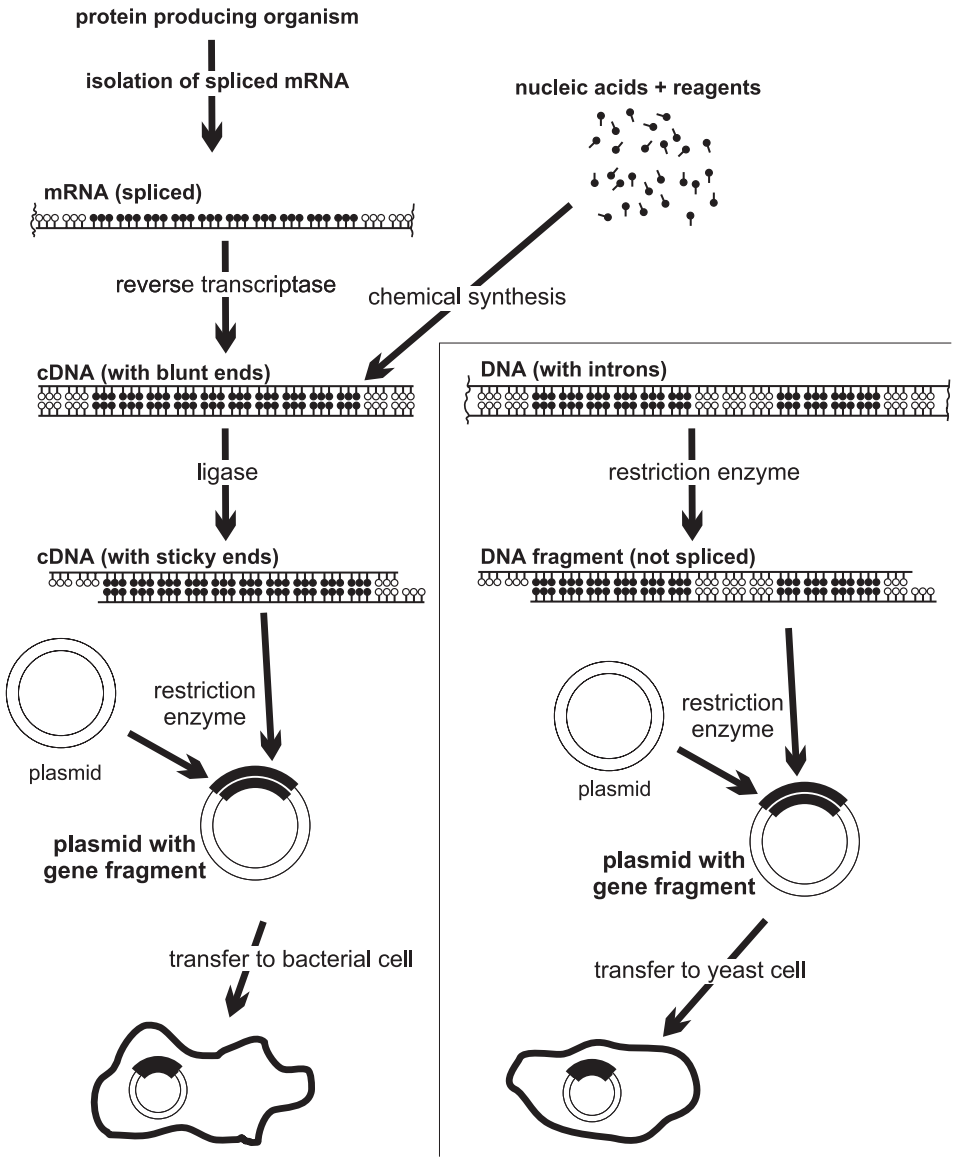


Fig. 9 Approaches to solve the problem of removing uninformative genetic material.

There are several factors to be considered while optimizing cloning and gene expression:

- The plasmid should have only one position for each restriction enzyme to prevent mismatching of the sequence in a plasmid.
- Further, the promoter should be strong so that the expression of the gene sequence is favored for production of high concentrations of the protein.
- The recombinant organism should be genetically stable.

4.2.4 Screening and mutation of biocatalysts

Screening for interesting enzymes can be directed by understanding biological pathways. If the biological reactions are known for several conversions of structural elements in the intermediates, the isolation of the corresponding catalyst can be started. If a catalyst can be isolated, purified and produced in small amounts for preliminary investigations of substrate spectrum and kinetics, the next step would be the sequencing of the amino acids and determination of the crystal structure to understand the mechanism of the biotransformation.

The enzymology of DNA, including the discovery and purification of restriciton enzymes and of DNA polymerases, have given the biotechnologist a new tool in the recent past for optimizing the protein, namely, **genetic engineering** or **protein engineering.** A cloned gene can now be overexpressed to manufacture relatively large quantities of the desired enzyme. Selective mutation of the gene – and, consequently, the amino acid sequence – leads to a modified enzyme with altered kinetic parameters or specificity.

Mutation can be carried out by several methods:

- physical (irradiation with high energy radiation)
- chemical (treatment with reactive molecules to change nucleic bases, insertion of analog bases, which results in mismatched bases pairing or fragments, which can be inserted during insertion to lead to a whole shift in the sequence of one chain)
- biochemical (enzymes to cut DNA-chains and to change selected nucleic bases)

However, the frequency of desired mutations and the possibility of combining desired properties in a recombination process are rather low due to the random nature of mutation and selection. Strain improvement depends, therefore, to a very large extent on proper and efficient selection methods which can detect and isolate one mutant among several thousands of cells.

Genetic engineering techniques also allow the production of enzymes of higher organisms by microorganisms by placement of the corresponding gene into the latter. Several examples are shown in chapter 5 where mutation improves dramatically K_M-values, activity and stability. The main objective of mutation and selection is to achieve higher overall productivity when using the biocatalyst, thus making biotransformations economically feasible.

4.2.5 Optimization of reaction conditions

Optimization of the reaction conditions is a very important point with regard to catalyst consumption, product specific catalyst costs and productivity.

In industrial biotransformations often the immobilization on a support is chosen to enhance the stability as well as to simplify the biocatalyst recovery. An alternative approach for an easy biocatalyst recovery is the use of a filtration unit [9,10,11]. For the separation of suspended whole cells or solubilized enzymes, membranes are often applied as filters. In membrane filtration, the specific pore

size and charge of a membrane are used to separate different compounds by their physical size. Figure 10 shows the classification of different filtration types in the order of the pore sizes or cut off values.

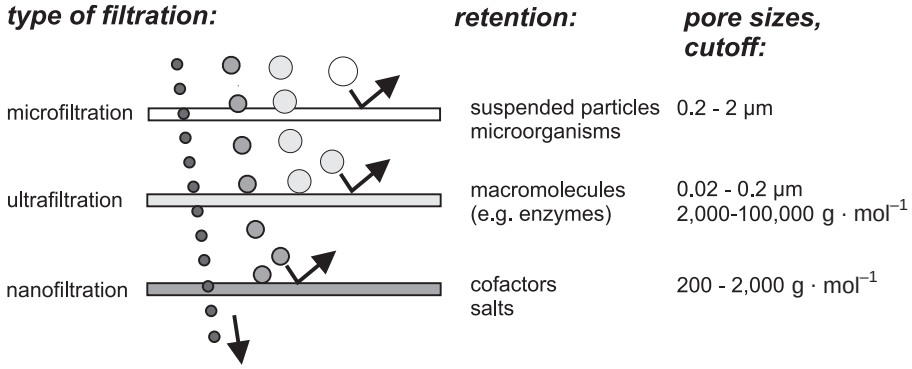


Fig. 10 Classification of different filtration types.

Microfiltration membranes are applied for the separation of whole cells (for examples see the processes on pages 137, 253, 369). Due to the lower molecular weight and physical size of enzymes, ultrafiltration membranes have to be used to retain them [12,13] (for examples see the processes on pages 103, 113, 125).

4.2.5.1 Immobilization of biocatalysts

If a good biocatalyst is found for a specific reaction, one possibility for further improvement of its properties is immobilization. The best way in which the biocatalyst can be immobilized has to be found by experiment. This is dependent on the reaction, the stability of the biocatalyst, the possibilities for the immobilization of the biocatalyst and the activity of the immobilized biocatalyst. No straightforward plan for testing immobilization is known, but at least the biocatalyst structure should be considered.

The main advantages of immobilization are:

- easy separation of biocatalyst,
- lower down stream processing costs,
- possibility of biocatalyst recycling,
- better stability, especially towards organic solvents and heat,
- use of fixed bed reactors and,
- easier realization for continuous production.

The main disadvantages of immobilization are:

- loss of absolute activity due to immobilization process,
- lower activity of immobilized biocatalyst compared to non-immobilized biocatalyst as used in processes with membrane filtration,
- additional costs for carrier or immobilization matrix and immobilization procedure,

- carrier or matrix cannot be recycled and
- diffusion limitations lowering reaction rates.

In spite of these disadvantages, immobilization has become an indispensable part of industrial biotransformations. The most common methods for the immobilization are entrapment in matrices, cross-linking and covalent binding (figure 11):

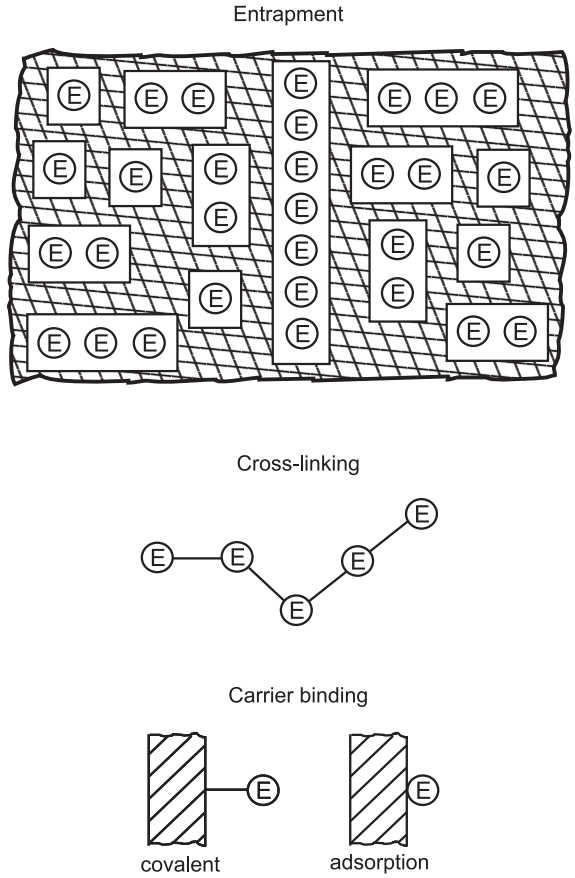


Fig. 11 Common immobilization methods.

Entrapment

The biocatalyst can be entrapped in natural or synthetic gel matrices. A very simple method is the entrapment in sodium alginate, a natural polysaccharide. The water soluble alginate is mixed with the biocatalyst solution and dropped into a calcium chloride solution in which water-insoluble alginate beads are formed (figure 12):

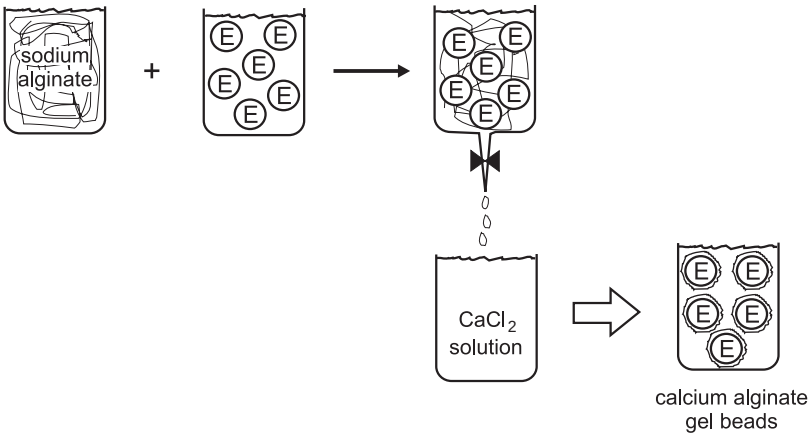


Fig. 12 Alginate gel formation.

Another naturally-occuring polysaccharide widely used for immobilization is κ-carrageenan. In a manner similar to the alginate method, a mixture of κ-carrageenan in saline and biocatalyst solution (or suspension) is dropped into a solution of a gelling reagent like potassium chloride. Ammonium, calcium and aluminum cations also serve as good gelling reagents. The gel can be hardened by glutaraldehyde, hexamethylenediamine or other cross-linking reagents, often enhancing biocatalyst stability (figure 13):

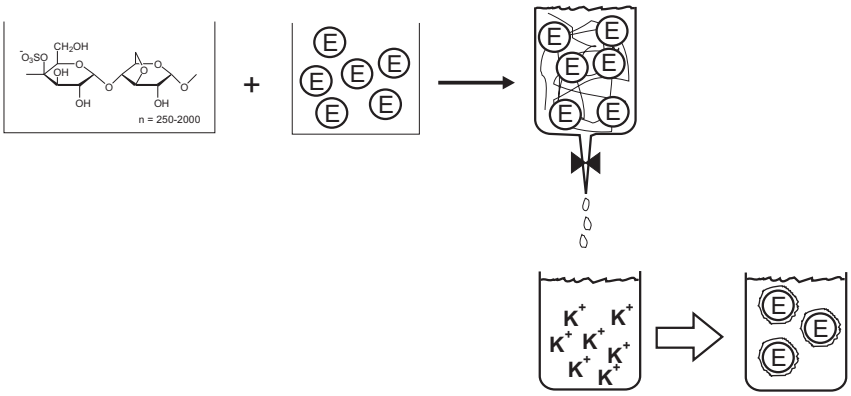
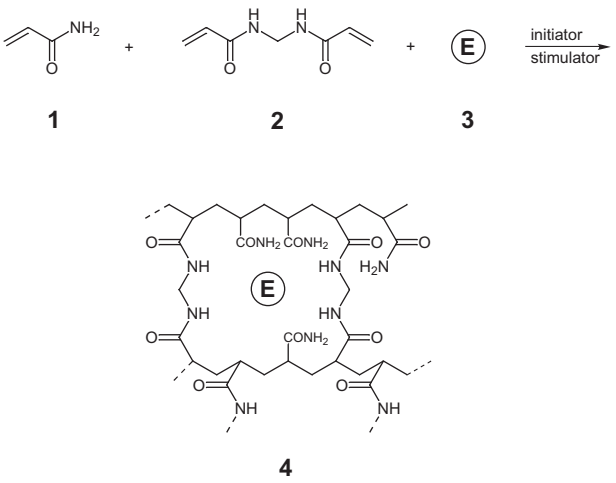


Fig. 13 κ-Carrageenan gel formation.

An often-used synthetic immobilization method employs polyacrylamide gel. The biocatalyst, a monomer (acrylamide) and a cross-linker (e.g. N,N′-methylenebisacrylamide) are mixed and polymerized by starting the reaction with an initiator (e.g. potassium persulfate) in the presence of a stimulator (e.g. 3-dimethylamino propionitrile) (figure 14):



1 = acrylamide
2 = N,N'-methylenebisacrylamide
3 = enzyme
4 = polyacrylamide gel

Fig. 14 Polyacrylamide gel formation.

Cross-linking

The most popular cross-linking reagent is glutaraldehyde, although other bi- or multifunctional reagents can be used instead. The single biocatalyst (protein molecule or cell) is cross-linked to insoluble macromolecules or cell pellets (figure 15):

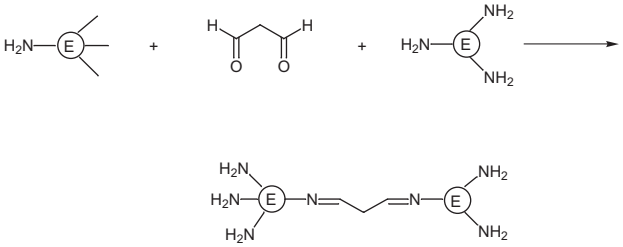


Fig. 15 Cross-linking with glutaraldehyde.

Covalent binding

The biocatalyst, in this case usually an isolated enzyme, can be attached to a carrier by a reaction of amino- or acid-groups of the proteins. Generally, amino acid residues, which are not involved in the active site or in the substrate-binding

site of the enzyme, can be used for covalent binding with carriers. Usually the carrier is a polymer (polysaccharide, polysiloxane, polyacrylamide etc.) bearing hydroxy groups or amino groups on its surface. To combine the enzyme with these groups, different activation methods can be applied. These activated carriers are commercially available. Examples are spacers with epoxy groups, which are activated by cyanogen bromide or other activating groups such as acid azide, leading to a spontaneous reaction with the amino group of the biocatalyst (figure 16):

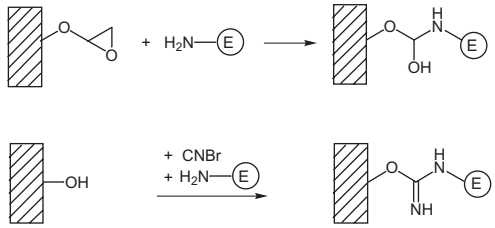


Fig. 16 Two examples for carrier-coupling using the amino group of an enzyme.

If the acid group is to be coupled to a carrier, the enzyme has to be activated too, e.g. by reaction with a diimide. The activated carrier and enzyme can now be coupled (figure 17):

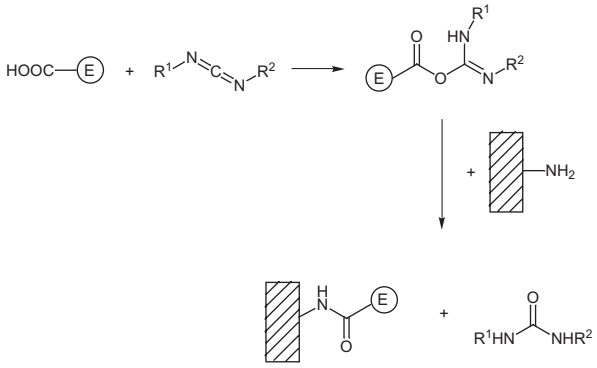


Fig. 17 Example for carrier-coupling using the acid group of an enzyme.

4.2.5.2 Reaction parameters

The reaction parameters have to be optimized for the reaction with respect to high space-time yields and high stability of the biocatalyst, meaning low production costs.

Parameters that can be varied are:

- pH,
- temperature,
- solvents,

- buffer salts,
- cofactors,
- immobilization methods,
- substrate and product concentrations,
- addition of antioxidants or stabilizers,
- reactor material or coating and
- physical treatment (stirring, pumping, gas-liquid phases, etc.).

During optimization of the space-time yield it is necessary to consider catalyst costs. Especially the combination of stability and activity has to be considered. Sometimes it is desirable to work at very low temperatures with low reaction rates, which have to be compensated by a high amount of biocatalyst if the turnover number can nevertheless be increased. In other cases the turnover number will have a lower priority since as much product as possible is to be synthesized.

No pragmatic rule exists for the best strategy to optimize reaction conditions. Empirical methods based on statistical methods have good chances of being successful (e.g. genetic algorithms).

The most important improvements can be made in finding and constructing an optimal catalyst.

4.3 Characteristics of the different enzyme classes

For selecting reaction conditions and deciding on the reaction layout it is also important to consider the special properties and limitations that are specific for the different enzyme classes. In the following paragraphs a brief overview is given. References pointing to the related industrial biotransformations in chapter 5 are inserted for illustration.

EC 1: Oxidoreductases

Oxidoreductases are all cofactor dependent. The reducing or oxidizing equivalent is either supplied or taken by the cofactor. The most commonly needed cofactors are NADH/NAD^+, NADPH/$NADP^+$, FADH/FAD^+, ATP/ADP and PQQ [14–16]. Since some of them like NADH or NADPH are quite expensive, an effective cofactor regeneration system is required to design a cost-effective process. In the literature and in industry mainly three applied approaches can be found to solve this problem. If working with isolated enzymes, either a second enzyme can be used (in the case of NADH the best approach is to use a formate dehydrogenase that utilizes formate and produces CO_2 (see page 103 and 125)) or the cofactor can be regenerated by applying a second substrate (figure 18).

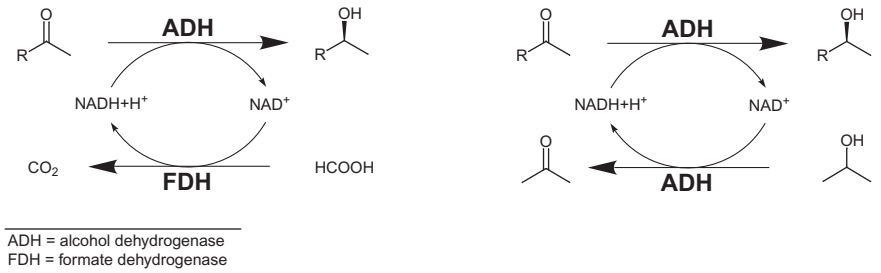


Fig. 18 Different approaches to cofactor recycling.

Another approach is the application of whole cells with glucose for example as a C-source (see page 121). Through this approach the multicatalyst-system of the whole cell itself is used for the regeneration. There are also electrochemical regeneration methods known which have not yet made it to an industrial process [17].

EC 2: Transferases

In nature, transferases play a far larger role than in industrial biotransformations. Here only a few are known. This may be due to the fact that often equilibrium reactions impede quantitative yields (see page 175), coupling reactions arise and the group-transferring substrates are very expensive or their corresponding products are not easily recycled. Nonetheless, these reactions could gain importance in future, should the latter mentioned problems be solved in a chemoenzymatic synthesis. The very high regio- and stereoselectivities in transferase-catalyzed reactions are the main reasons for their increasing utility in synthesis (see page 179). This property leads to the *one enzyme – one linkage* principle.

EC 3: Hydrolases

If hydrolases are used for the kinetic resolution of a racemate, the maximal yield is limited to 50% by the enzyme itself (figure 19a). There are different possible ways to increase the yield up to 100%. If the target compound is the preferentially formed product enantiomer the dynamic resolution process can be applied (figure 19b) [18].

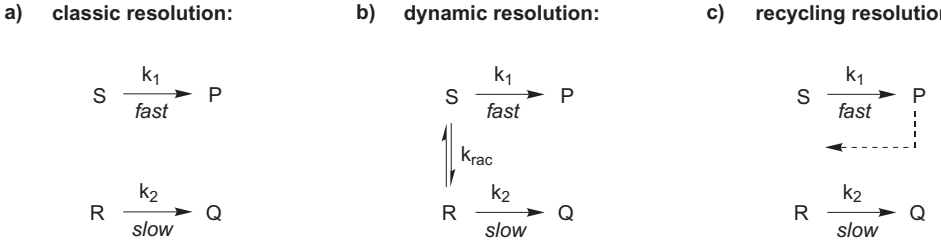


Fig. 19 Different methods of kinetic resolution.

The strategy is a fast isomerization of the substrate enantiomers ($k_{rac} >> k_1$) [19]. In the case of the *in situ* racemization of amino acid esters the racemization can readily be induced by the addition of pyridoxal-5-phosphate [20]. Alternatively, racemases can be applied (see page 314) [21]. A dynamic resolution is also indicated if the substrate is chirally labile [22]. If the target compound is the more slowly converted substrate enantiomer (figure 19c), one possibility is the recycling of the product under chemical conditions leading to racemization (see page 300). This is only applicable if $k_2 << k_1$.

There are also methods available employing chemical stereo-inversion for the production of chiral alcohols, e.g. the synthetic pyrethroid insecticide (*S*)-4-hydroxy-3-methyl-2-prop-2-ynyl-cyclopent-2-enone (see page 208). The lipase-catalyzed kinetic resolution is carried out by hydrolyzing the acylated (*S*)-enantiomer. Subsequently, the cleaved alcohol is sulfonated in the presence of the acylated compound with methanesulfonyl chloride (figure 20) [23].

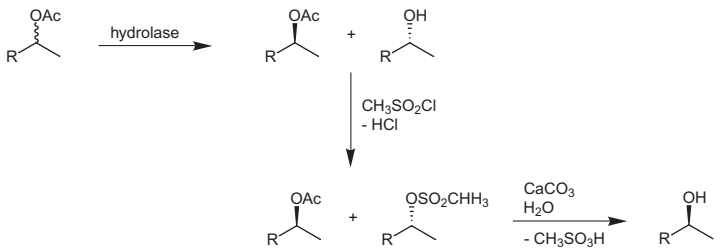


Fig. 20 Resolution coupled to chemical stereo-inversion.

Of key importance is the fact that the hydrolysis of the sulfonated enantiomer in the presence of small amounts of calcium carbonate takes place under inversion of the chiral center in contrast to the hydrolysis of the acylated enantiomer, which is carried out under retention. By this means, an enantiomeric excess of 99.2% and a very high yield are achieved for the (*R*)-alcohol.

EC 4: Lyases

Lyases are of growing significance for industrial biotransformations, since the predominant bond-breaking (lyase) reactions taking place in nature can be reversed (bond formation, synthetase) under non-natural conditions (i.e. high reactant concentrations). Whenever the use of highly concentrated reactants becomes feasible (2.5 M aspartic acid, see page 334) or a product can be withdrawn from the reaction equilibrium (for instance, in a successive reaction) these reactions can be made to run quantitatively. Often a chiral center is generated during bond formation (see page 344). Even the simultaneous formation of two chiral centers is possible. The synthetase reaction leads to the construction of new bonds and is therefore of great importance in synthesis. Especially as a reasult of the technical evolution of enzymes their corresponding substrate spectra are currently being expanded.

EC 5: Isomerases

Racemases that can be classified as isomerases are of particular significance in kinetic racemic resolution, whenever it is possible to carry out the racemization under similar conditions as those present in a racemic resolution reaction. In this way, kinetic racemic resolutions can lead to yields of up to 100 %. This is the case in the production of D-amino acids and, since recent times, also in the production of L-amino acids (see page 314). Suitable racemases for other amino acid derivatives in technical applications are hardly known. Due to the above mentioned reasons, racemases will probably become the working field of choice for the technical evolution of enzymes. The most renowned enzyme of this group is certainly glucose isomerase (see page 387). In this case the isomerisation leads to an increase in value without the addition of further substrate. In this way, isomerases make it possible to use cheaper substrates (e.g. *N*-acetylglucosamine instead of acetylmannosamine in the preparation of neuraminic acid (see page 385)). Since the isomerization with epimerases does not necessarily give yield of 50 %, it is essential to examine if and how the problem of an undesirable state of equilibrium can be solved by appending a successive reaction.

EC 6: Ligases

For ligases in a narrower sense there are no known industrial biotransformations being carried out at a kg scale. Nonetheless, ligases play a significant role in nature, for instance in ribosomal peptide synthesis, in repairing DNA fragments and in genetic engineering (DNA-ligases).

Additionally, there are some issues that arise for the majority of all biotransformations. These are:

- Low solubility of reactants or products,
- Limited stability of biocatalysts.

With regard to the first point there are different solutions possible. One of the easiest is working in an aqueous-organic two-phase system. However, due to the limited stability of some enzymes in the presence of an interface or organic solvents this is not always possible. A more biocompatible approach is the addition of complexing agents like dimethylated cyclodextrins or adsorbing materials like XAD-7 resins used by Eli Lilly (see page 110). A solution based on reaction engineering is the membrane-stabilized interface as used by Sepracor, USA, in the case of the kinetic resolution of esters (see page 202) or the continuous extraction of reaction products as applied by the Research Center Juelich (see page 103).

There is a wide variety of methods available to increase the catalyst stability. These include the addition of antioxidants (e.g. dithiothreitol), the immobilization on supports, crosslinking of enzyme crystals, separation from deactivating reagents, variation of reaction conditions and optimization of the biocatalyst by the methods of genetic engineering. In an industrial environment it is more often the time that is limiting to find the proper method of catalyst stabilization.

4.4 Kinetics

In this chapter the fundamentals of enzyme kinetics will be discussed in brief. For a detailed description of enzyme kinetics and discussion of the different kinetic models please refer to the following publications: [24–28].

The determination of the kinetic parameters can be carried out in two different ways: either by measurement of the initial reaction rate at different reaction conditions or by batch experiments. In both cases a kinetic model needs to exist describing the reaction rate as a function of the concentrations of the different reaction components. The two methods differ in the number of variable components. In the case of the initial reaction rate determination, only the concentration of one compound is altered, whereby all others are constant. On the contrary, in the case of batch reactions, the time course of all concentrations of all (!) components is measured. Therefore, all mass balances (see equations 23 and 24) are needed for the determination of the kinetic parameters that form a system of coupled differential equations. The values of the kinetic parameters are determined by fitting the kinetic equations to the measured data by non-linear regression (figure 21). In the case of batch experiments this is supplemented by numerical integration of the reaction rate equations. An appropriate test of the kinetic model and the kinetic parameters is the simulation of the time-courses of batch reactor experiments with different starting concentrations of substrate. These are then compared to the actual batch experiments.

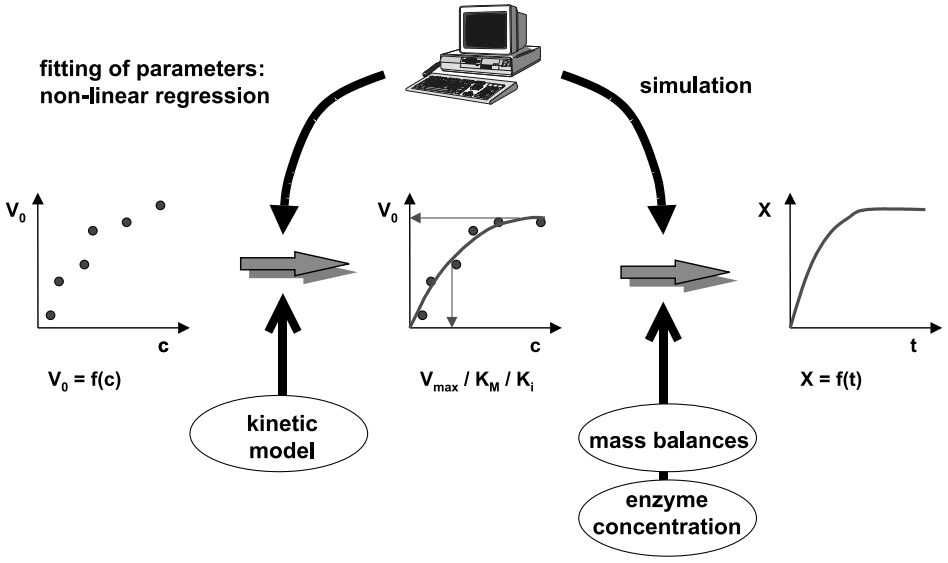


Fig. 21 Determination of kinetic parameters.

The fundamental description of enzyme kinetics dates back to Michaelis and Menten [24]. In 1913, they postulated in their theory on enzyme catalysis the existence of an enzyme-substrate (ES) complex that is formed in a reversible reaction out of substrate (S) and enzyme (E).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

The rate limiting step is the dissociation of the ES complex ($k_{-1} >> k_2$). The reaction rate is proportional to the 'rapid, preceding equilibrium'. As a consequence of the latter assumptions the following reaction rate equation is derived (equation 17):

$$v = \frac{V_{\max} \cdot [S]}{K + [S]} \quad with: \quad K = K_S = \frac{k_{-1}}{k_1} \tag{17,18}$$

v reaction rate ($U \cdot mg^{-1}$)
V_{max} maximum reaction rate ($U \cdot mg^{-1}$)
K dissociation constant of ES complex (mM)

k_x reaction rate constant of reaction step x (min^{-1})
$[S]$ substrate concentration (mM)

Here K is identical to the dissociation constant K_S of the ES complex. Briggs and Haldane extended this theory in 1925 [29]. They substituted the assumption of the 'rapid equilibrium' by a 'steady state assumption'. This means that after starting the reaction a nearly steady state level of the ES complex is established in a very short time. The concentration of the ES complex is constant in time (d[ES]/dt = 0). In this assumption the constant K has to be enlarged by k_2, resulting in the Michaelis-Menten constant K_M.

$$K = K_M = \frac{k_{-1} + k_2}{k_1} \tag{19}$$

K_M Michaelis-Menten constant (mM)

The Michaelis-Menten constant does not describe any more the dissociation but is rather a kinetic constant. It denotes the special substrate concentration where half of the maximal activity is reached. Since the Michaelis-Menten constant approaches the dissociation constant K_S of the ES complex, it is valuable for estimating individual reaction kinetics. K_M values usually range from 10 mM to 0.01 mM. A low K_M value implies a high affinity between enzyme and substrate.

The function $v = f(S)$ is shown in figure 22:

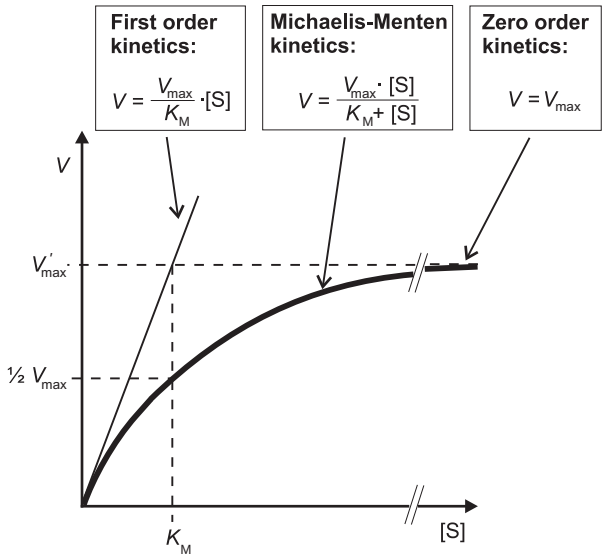


Fig. 22 Typical Michaelis-Menten-curve.

Here, two borderline cases have to be discussed. If the substrate concentration $[S]$ is far below the K_M-value a linear first-order kinetics results. The active sites of the enzyme are almost all free and the substrate concentration is rate limiting. If the substrate concentration is so high that all active sites are saturated, zero-order kinetics results.

By the above given Michaelis-Menten equation one-substrate reactions can be described. If a two-substrate reaction is to be addressed, two reaction rate terms are connected by multiplication. For a simple two-substrate reaction of A + B the double substrate kinetics for the forward reaction are:

$$v_{forward} = \frac{V_{\max}^{forward} \cdot [A] \cdot [B]}{(K_{MA} + [A]) \cdot (K_{MB} + [B])} \tag{20}$$

A corresponding equation for the reverse reaction can be set up as well. The resulting total reaction rate equals the difference of forward and reverse reactions.

$$v = \frac{d[P]}{dt} = v_{forward} - v_{reverse} \tag{21}$$

The easiest way to describe a double substrate reaction is the already described kinetics (equations 20 and 21) derived from the single substrate Michaelis-Menten kinetics (equation 17). The disadvantages of this approach are:

- No information about the mechanism is included.
- Forward and reverse reactions are addressed as two totally independent reactions. No information about the equilibrium is included.

But opposite to these disadvantages there are also significant advantages of the Michaelis-Menten kinetics:

- Over broad ranges real reactors can be described with this simple type of kinetics.
- Kinetic parameters are independent of the definition of reaction direction.
- All parameters possess a graphical meaning.

A mechanistically correct description of the total reaction is only possible with a more complex model, e.g. ordered bi-bi, random bi-bi, ping-pong, etc [26]. In these models all equilibria leading to the formation of transition states are individually described. The single kinetic parameters do not have any more a descriptive meaning. The advantage of these mechanistic models is the exact description of the individual equilibria.

4.5 Basic reactor types and their mode of operation

While designing and selecting reactors for biotransformations, certain characteristic features of the biocatalysis have to be considered.

- Materials are processed in each active microbial cell, so that the main function of the bioreactor should be to provide and maintain the optimal conditions for the cells to perform the biotransformation.
- The performance of the biocatalysts depends on concentration levels and physical needs (such as salts and proper temperature, respectively). Microorganisms can adapt the structure and activity of their enzymes to the process condition, unlike isolated enzymes.
- The microbial mass can increase significantly as the biotransformation progresses, leading to a change in rheological behaviour. Also, metabolic products of cells may influence the performance of the biocatalyst.
- Microorganisms are often sensitive to strong shear stress.
- Bioreactors generally have to function under sterile conditions to avoid microbial contamination, so they must be designed for easy sterilization.
- In the case of both enzymes and whole cells as biocatalysts, the substrate and/or the product may inhibit or deactivate the biocatalyst, necessitating special reactor layouts.
- Biotransformations with enzymes are usually carried out in a single (aqueous) or in two (aqueous/organic) phases, whereas the whole cells generally catalyze in gas-liquid-solid systems. Here, the liquid phase is usually aqueous.
- Foam formation is undesirable but often unavoidable in the case of whole cell biotransformations, where most processes are aerobic. Due consideration must be given to this aspect while designing or selecting a bioreactor.

Here only the three basic types of reactors are presented. All others are variations or deductions therefrom:

- Stirred-tank reactors (STRs) or batch reactors
- Continuously operated stirred-tank reactors (CSTRs)
- Plug-flow reactors (PFRs)

In contrast to the stirred tank reactor that is operated batchwise, the latter ones are operated continuously. By knowing the main characteristics of these fundamental reactors and some of their variations, it is possible to choose the appropriate reactor for a specific application [30]. This is especially important when dealing with a kinetically or thermodynamically limited system. In the following only the basic terms are explained. For further reading, the reader is referred to textbooks [31–40].

The *stirred tank reactor* is operated in a non-stationary way (figure 23). Assuming ideal mixing, the concentration is the same in every volume element as a function of time. With advancing conversion the substrate concentration decreases and the product concentration increases.

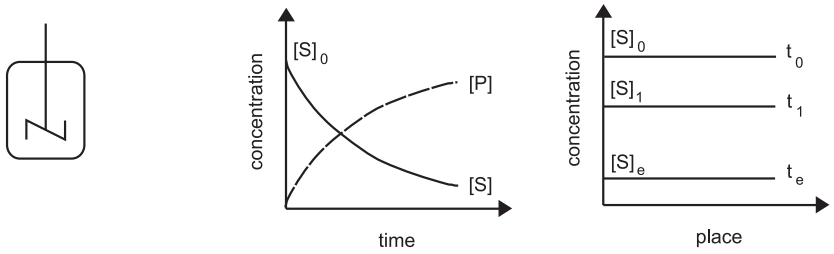


Fig. 23 Concentration-time and concentration-place profile for a stirred tank reactor.

This reactor type is widely used on an industrial scale. A variation is operation as repetitive batch or fed batch. *Repetitive batch* means that the catalyst is separated after complete conversion by filtration or even decantation. New substrate solution is added and the reaction is started again. *Fed batch* means that one reaction compound, in most cases the substrate, is fed to the reactor during the conversion.

The *continuously operated stirred tank reactor* works under product outflow conditions, meaning that the concentrations in every volume element are the same as those at the outlet of the reactor (figure 24).

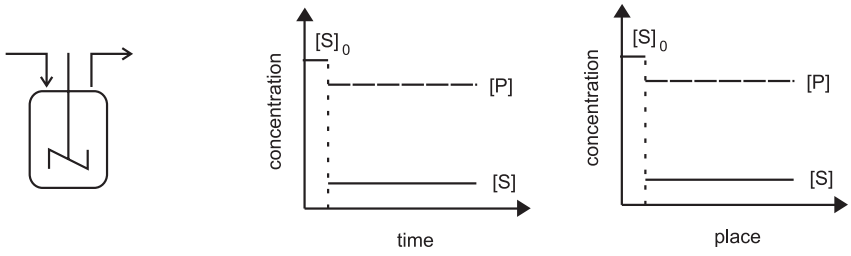


Fig. 24 Concentration-time and concentration-place profile for a continuously operated stirred tank reactor.

If the steady state is reached, the concentrations are independent of time and place. The conversion is controlled by the catalyst concentration and the residence time τ:

$$\tau = \frac{V}{F} \tag{22}$$

τ residence time (h)

V total reactor volume (L)

F substrate feed rate ($L \cdot h^{-1}$)

One very common application of the CSTR is the *cascade of n CSTRs* (figure 25). With increasing number *n* of reaction vessels the cascade is approximating the plug flow reactor. The product concentration increases stepwise from vessel to vessel.

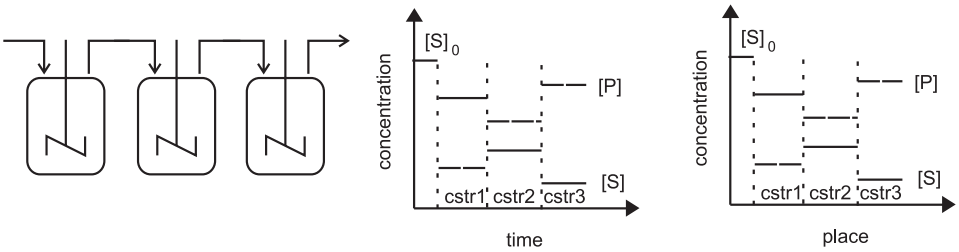


Fig. 25 Concentration-time and concentration-place profile for a cascade of continuously operated stirred tank reactors.

In the *plug flow reactor* the product concentration increases slowly over the length of the reactor (figure 26). Therefore, the average reaction rate is faster than in the continuously operated stirred tank reactor. In each single volume element in the reactor the concentration is constant in the steady state. In other words, the dimension of time is exchanged with the dimension of place in comparison to the stirred tank reactor.

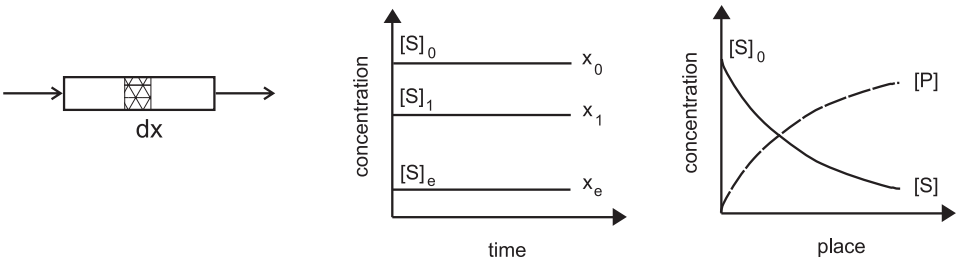


Fig. 26 Concentration-time and concentration-place profile for a plug-flow reactor.

If a reaction is limited by a substrate surplus or product inhibition the choice of the right reactor is important to yield a high reaction rate:

- Any reaction exhibiting *substrate surplus inhibition* should not be carried out in a batch reactor setup, since this results in longer reaction times. The high substrate concentration at startup lowers the reaction velocity. Here, a continuously operated stirred tank reactor is preferred. By establishing a high conversion in the steady state a low substrate concentration is achieved. Also the use of a fed batch results in a small substrate concentration.
- If *product inhibition* occurs either a stirred tank reactor, a plug flow reactor or a cascade of n continuously operated stirred tank reactors should be chosen. In all these reactors the product concentration increases over time. Alternatively a differential reactor with integrated product separation can be used.

4.5.1 Mass balances

The performance of the different reactor types concerning one reaction can be simulated mathematically. This is also the verification of the kinetic model of the reaction, since it should describe the course of the concentration for each compound with only a small error. The main part of the simulation model is the coupled system of differential equations of the first order, which are the mass balances of all reactants and products. The change in the concentration of one compound in time and in a volume element (= 'accumulation') is the sum of convection, reaction and diffusion.

$$\text{accumulation} = \text{convection} + \text{reaction} + \text{diffusion} \tag{23}$$

The convection term describes the change in the concentration of one compound in the reactor as the difference of the influx into the reactor and the efflux. The reaction term describes, by use of the kinetic model, the change of the concentration of one compound as a result of the reaction. The reaction velocity v is the sum of the individual reaction velocities describing consumption of a substrate or formation of a product. Diffusion is only given in the case where no ideal mixing is stated.

Depending on the reactor type chosen, the mass balance can be simplified, stating ideal mixing:

4.5.1.1 Stirred tank reactor:

The mass balance of each compound is defined by the reaction rate only, since no fluid enters or leaves the reactor. At a defined time the concentrations are the same in every volume element (diffusion = 0). There is no influx or efflux of substrate or products to a single volume element in time (convection = 0).

The mass balance is simplified to:

$$-\frac{d[S]}{dt} = v \tag{24}$$

The time t that is necessary to reach a desired conversion X can be determined by integrating the reciprocal rate equation from zero to the desired conversion X.

$$dt = -\frac{d[S]}{v} = \frac{[S]_0 \cdot dX}{v} \quad \Rightarrow \quad t = [S]_0 \cdot \int_0^X \frac{1}{v} \cdot dX \tag{25}$$

4.5.1.2 Plug-flow reactor:

The change of reaction rate within a unit volume passing the reactor length is equivalent to a change corresponding to the residence time τ within the reactor. Diffusion is neglected in an ideal plug-flow reactor (diffusion = 0) and all the concentrations will not change with time in the steady state (accumulation = 0).

Just by exchanging t for τ equation 25 can be also used for the plug flow reactor to determine the residence time τ necessary to reach a desired conversion X.

$$\tau = [S]_0 \cdot \int_0^X \frac{1}{v} \cdot dX \tag{26}$$

4.5.1.3 Continuously operated stirred tank reactor:

The concentration of substrate S within the reactor is effected by convection as well as by reaction. There is no diffusion between different volume elements (diffusion = 0) and in the steady state the concentrations will not change with time (accumulation = 0).

The mass balance is simplified to:

$$0 = -\frac{d[S]}{dt} = \frac{[S]_0 - [S]}{\tau} + v_S \tag{27}$$

The residence time τ, that is necessary to reach a desired conversion, can be determined by equation 28:

$$\tau = [S]_0 \cdot \frac{1}{v} \cdot dX \tag{28}$$

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5 Processes

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In this chapter you will find industrial biotransformations sorted in the order of the enzyme classes (EC). One type of biotransformation is often carried out by several companies leading to identical or the same class of products. Here only one exemplary process is named. Only in cases where the reaction conditions differ fundamentally, resulting in a totally different process layout, these are listed separately (e.g. L-aspartic acid).

It is difficult to judge which processes are applied on an industrial scale. But even if not all of the following processes are used in the ton scale, they are at least performed by industrial companies to produce compounds for research or clinical trials in kg scale.

If you know of any new biotransformation carried out on an industrial scale, or you notice that we missed any important one, we would be pleased if you could supply us with the appropriate information. For your convenience you will find a form at the end of this book.

On the next pages you will find a process example with all necessary explanations for an easy understanding of all used parameters and symbols in the flow sheets.

By reading the example you will also see the maximum number of parameters we have tried to find for each process.

X.X.X.X = enzyme nomenclature number

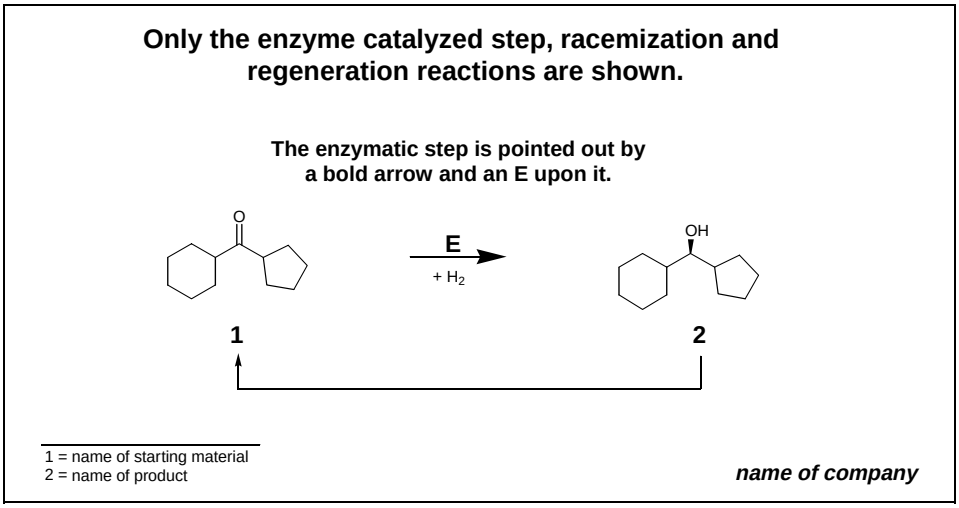


Fig. X.X.X.X – 1

1) Reaction conditions

[**N**]:	molar concentration, mass concentration [molar mass] of component **N**
pH:	pH of reaction solution
T:	reaction temperature in °C
medium:	type of reaction medium: in most cases aqueous, but can also be several phases in combination with organic solvents
reaction type:	suggestion of enzyme nomenclature for the type of enzymatic catalyzed reaction
catalyst:	application of catalyst: solubilized / immobilized enzyme / whole cells
enzyme:	systematic name (alternative names)
strain:	name of strain
CAS (enzyme):	[CAS-number of enzyme]

2) Remarks

- Since in the chemical drawing on top of the page only the enzymatic step is shown, prior or subsequent steps, which might be part of the industrial process can be found here.
- Since it is often difficult to gain knowledge of the true industrial process conditions, those published in the past for the same reaction system are given.
- Beside the already mentioned topics you will find additional information regarding the discussed biotransformation, e.g. substrate spectrum, enzyme improvement, immobilization methods, and all other important information which does not fit to another category.
- If an established synthesis is replaced by a biotransformation, the classical, chemical synthesis can be found here as well.

3) Flow scheme

- The flow schemes are reduced to their fundamental steps. A list and explanation of the symbols is given in the next figures:

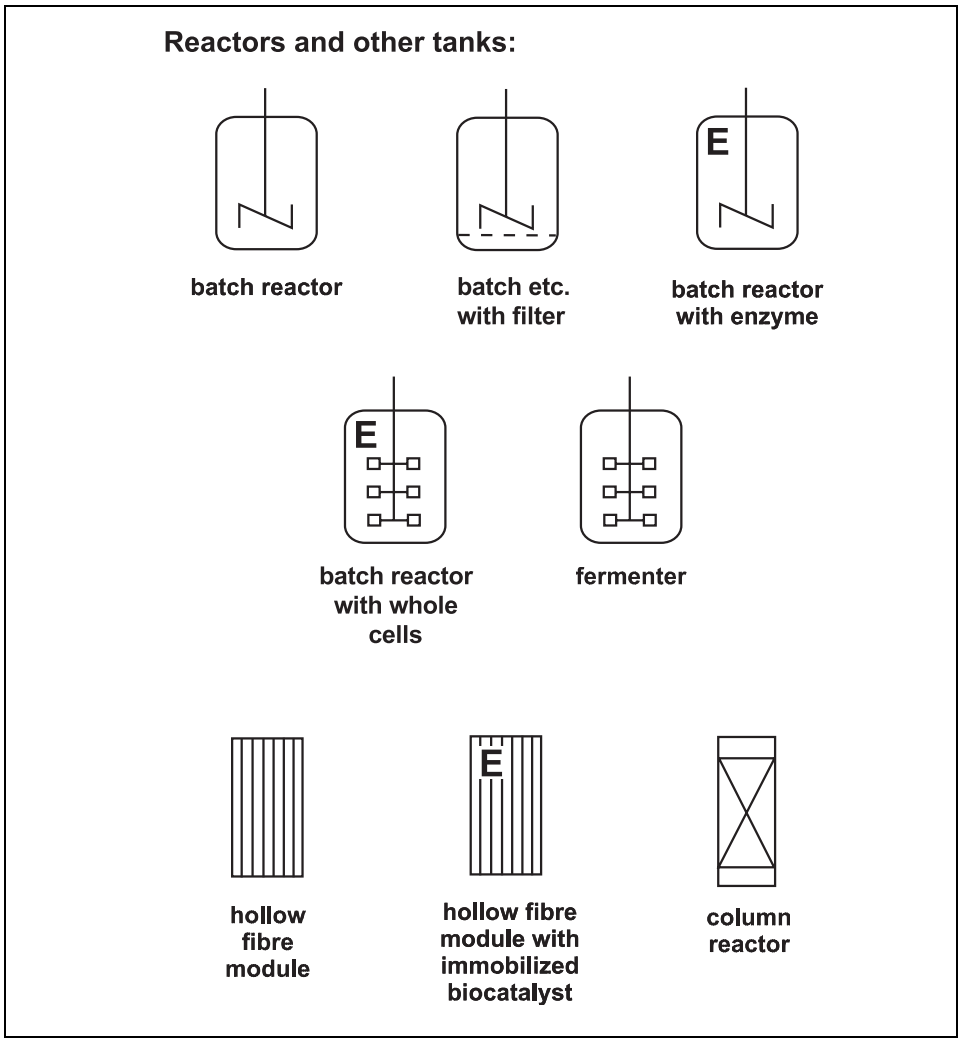


Fig. X.X.X.X – 2

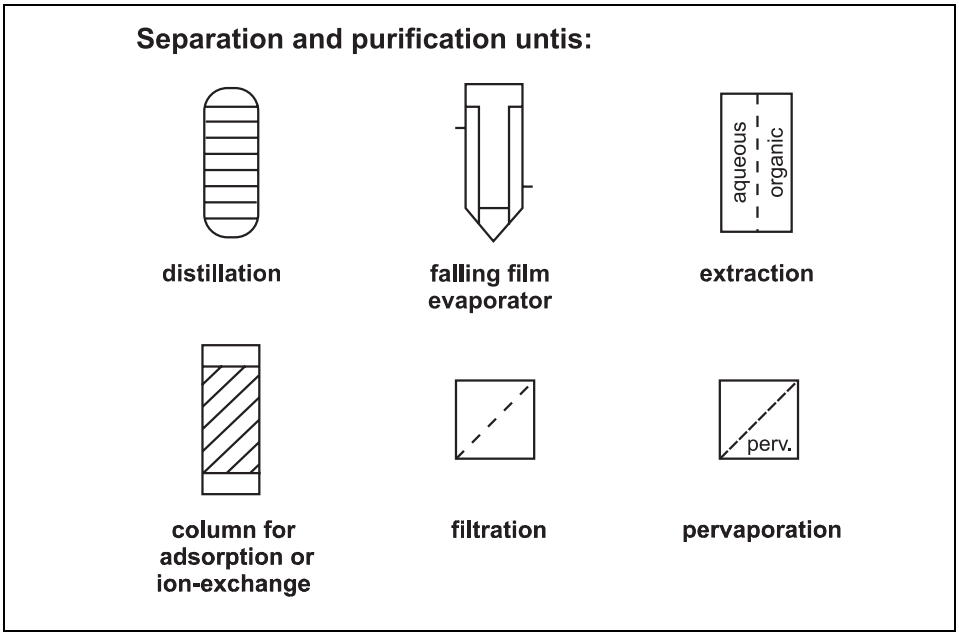


Fig. X.X.X.X – 3

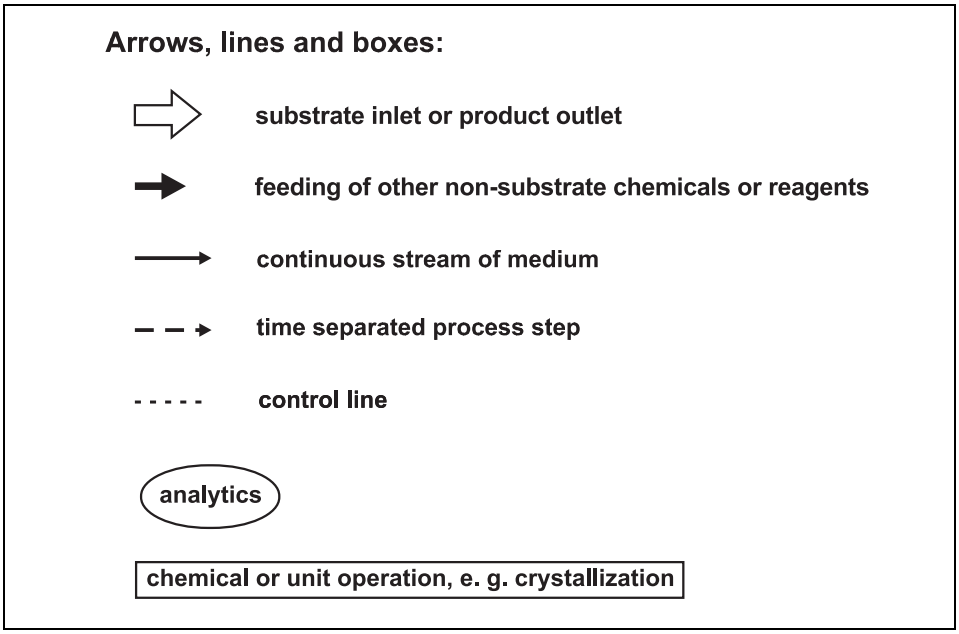


Fig. X.X.X.X – 4

4) Process parameters

conversion:	molar conversion in %
yield:	molar yield in %
selectivity	molar selectivitiy in %
ee:	enantiomeric excess in %
chemical purity:	purity of component in %
reactor type:	fed or repetitive batch, CSTR, plug flow reactor
reactor volume:	reactor volume in L
capacity:	mass of product per year in $t \cdot a^{-1}$
residence time:	time for one batch reaction or residence time in continuous operated reactor in hours
space-time-yield:	mass of product per time and reactor volume in $kg \cdot L^{-1} \cdot d^{-1}$
down stream processing:	purification of raw material after reaction, e. g. crystallization, filtration, distillation
enzyme activity:	in U(nits = $\mu mol \cdot min^{-1}$) per mass of protein (mg) or volume of reaction solution (L)
enzyme consumption:	amount of consumed enzyme per mass of product
enzyme supplier:	company, country
start-up date:	start of production
closing date:	end of production
production site:	company, country
company:	company, country

5) Product application

- The application of the product as intermediate or the end-product are given here.

6) Literature

- Cited literature you will find here. Often a personal communication or direct information of the company provided us neccessary information.

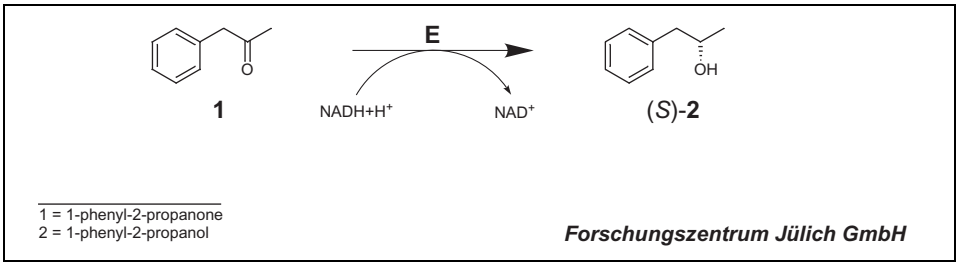


Fig. 1.1.1.1 – 1

1) Reaction conditions

[**1**]:	0.015 M, 1.95 g · L^{-1} [134,18 g · mol^{-1}]
pH:	6.7
T:	25 °C
medium:	aqueous
reaction type:	redox reaction
catalyst:	solubilized enzyme
enzyme:	alcohol-NAD^+ oxidoreductase (alcohol dehydrogenase)
strain:	*Rhodococcus erythropolis*
CAS (enzyme):	[9031–72–5]

2) Remarks

- The cofactor regeneration is carried out with a formate dehydrogenase from *Candida boidinii* (FDH = formate dehydrogenase, EC 1.2.1.2) utilizing formate that is oxidized to CO_2:

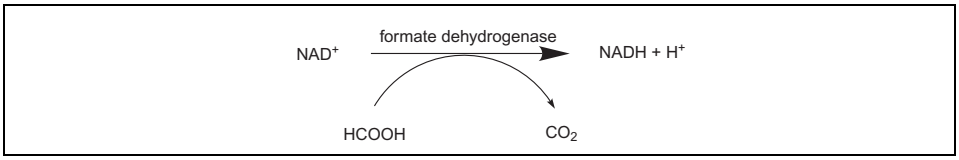
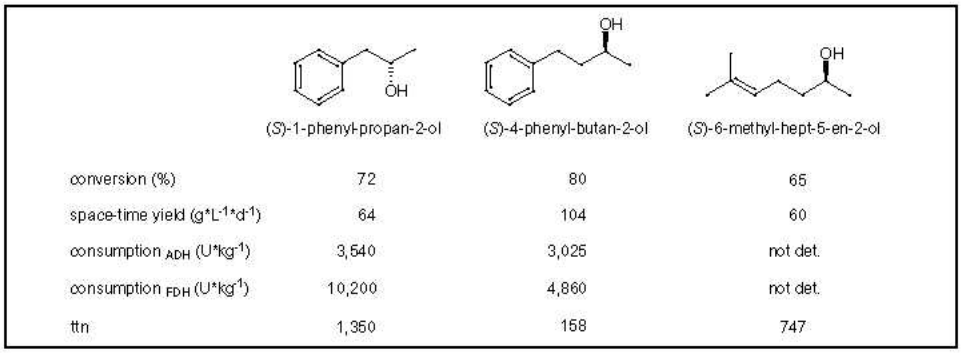


Fig. 1.1.1.1 – 2

- This reactor concept is especially attractive for starting materials of low solubility. The starting materials are directly titrated into the aqueous phase. The process consists of three loops: I: aqueous loop with a hydrophilic ultra-filtration membrane retaining the enzymes; II: permeated aqueous reaction solution products, starting materials and cofactors are passed through the tube phase of the extraction module; III: organic solvent phase, containing extracted products and starting materials.
- The charged cofactors (NAD^+/NADH) remain in the aqueous loops I and II. Therefore only deactivated cofactor needs to be replaced resulting in an economically high total turnover number (= ttn).
- The extraction module consists of microporous, hydrophobic hollow-fiber membranes. The organic extraction solvent is recycled by continuous distillation. The product remains in the bottom of the distillation column.

- Using this method very good space-time yields are obtainable in spite of the low substrate solubilities:



	(*S*)-1-phenyl-propan-2-ol	(*S*)-4-phenyl-butan-2-ol	(*S*)-6-methyl-hept-5-en-2-ol
conversion (%)	72	80	65
space-time yield ($g*L^{-1}*d^{-1}$)	64	104	60
consumption $_{ADH}$ ($U*kg^{-1}$)	3,540	3,025	not det.
consumption $_{FDH}$ ($U*kg^{-1}$)	10,200	4,860	not det.
ttn	1,350	158	747

Fig. 1.1.1.1 – 3

3) Flow scheme

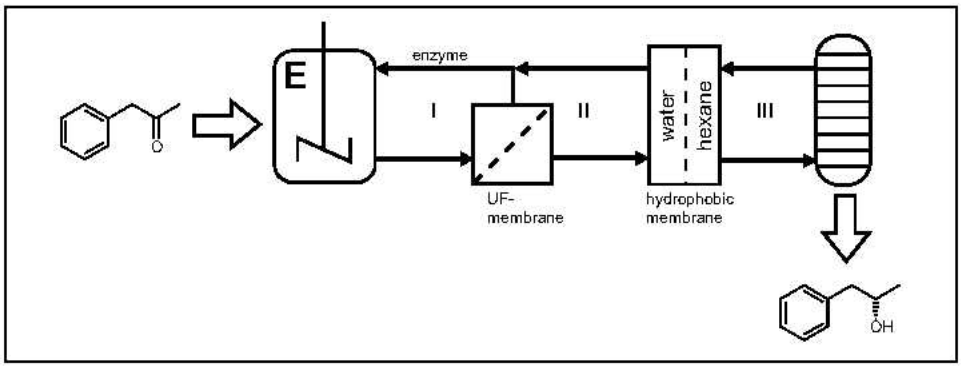


Fig. 1.1.1.1 – 4

4) Process parameters

conversion:	72 %
yield:	72 %
selectivity:	100 %
ee:	>99 %
reactor type:	CSTR (enzyme bimembrane reactor)
reactor volume:	0.05 L
residence time:	0.33 h
space-time-yield:	63.5 $g \cdot L^{-1} \cdot d^{-1}$
down stream processing:	distillation
enzyme activity:	0.95 $U \cdot mL^{-1}$
enzyme consumption:	3.5 $U \cdot g^{-1}$
enzyme supplier:	Institute of Enzyme Technology, University of Düsseldorf, Germany
start-up date:	1996
production site:	Jülich, Germany
company:	Forschungszentrum Jülich GmbH, Germany

5) Product application

- The trivial name for (*S*)-6-methyl-5-hepten-2-ol is (*S*)-(+)-sulcatol, a pheromone from the scolytid beetle *Gnathotrichus sulcatus / Gnathotrichus retusus.*
- (*S*)-1-Phenyl-2-propanol is used as an intermediate for the synthesis of amphetamines (sympathomimetics):

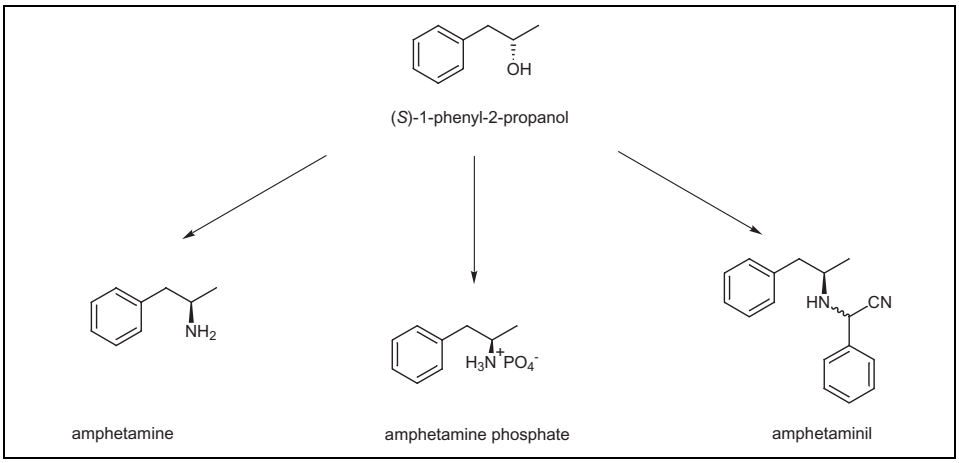


Fig. 1.1.1.1 – 5

- (*S*)-4-Phenyl-2-butanol is used as a precursor for anti-hypertensive agents and spasmolytics or anti-epileptics:

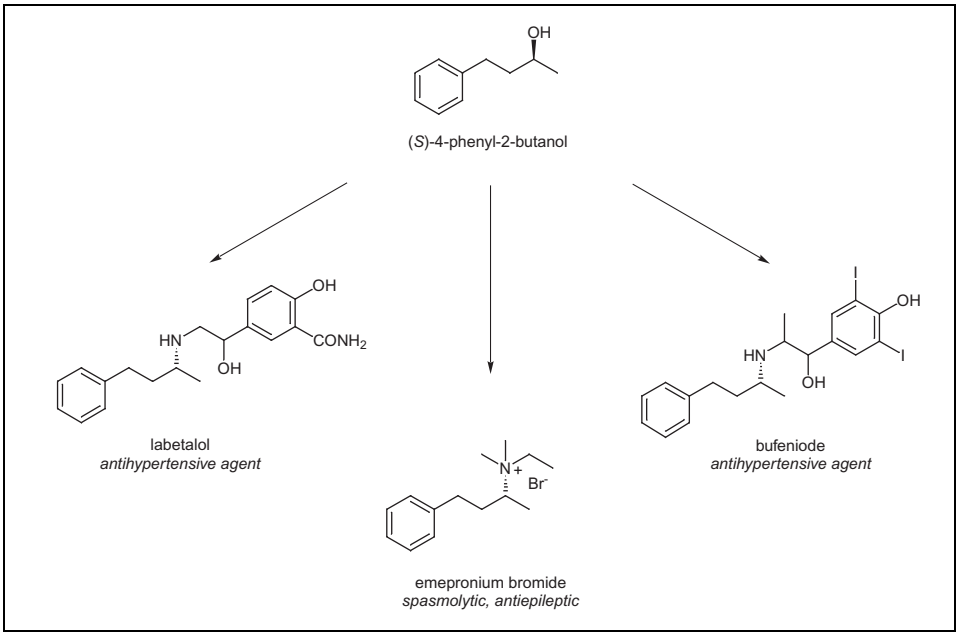


Fig. 1.1.1.1 – 6

6) Literature

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- Johnston, B., Slessor, K. (1979) Facile synthesis of the enantiomers of sulcatol, Can. J. Chem. **57**, 233–235
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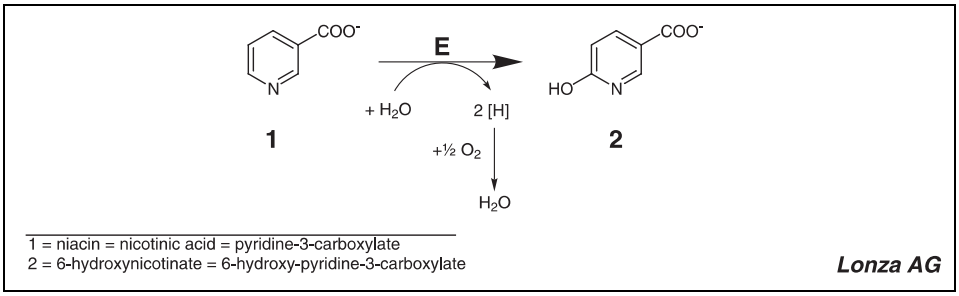

Fig. 1.5.1.13 – 1

1) Reaction conditions

[**1**]:	0.533 M, 65 g · L^{-1} [122.06 g · mol^{-1}]
pH:	7.0
T:	30 °C
medium:	aqueous
reaction type:	redox reaction (hydroxylation)
catalyst:	suspended whole cells
enzyme:	nicotinate: $NADP^+$6-oxidoreductase (nicotinic acid hydroxylase, nicotinate dehydrogenase)
strain:	*Achromobacter xylosoxidans*
CAS (enzyme):	[9059-03–4]

2) Remarks

- The 6-hydroxynicotinate producing strain was found by accident, when in the mother liquor of a niacin producing chemical plant precipitating white crystals of 6-hydroxynicotinate were found.
- At niacin concentrations higher than 1 % the second enzyme of the nicotinic acid pathway, the decarboxylating 6-hydroxynicotinate hydroxylase gets strongly inhibited, whereas the niacin hydroxylase operates unaffected:

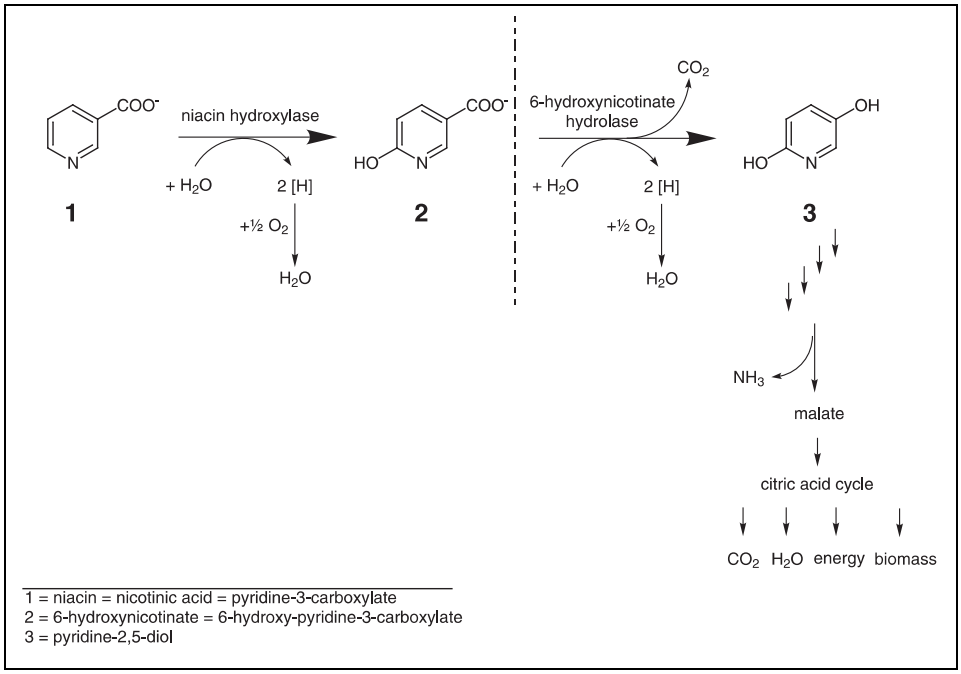

Fig. 1.5.1.13 – 2

- The process takes place in two phases (see flow scheme):
  1) Growing of cells in a fermenter (chemostat) on niacin and subsequent storage of biomass in cooled tanks.
  2) Addition of biomass to niacin solution, incubation, separation of biomass and purification of product.
- The product is precipitated by the addition of acid.
- Alternatively, the integration of the two phases into an one reaction vessel fed-batch operation is possible (product concentration of 75 $g \cdot L^{-1}$ in 25 h). This procedure is not used on an industrial scale.
- Also, a continuous process was developed as 'pseudocrystal fermentation'. The substrate is added in its solid form and the product crystallizes out of the reaction solution. The process takes advantage of the fact that the Mg-salt of niacin is 100 times more soluble in H_2O at neutral pH than Mg-6-hydroxynicotinate. The pH is titrated to 7.0 with nicotinic acid. The concentration of Mg-nicotinate is regulated to 3 % using conductivity measurement techniques and direct addition of the salt. Mg-6-hydroxynicotinate is collected in a settler.
- Niacin hydroxylase works only in the presence of electron-transmitting systems such as cytochrome, flavine or $NADP^+$, and therefore air needs to be supplied to facilitate the cofactor regeneration. The oxygen-transfer rate limits the reaction.
- In contrast to the biotransformation the chemical synthesis of 6-substituted nicotinic acids is difficult and expensive due to the separation of by-products.

3) Flow scheme

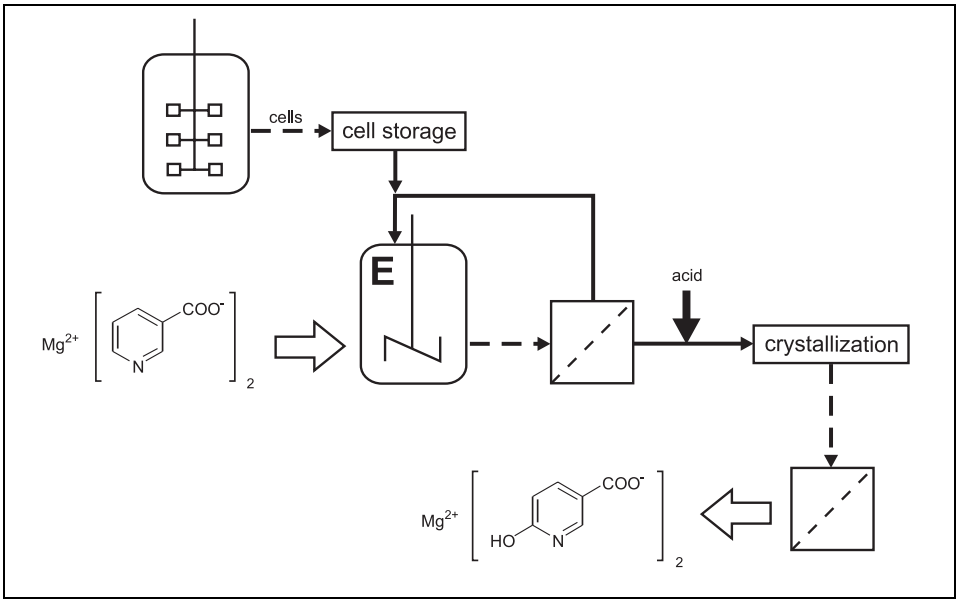

Fig. 1.5.1.13 – 3

4) Process parameters

conversion:	> 90 %
yield:	> 90 % (overall)
selectivity:	high
chemical purity:	> 99 %
reactor type:	batch
reactor volume:	12,000 L
capacity:	several tons
residence time:	12 h
down stream processing:	precipitation, centrifugation and drying
production site:	Visp, Switzerland
company:	Lonza AG, Switzerland

5) Product application

- Versatile building block chiefly in the synthesis of modern insecticides.
- By using common chemistry methods the product is converted into interesting building blocks:

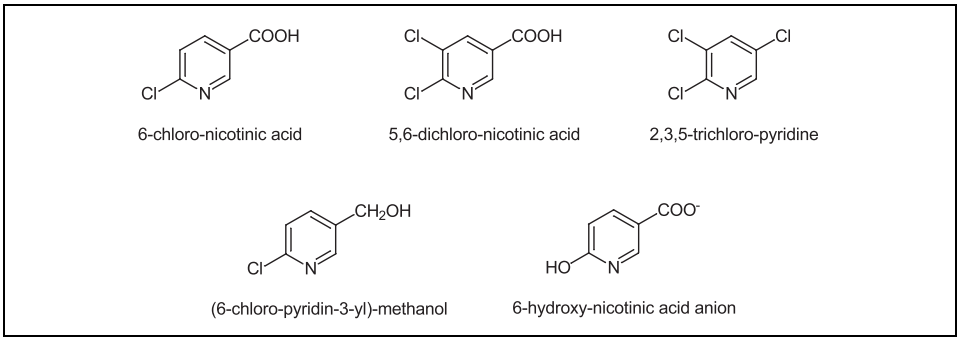

Fig. 1.5.1.13 – 4

6) Literature

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- Cabral, J., Best, D., Boross, L., Tramper, J. (1994) Applied Biocatalysis, Harwood Academic Publishers, Chur, Switzerland
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- Sheldon, R.A. (1993) Chirotechnology, Marcel Dekker Inc., New York
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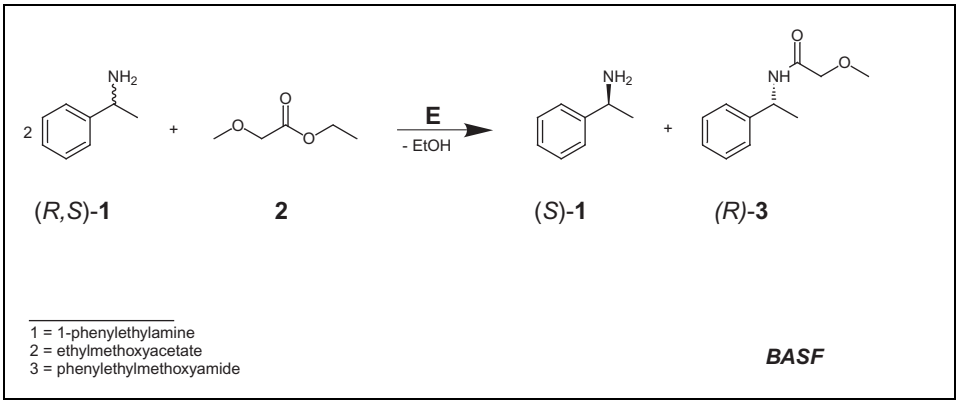

Fig. 3.1.1.3 – 1

1) Reaction conditions

[(R/S)-**1**]:	1.65 M, 200 g · L^{-1} [121.18 g · mol^{-1}] in MTBE (= methl-*tert*-butylether)
[(S)-**1**]:	1.4 M, 170 g · L^{-1} [121.18 g · mol^{-1}] in MTBE
pH:	8.0–9.0
T:	25 °C
medium:	MTBE-ethylmethoxyacetate
reaction type:	carboxylic ester hydrolysis
catalyst:	immobilized enzyme
enzyme:	triacylglycerol acylhydrolase (triacylglycerol lipase)
strain:	*Burkholderia plantarii*
CAS (enzyme):	[9001–62–1]

2) Remarks

- The lipase is immobilized on polyacrylate.
- The lowering in activity caused by the use of organic solvent can be offset (about 1,000 times and more) by freeze-drying a solution of the lipase together with fatty acids (e.g. oleic acid).
- The E-value of the reaction is above 500.

- The (*R*)-phenylethylmethoxy amide can be easily hydrolyzed to get the (*R*)-phenylethylamine:

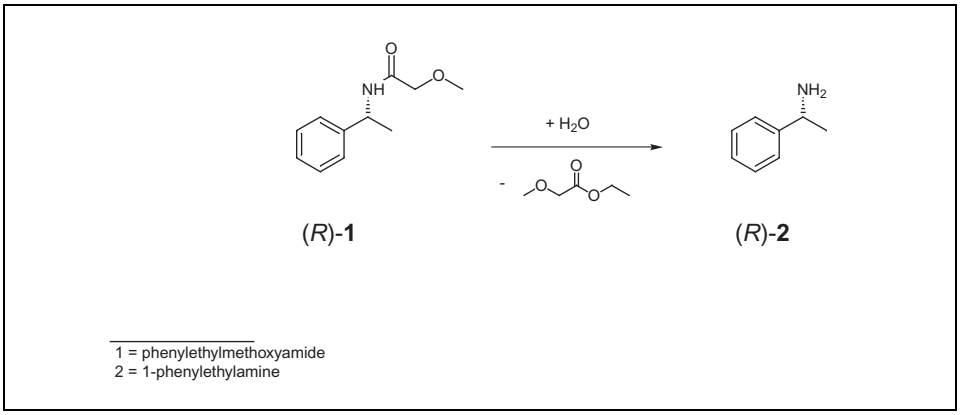

Fig. 3.1.1.3 – 2

- The (*S*)-enantiomer can be racemized using a palladium catalyst.
- The following amines can also be used in this process:

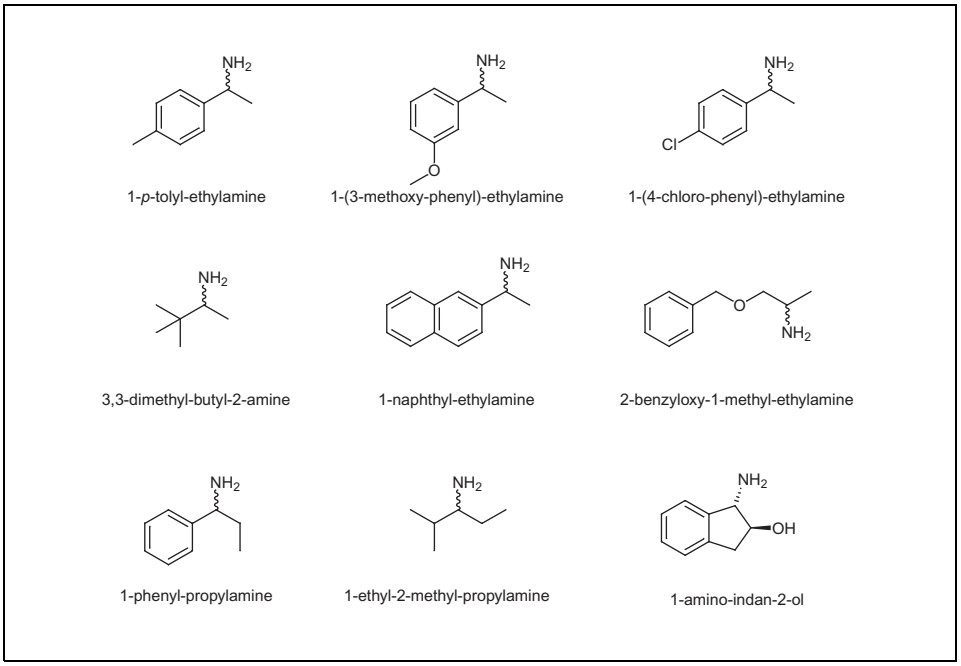

Fig. 3.1.1.3 – 3

3) Flow scheme

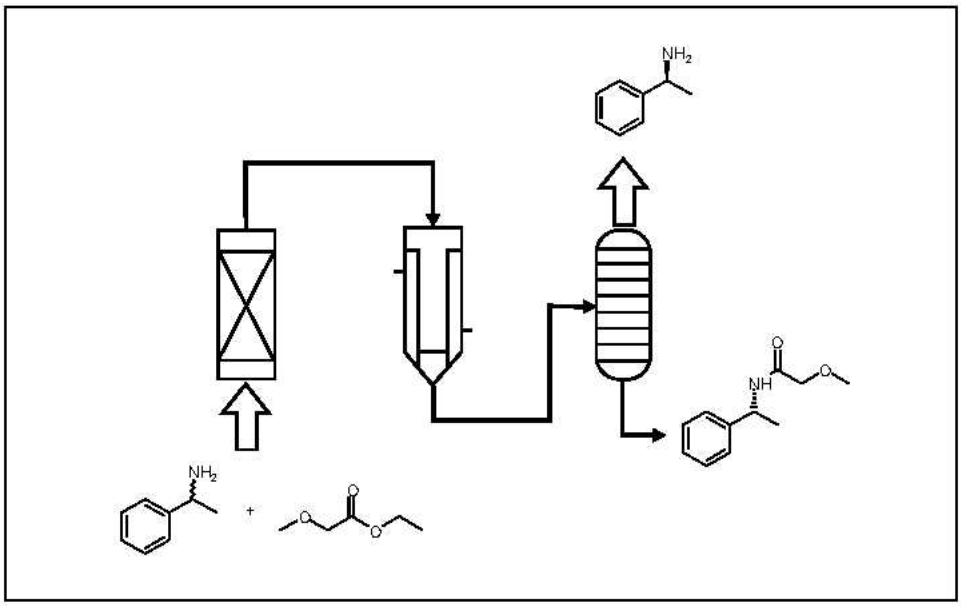

Fig. 3.1.1.3 – 4

4) Process parameters

conversion:	50 %
yield:	> 90 %
ee:	> 99 % (*S*); 93 % (*R*)
reactor type:	plug-flow reactor or batch
capacity:	> 100 t · a^{-1}
residence time:	5–7 h
down stream processing:	distillation or extraction
production site:	Ludwigshafen, Germany
company:	BASF, Germany

5) Product application

- Products are intermediates for pharmaceuticals and pesticides.
- They can also be used as chiral synthons in asymmetric synthesis.

6) Literature

- Balkenhohl, F., Hauer, B., Lander, W., Schnell, U., Pressler, U., Staudemaier H.R. (1995) Lipase katalysierte Acylierung von Alkoholen mit Diketenen, BASF AG, DE 4329293
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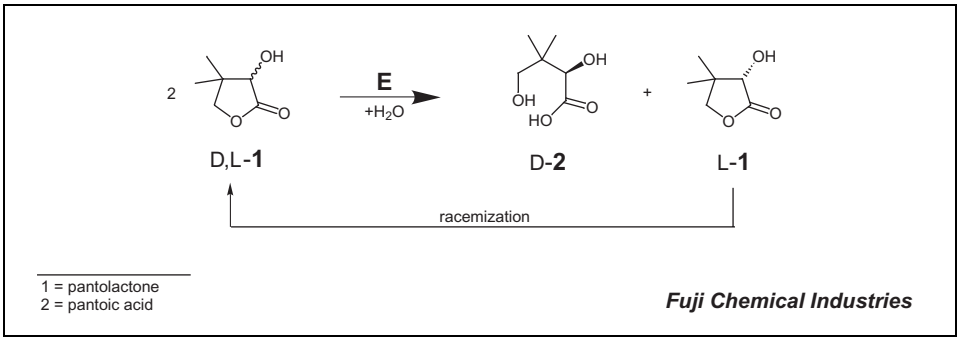

Fig. 3.1.1.25 – 1

1) Reaction conditions

[**1**]:	2.69 M, 350 g · L^{-1} [130.14 g · mol^{-1}]
pH:	6.8 – 7.2
T:	30 °C
medium:	aqueous
reaction type:	carboxylic ester hydrolysis
catalyst:	immobilized whole cells
enzyme:	1,4-lactone hydroxyacylhydrolase (γ-lactonase)
strain:	*Fusarium oxysporum*
CAS (enzyme):	[37278–38–9]

2) Remarks

- The reverse reaction, the lactonization of aldonic acid, is catalyzed under acidic conditions. The reverse reaction does not take place with aromatic substrates.
- The lactonase from *Fusarium oxysporum* has a very broad substrate spectrum:

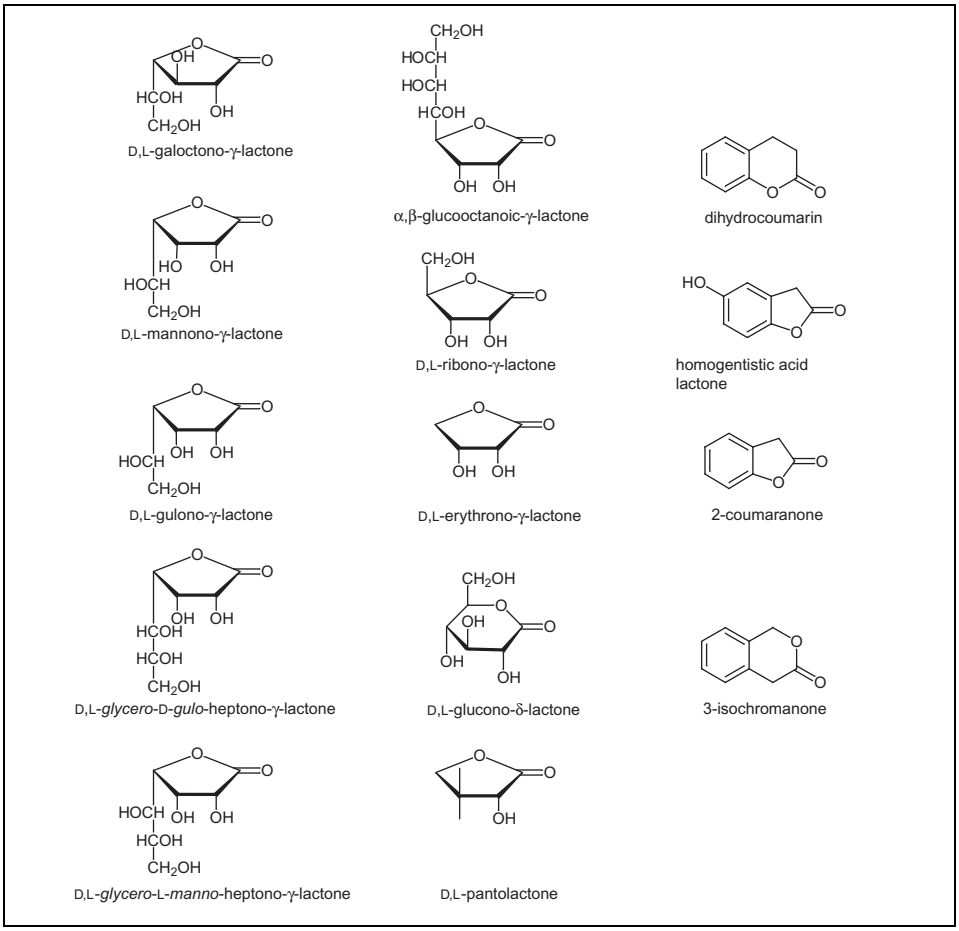

Fig. 3.1.1.25 – 2

- For the synthesis whole cells are immobilized in calcium alginate beads and used in a fixed bed reactor.
- The immobilized cells retain more than 90 % of their initial activity even after 180 days of continuous use.
- At the end of the reaction L-pantolactone is extracted and reracemized to D,L-pantolactone that is recycled into the reactor. The D-pantoic acid is chemically lactonized to D-pantolactone and extracted:

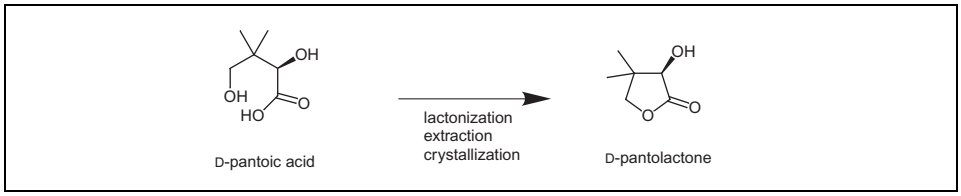

Fig. 3.1.1.25 – 3

- The biotransformation skips several steps that are necessary in the chemical resolution process:

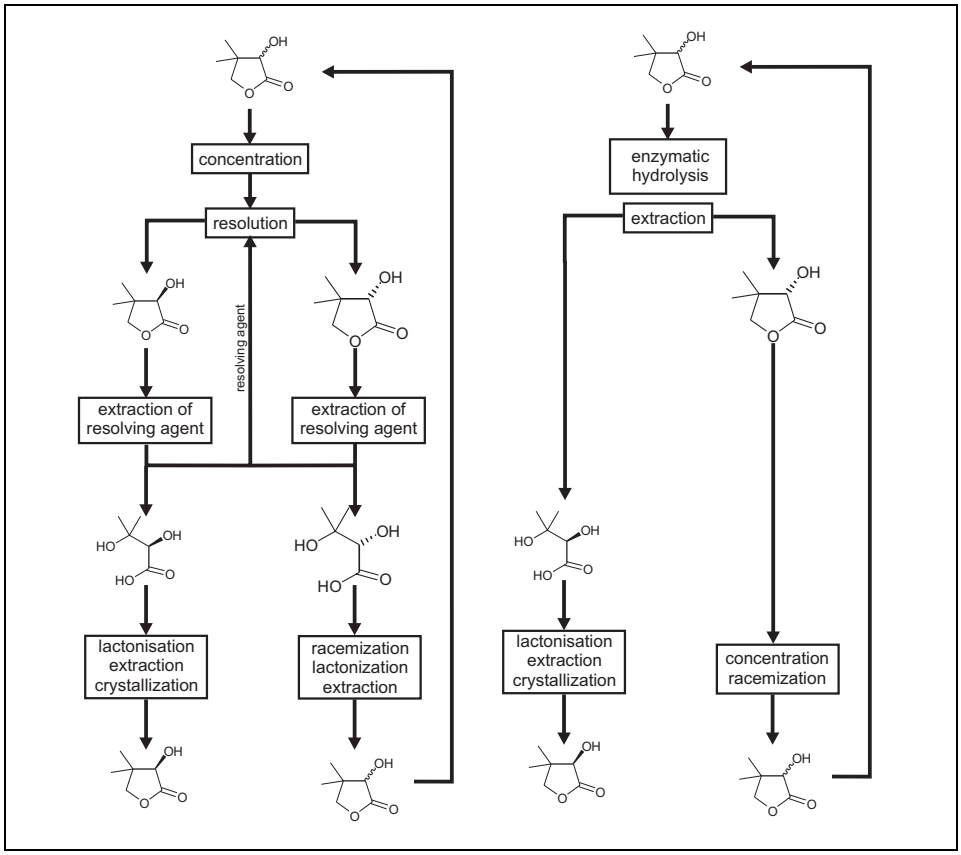

Fig. 3.1.1.25 – 4

- By using the lactonase from *Brevibacterium protophormia* L-lactones are available:

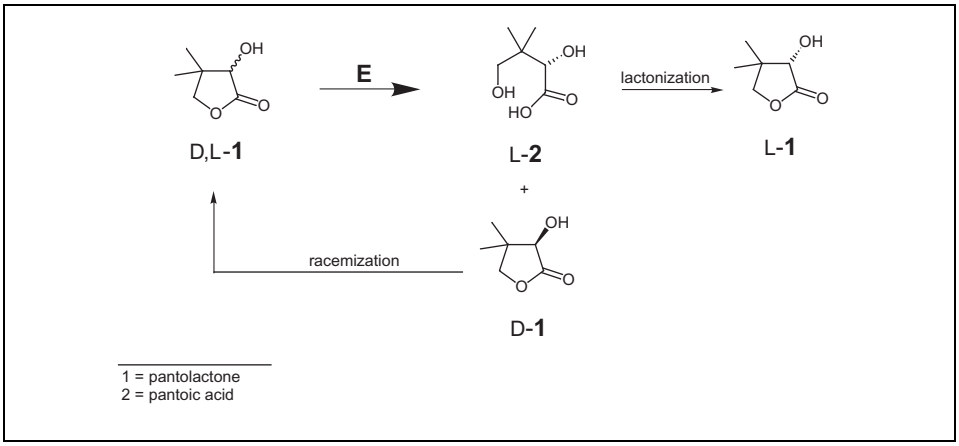

Fig. 3.1.1.25 – 5

3) Flow scheme

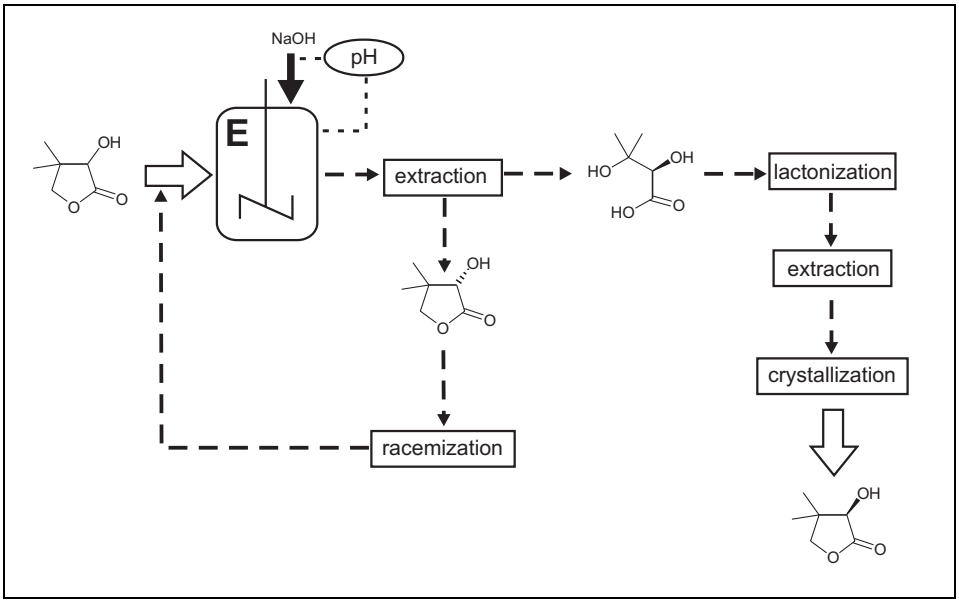

Fig. 3.1.1.25 – 6

4) Process parameters

conversion:	90–95 %
ee:	90–97 %
reactor type:	plug-flow reactor
residence time:	21 h
down stream processing:	lactonization, extraction and crystallization
production site:	Takaoka, Toyama Prefecture, Japan
company:	Fuji Chemical Industries, Japan

5) Product application

- The pantoic acid is used as a vitamin B_2-complex.
- D- and L-pantolactones are used as chiral intermediates in chemical synthesis.

6) Literature

- Simizu, S., Ogawa, J., Kataoka, M., Kobayashi. M. (1997) Screening of novel microbial enzymes for the production of biologically and chemically useful compounds, in: New Enzymes for Organic Synthesis (Scheper, T., ed.) pp. 45–88, Springer, New York

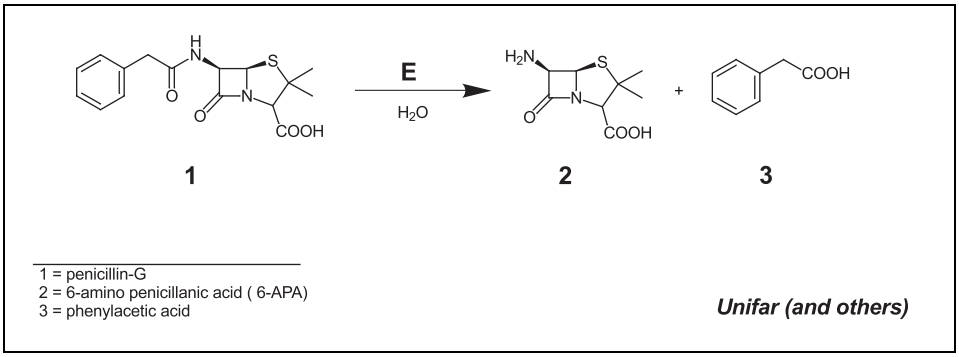

Fig. 3.5.1.11 – 1

1) Reaction conditions

[**1**]:	0.24 M, 80 g · L^{-1} [334.39 g · mol^{-1}]
pH:	8.0
T:	30–35 °C
medium:	aqueous
reaction type:	carboxylic acid amide hydrolysis
catalyst:	immobilized enzyme
enzyme:	penicillin amidohydrolase (penicillin acylase, penicillin amidase)
strain:	*Escherichia coli* and others (e.g. *Bacillus megaterium*)
CAS (enzyme):	[9014-06–6]

2) Remarks

- The enzyme is isolated and immobilized on Eupergit-C (Röhm, Germany).
- The production is carried out in a repetitive batch mode. The immobilized enzyme is retained by a sieve with a mesh size of 400.
- The time for filling and emptying the reactor is approximately 30 min.
- The residual activity of biocatalyst after 800 batch cycles, which is one production campaign, is about 50 % of the initial activity.
- The hydrolysis time after 800 batch cycles increases from the initial 60 min to 120 min.
- Phenylacetic acid is removed by extraction and 6-APA can be crystallized.
- The yield can be increased by concentrating the split-solution and/or the mother liquor of crystallization via vacuum evaporation or reverse osmosis.
- The production operates for 300 days per year with an average production of 12.8 batch cycles per day (production campaigns of 800 cycles per campaign).
- Several chemical steps are replaced by a single enzyme reaction. Organic solvents, the use of low temperature (–40 °C) and the need for absolutely anhydrous conditions, which made the process difficult and expensive, are no longer necessary in the enzymatic process:

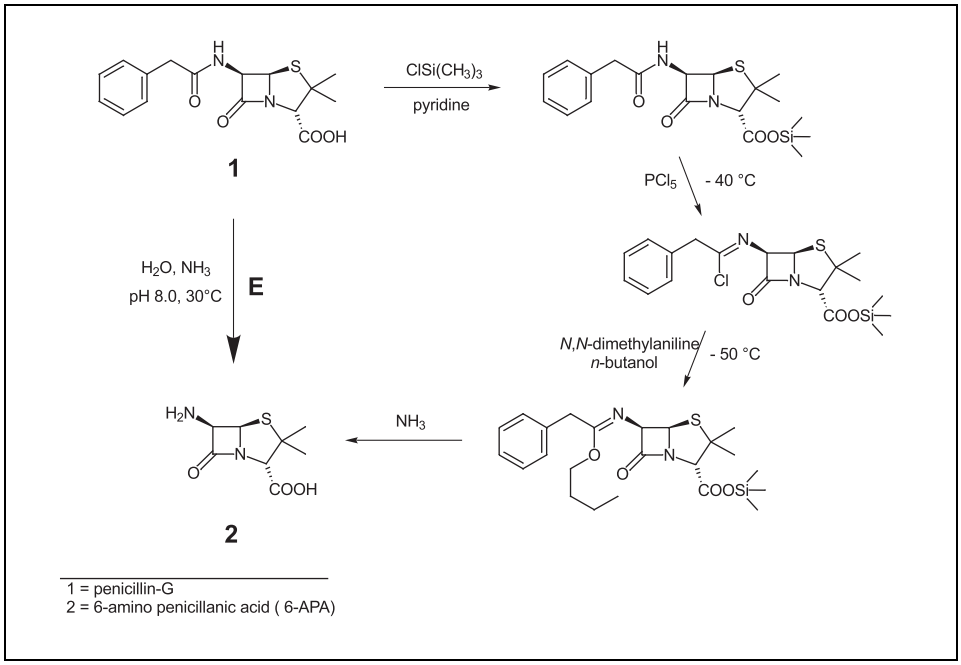

Fig. 3.5.1.11 – 2

3) Flow scheme

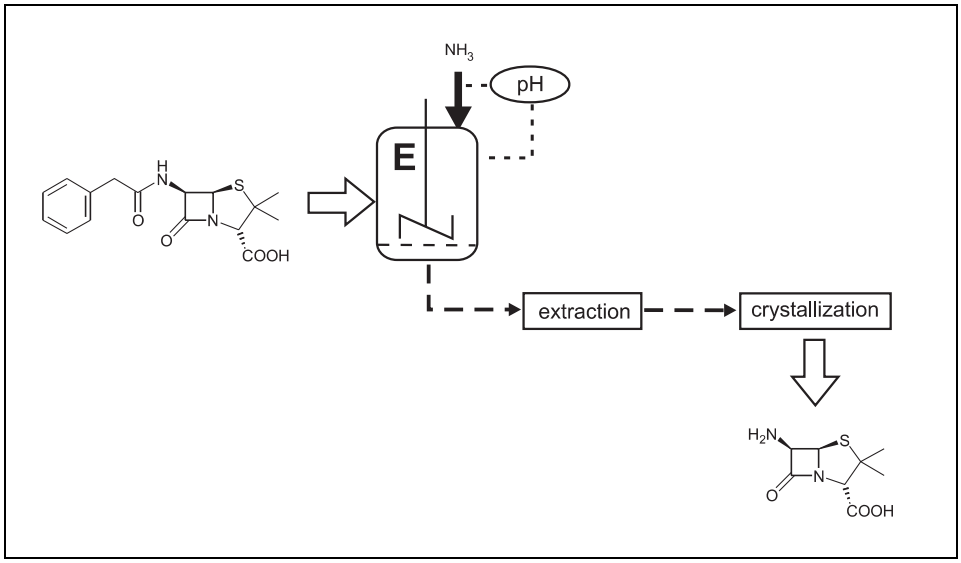

Fig. 3.5.1.11 – 3

4) Process parameters

conversion:	98 %
yield:	86 % (in reaction: 97 %)
selectivity:	> 99 %
chemical purity:	99 %
reactor type:	repetitive batch
reactor volume:	3,000 L
capacity:	300 $t \cdot a^{-1}$
residence time:	1.5 h (average over 800 cycles; initial: 1 h)
space-time-yield:	445 $g \cdot L^{-1} \cdot d^{-1}$ (which is the average for a production campaign of 800 batch cycles)
down stream processing:	extraction, crystallization (see remarks)
enzyme activity:	22 M U, corresponding to approx. 100 kg wet biocatalyst (27.5 kg of dry Eupergit-C)
enzyme consumption:	345 $U \cdot kg^{-1}$ (6-APA)
start-up date:	1973
production site:	Unifar, Turkey (and elsewhere)
company:	Unifar, Turkey (and others: Fujisawa Pharmaceutical Co., Japan, Gist-Brocades/DSM, The Netherlands, Novo-Nordisk, Denmark, Pfizer, USA)

5) Product application

- 6-APA is used as an intermediate for the manufacture of semi-synthetic penicillins.

6) Literature

- Cheetham, P. (1995) The application of enzymes in industry, in: Handbook of Enzyme Biotechnology (Wiseman, A. ed.), pp. 493–498, Ellis Harwood, London
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- Matsumoto, K. (1993) Production of 6-APA, 7-ACA, and 7-ADCA by immobilized penicillin and cephalosporin amidases, in: Industrial Application of Immobilized Biocatalysts (Tanaka, A, Tosa, T., Kobayashi, T. eds.) pp. 67–88, Marcel Dekker Inc., New York
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- Verweij, J, Vroom, E, (1993) Industrial transformations of penicillins and cephalosporins, Rec. Trav. Chim. Pays-Bas, **112** (2) 66–81

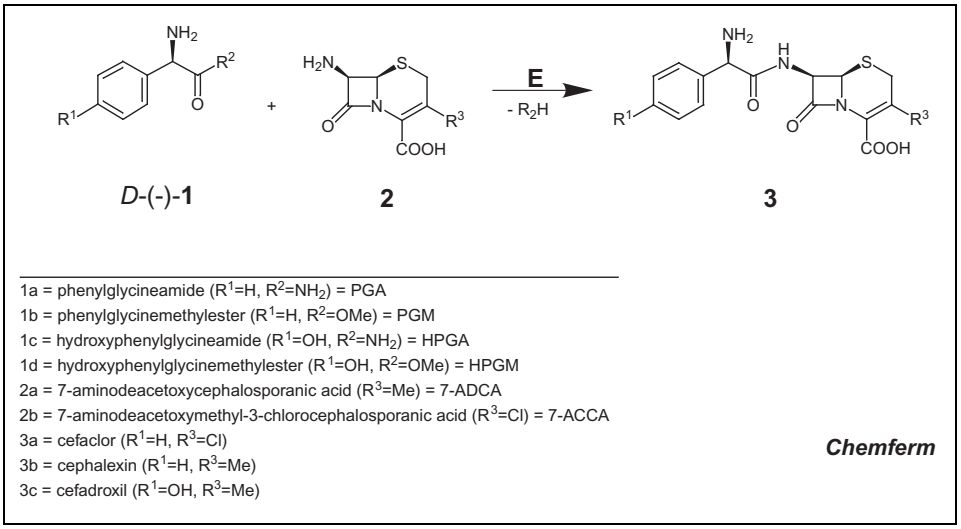

Fig. 3.5.1.11 – 1

1) Reaction conditions

medium:	aqueous
reaction type:	carboxylic acid amide hydrolysis
catalyst:	immobilized whole cells or enzyme
enzyme:	penicillin amidase (penicillin amidase, α-acylamino-β-lactam acylhydrolase)
strain:	*Escherichia coli* and others
CAS (enzyme):	[9014-06-06]

2) Remarks

- The established chemical synthesis started from benzaldehyde and included fermentation of penicillin. The process consists of ten steps with a waste stream of 30–40 kg waste per kg product. The waste contained methylene chlorid, other solvents, silylating agents and many by-products from side chain protection and acylating promoters.
- In comparison the chemoenzymatic route needs only six steps including three biocatalytic ones.
- The following figure compares the chemical and chemo-enzymatic routes (Bruggink, 1996):

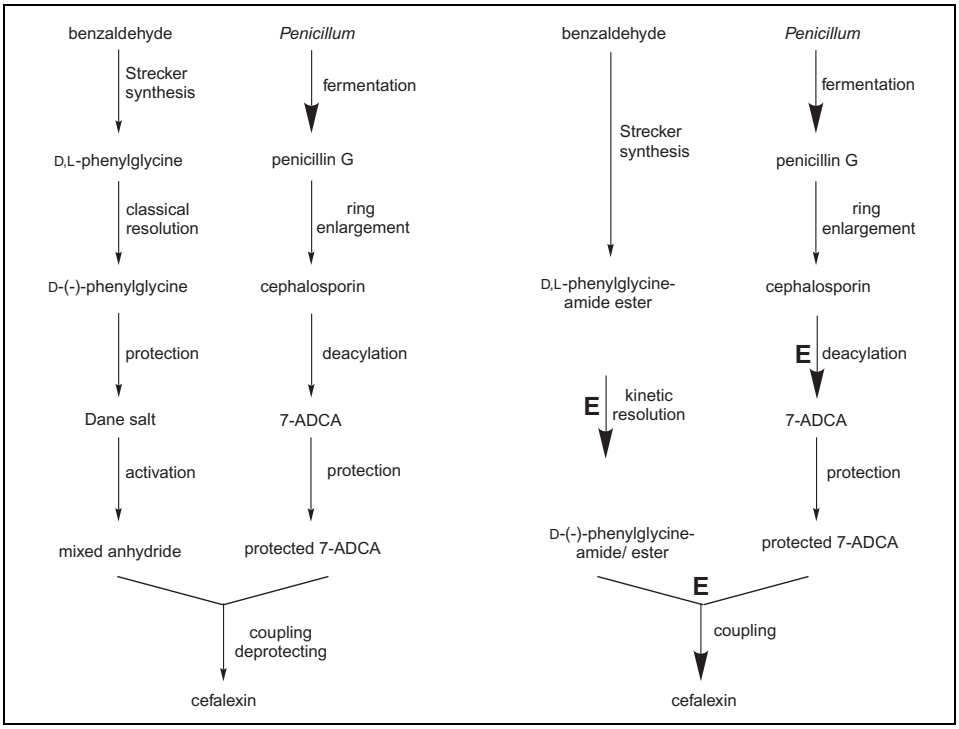

Fig. 3.5.1.11 – 2

- Beside *E. coli*, e.g., the strains *Klyveromyces citrophila* and *Bacillus megaterium* produce penicillin acylase.
- The penicillin acylases do not accept charged amino groups. Therefore phenylglycine itself cannot be used since at a pH value which the carboxyl function is necessarily uncharged the amino group will be charged.
- To reach non-equilibrium concentrations of the product, the substrate must be activated as an ester or amide. By this means the amino group can be partly uncharged at the optimal pH value of the enzyme. In biological systems the activation energy is delivered by ATP.
- The enzyme can be covalently attached on a gelatin-based carrier. Consequently the catalyst becomes water insoluble and can be easily separated from the reaction solution. Additionally the selectivity can be improved by choice of the right carrier composition. By-products resulting from hydrolysis of the educt can be avoided.
- To reach high conversions and high yields the educts and the by-products have to be added in a molar excess.
- Since the characteristics of the shown substances are different for each antibiotic, a special synthetic way had to be established.
- The production of **cefalexin** was the first successful application.
- If an excess of D-(–)PGA is used, surplus or non-converted D-(–)PGA has to be separated and recycled.

- The separation of D-(–)PGA can be done by addition of benzaldehyde and formation of the precipitating Schiff base which can be filtered off subsequent to separation of enzyme and solid products by filtration.
- Also the D-(–)-PGA is almost in-soluble in aqueous solution so that at the end of the reaction three solids (d-(–)-PGA, cefalexin, D-(–)-HPGA) have to be separated.
- One solution of this problem was the use of a special immobilized enzyme, which floats after stopping of the stirrer (Novo Nordisk, Denmark). The reaction solution can be removed from the bottom of the reactor containing the solid products.
- A better technique uses enzyme immobilizates with a defined diameter. At the end the reaction solution and solid substances can be removed from the reactor using a special sieve that is not permeable to the immobilized enzyme. This technique is shown in the flow scheme (Fig. 3.5.1.11–4).
- 7-ACCA (= 7-aminodeacetoxymethyl-3-chlorocephalosporanic acid) can be obtained by ozonolysis and chlorination of 3-methylene cephams. It is the precursor for the synthesis of cefaclor. Cefaclor is unstable at pH values above 6.5 while the solubility of 7-ACCA is very low at pH values under 6.5.
- One strategy, also established for cefalexin too, is to add a complexing agent (β-naphthol). The complex crystallizes and yields above 90 % are possible.
- Using this technique the concentration of product in the reaction mixture is very low, so that succeeding reactions can be suppressed and the mother liquor can be rejected. The disadvantage is the necessity of an organic solvent to yield a two phase system in which the decomplexation at low pH is possible.
- Using the same synthetic pathway alternatively to 7-ADCA and 7-ACCA also 6-APA derivatives can be synthesized:

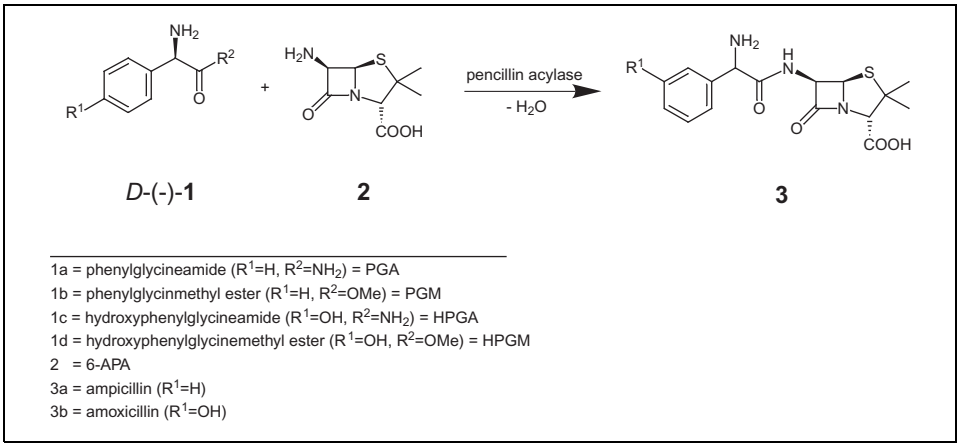

Fig. 3.5.1.11 – 3

- In contrast to cefalexin **ampicillin** has a better solubility, so that by using the recovery strategy of cefalexin too much product would be lost.

- Since the penicillanic acid derivatives are more sensitive towards degradation than cephalosporanic acid derivatives at almost all pH values, the conversion of 6-APA has to be complete and the product has to be recovered rapidly by crystallization (Fig. 3.5.1.11–5).
- The biocatalyst can be retained in the reactor by the sieve method analogous to the cephalexin procedure. Solubilized and precipitated product and D-(–)-PGA crystals are dissolved at acidic pH. Ampicillin is precipitated by adjusting the pH to its isoelectric point.
- In a similiar manner **amoxicillin** can be recovered. The advantage in this case is the low solubility of the product under reaction conditions so that hydrolysis of the product is suppressed since it precipitates at first. A semi-continuous reactor system with high substrate concentrations can be applied. This is shown in the last flow scheme (Fig. 3.5.1.11–6).

3) Flow scheme

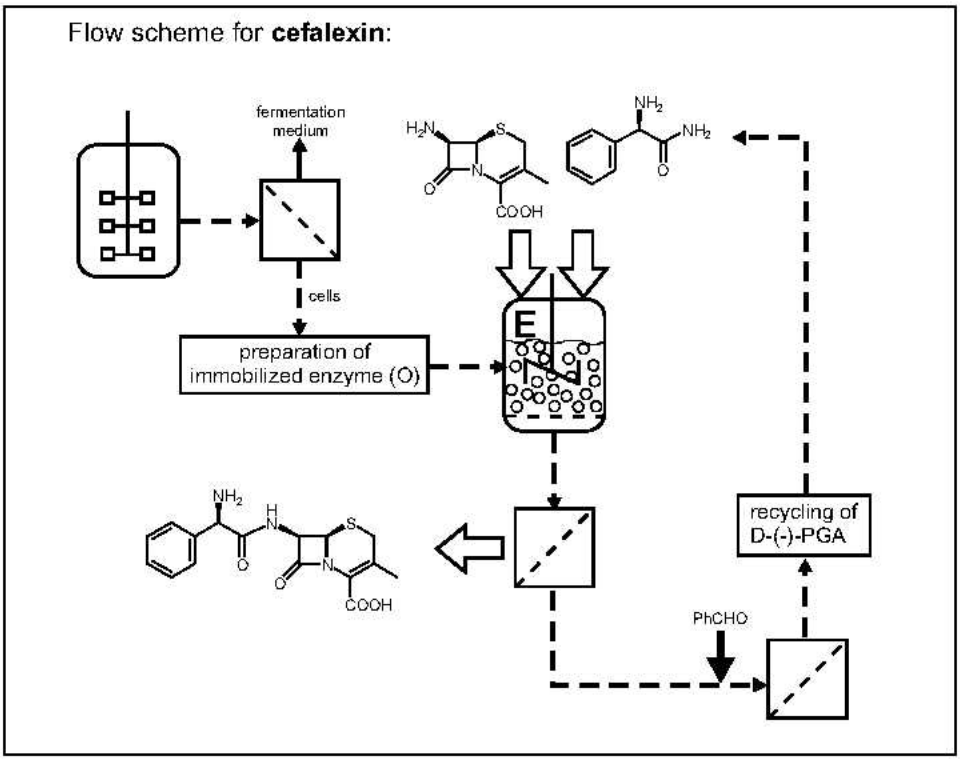

Fig. 3.5.1.11 – 4

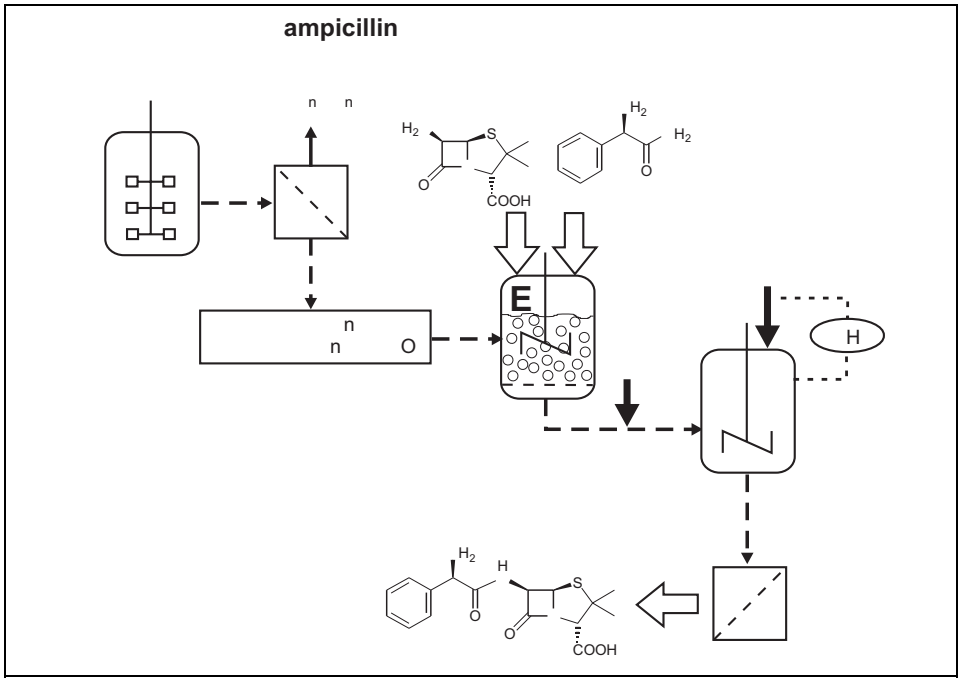

Fig. 3.5.1.11 – 5

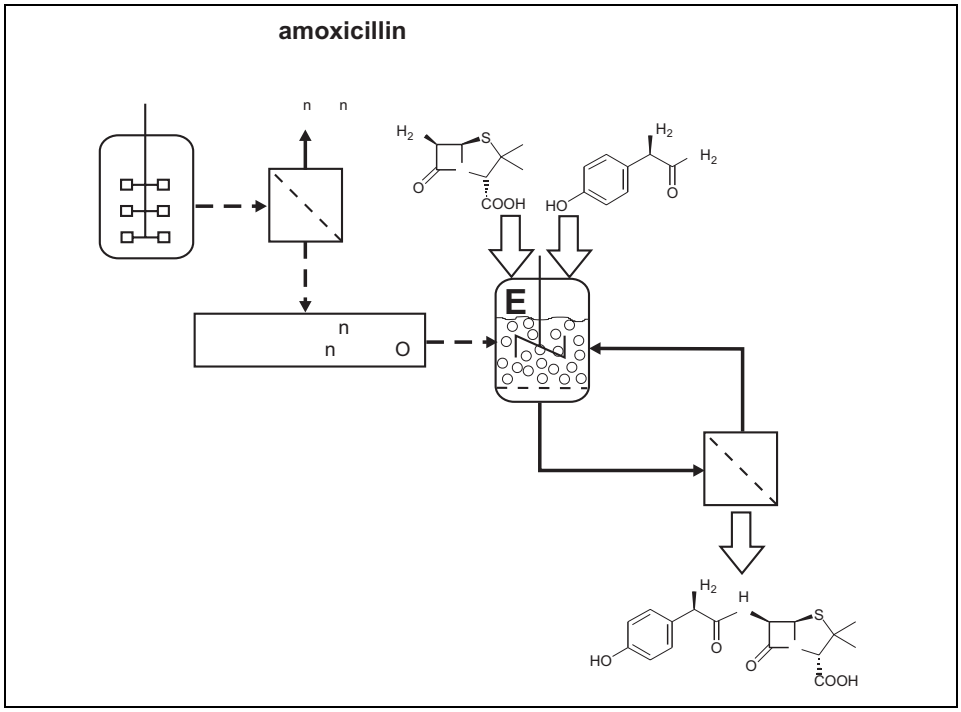

Fig. 3.5.1.11 – 6

4) Process parameters

yield:	> 90 %
selectivity:	> 95 %
ee:	> 99 %
reactor type:	repetitive batch (see flow schemes)
capacity:	2000 $t \cdot a^{-1}$ (worldwide)
down stream processing:	filtration
company:	Chemferm, The Netherlands (joint venture of Gist-Brocades and DSM Deretril, both The Netherlands); others

5) Product application

- The products are β-lactam antibiotics.

6) Literature

- Bruggink, A. (1996) Biocatalysis and process integration in the synthesis of semi-synthetic antibiotics, CHIMIA **50**, 431–432
- Bruggink, A., Roos, E.C., Vroom, E. de (1998) Penicillin acylase in the industrial production of β-lactam antibiotics, Org. Proc. Res. Dev. **2**, 128–133
- Clausen, K. (1995) Method for the preparation of certain β-lactam antibiotics, Gist-Brocades N. V., US 5,470,717
- Hernandez-Justiz, O., Fernandez-Lafuente, R., Terrini, Guisan, J. M. (1998) Use of aqueous two-phase systems for *in situ* extraction of water soluble antibiotics during their synthesis by enzymes immobilized on porous supports, Biotech. Bioeng. **59**, 1, 73–79

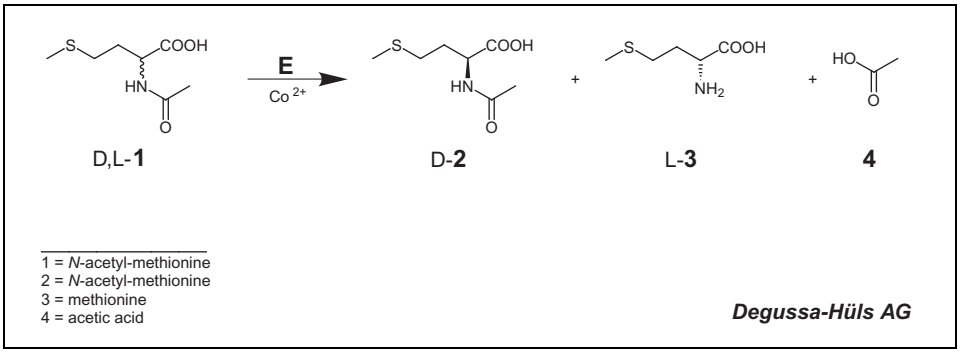

Fig. 3.5.1.14 – 1

1) Reaction conditions

[**1**]:	0.6 M, 97.96 g · L^{-1} [163.27 g · mol^{-1}]
[$\mathbf{Co^{2+}}$]:	$0.5 \cdot 10^{-3}$ M, 0.029 g · L^{-1} [58.93 g · mol^{-1}] (activator)
pH:	7.0
T:	37 °C
medium:	aqueous
reaction type:	hydrolysis
catalyst:	solubilized enzyme
enzyme:	*N*-acyl-L-amino-acid amidohydrolase (aminoacylase, acylase 1)
strain:	*Aspergillus oryzae*
CAS (enzyme):	[9012–37–7]

2) Remarks

- The *N*-acetyl-D,L-amino acid precursors are conveniently accessible through acetylation of D,L-amino acids with acetyl chloride or acetic anhydride under alkine conditions in a Schotten-Baumann reaction.
- As effector Co^{2+} is added to increase the operational stability of the acylase.
- The unconverted acetyl-D-methionine is racemized by acetic anhydride under alkaline conditions and the racemic acetyl-D,L-methionine is recycled.
- The racemization can also be carried out in a molten bath or by racemase.
- Product recovery of L-methionine is achieved by crystallization, because L-methionine is much less soluble than the substrate.
- A polyamide ultrafiltration membrane with a cutoff of 10,000 dalton is used.
- Several proteinogenic and non-proteinogenic amino acids are produced in the same way by Degussa-Hüls:

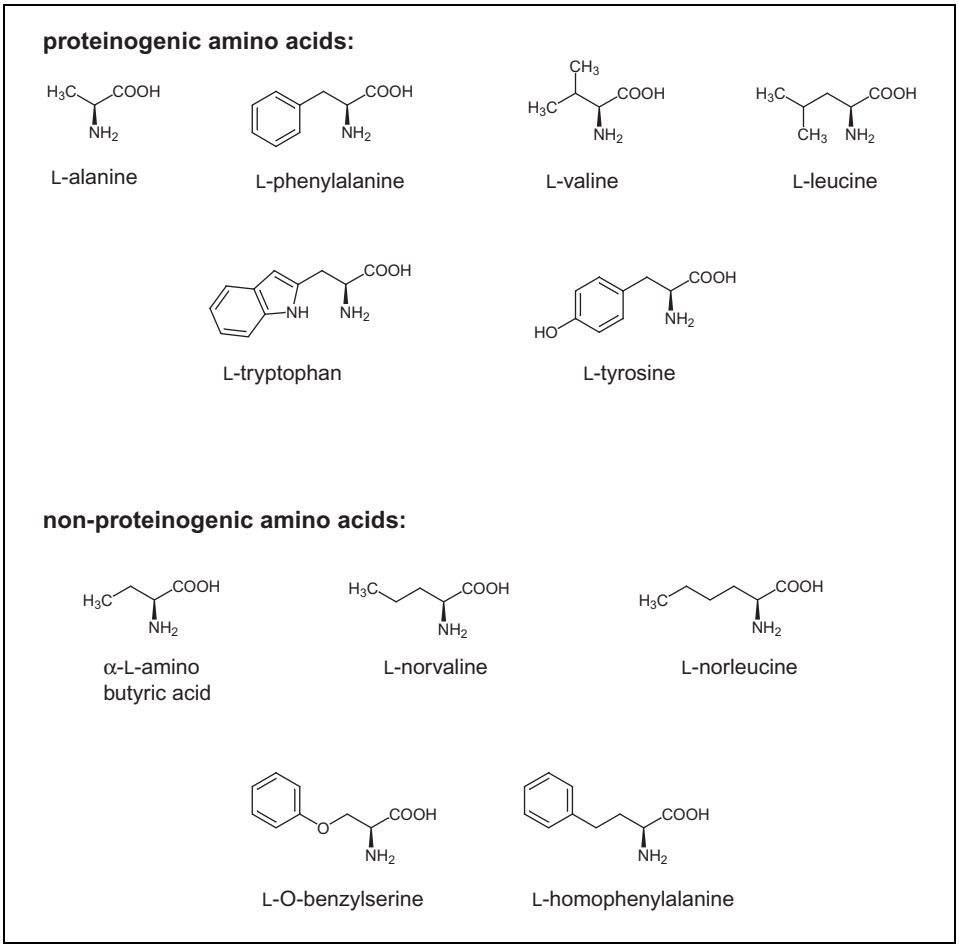
proteinogenic amino acids:
L-alanine
L-phenylalanine
L-valine
L-leucine
L-tryptophan
L-tyrosine
non-proteinogenic amino acids:
α-L-amino butyric acid
L-norvaline
L-norleucine
L-O-benzylserine
L-homophenylalanine

Fig. 3.5.1.14 – 2

3) Flow scheme

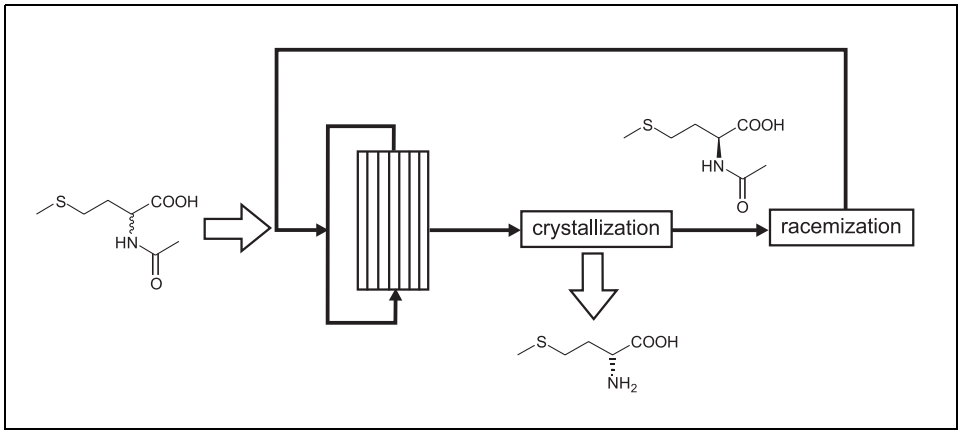

Fig. 3.5.1.14 – 3

4) Process parameters

yield:	80 %
ee:	99.5 %
reactor type:	cstr, UF-membrane reactor
capacity:	200 $t \cdot a^{-1}$
residence time:	2.9 h
space-time-yield:	592 $g \cdot L^{-1} \cdot d^{-1}$
down stream processing:	crystallization
enzyme activity:	1,067 $U \cdot g^{-1}_{protein}$
enzyme consumption:	2,067 $U \cdot kg^{-1}$
enzyme supplier:	Amano Corp., Nagoya, Japan
company:	Degussa-Hüls AG, Germany

5) Product application

- L-Amino acids are used for parenteral nutrition (infusion solutions), feed and food additives, cosmetics, pesticides and as intermediates for pharmaceuticals as well as chiral synthons for organic synthesis.

6) Literature

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- Chenault, H.K., Dahmer, J., Whitesides, G.M. (1989) Kinetic resolution of unnatural and rarely occuring amino acids: enantioselective hydrolysis of *N*-acyl amino acids catalyzed by acylase, J. Am. Chem. Soc. **111**, 6354–6364

- Leuchtenberger, W., Karrenbauer, M., Plöcker, U. (1984) Scale-up of an enzyme membrane reactor process for the manufacture of L-enantiomeric compounds, Enzyme Engineering 7, Ann. N. Y. Acad. Sci. **434**, 78
- Takahashi, T., Izumi, O., Hatano, K. (1989) Acetylamino acid racemase, production and use thereof, Takeda Chemical Industries, Ltd., EP 0 304 021 A2
- Wandrey, C., Flaschel, E. (1979) Process development and economic aspects in enzyme engineering. Acylase L-methionine system. In: Advances in Biochemical Engineering 12 (Ghose, T.K., Fiechter A., Blakebrough, N., eds.),pp. 147–218, Springer-Verlag, Berlin
- Wandrey, C., Wichmann, R., Leuchtenberger, W., Kula, M.R. (1981) Process for the continuous enzymatic change of water soluble α-ketocarboxylic acids into the corresponding amino acids, Degussa AG, US 4,304,858

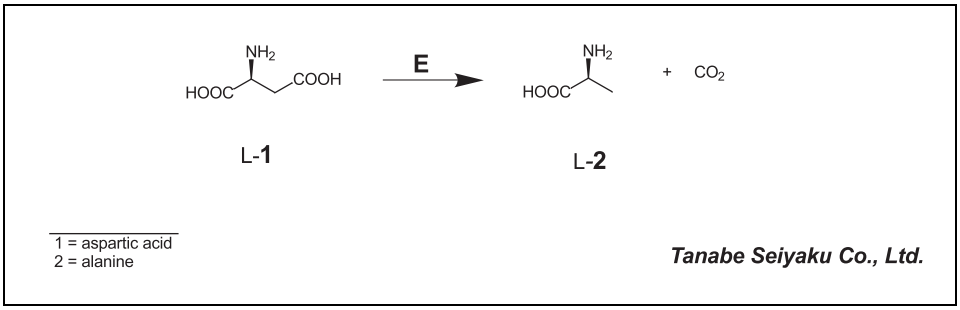


Fig. 4.1.1.12 – 1

1) Reaction conditions

[**1**]:	2.5 M, 332.75 g · L^{-1} [133.1 g · mol^{-1}]
pH:	6.2
T:	37 °C
medium:	aqueous
reaction type:	decarboxylation
catalyst:	immobilized whole cells
enzyme:	L-aspartate 4-decarboxylase (L-aspartate β-decarboxylase)
strain:	*Pseudomonas dacunhae*
CAS (enzyme):	[9024–57–1]

2) Remarks

- L-Alanine is produced industrially by Tanabe Seiyaku, Japan, since 1965 via a batch process with L-aspartate β-decarboxylase from *Pseudomonas dacunhae.*
- To improve the productivity a continuous production was established in 1982. Here the formation of carbon dioxide was the main problem in comparison to the catalyst stability and the microbial enzyme activity. The production of CO_2 occurs stoichiometricaly (nearly 50 L of CO_2 for each liter of reaction mixture with 2 M aspartate). The consequences are difficulties in obtaining a plug-flow condition in fixed bed reactors and the pH shift that takes places due to formation of CO_2. Therefore a pressurized fixed bed reactor with 10 bar was designed.
- The enzyme stability is not affected by the elevated pressure.
- The main side reaction, the formation of L-malic acid, can be completely avoided.
- To improve the yield of L-alanine the alanine racemase and fumarase activities can be destroyed by acid treatment of the microorganisms (pH 4.75, 30 °C). The L-aspartate β-decarboxylase activity is stabilized by the addition of pyruvate and pyridoxal phosphate.
- The process is often combined with the aspartase catalyzed synthesis of L-aspartic acid from fumarate (see page 381) in a two step biotransformation (Fig. 4.1.1.12 – 4). The main reason for the separation in two reactors is the difference in pH optimum for the two enzymes (aspartase from *E. coli*: pH 8.5, L-aspartate β-decarboxylase: pH 6.0). This is the first commercialized system of a sequential enzyme reaction using two kinds of immobilized microbial cells:

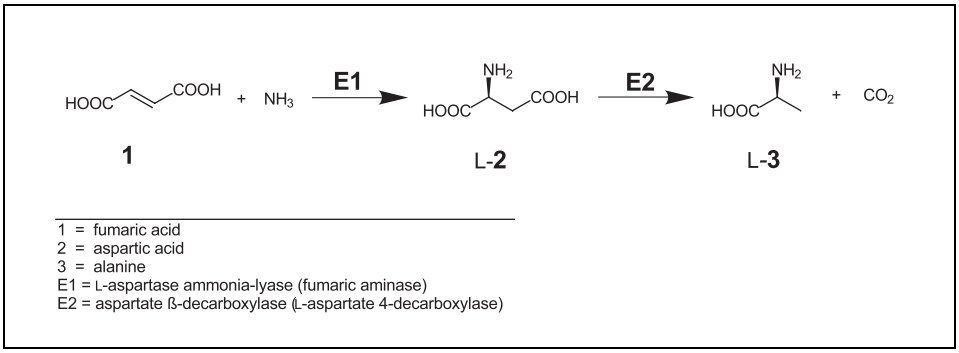


Fig. 4.1.1.12 – 2

- In this combination L-alanine can efficiently be produced by co-immobilization of *E. coli* and *Pseudomonas dacunhae* cells.
- If D,L-aspartic acid is used as a substrate for the reaction, L-aspartic acid is converted to L-alanine and D-aspartic acid remains unchanged in one resolution step. Both products can be separated after crystallization by addition of sulfuric acid. The continuous variant of the L-alanine and D-aspartic acid production is commercially in operation since 1988 (Fig. 4.1.1.12 – 5).

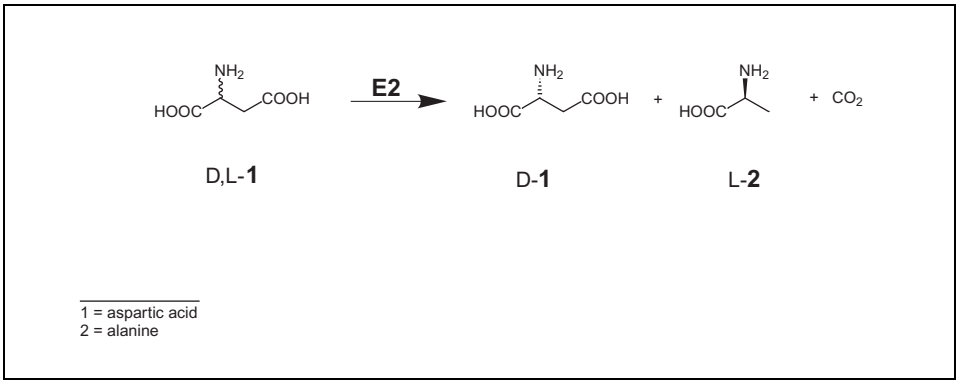


Fig. 4.1.1.12 – 3

3) Flow scheme

- Production of L-aspartic acid from fumarate in a two step biotransformation:

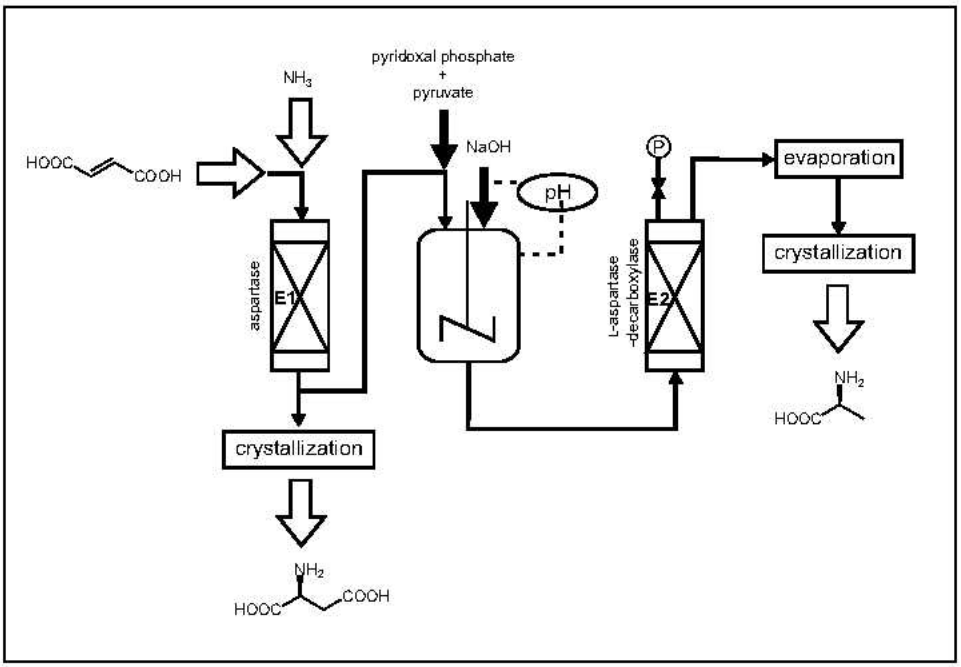


Fig. 4.1.1.12 – 4

- Production of L-alanine and D-aspartic acid:

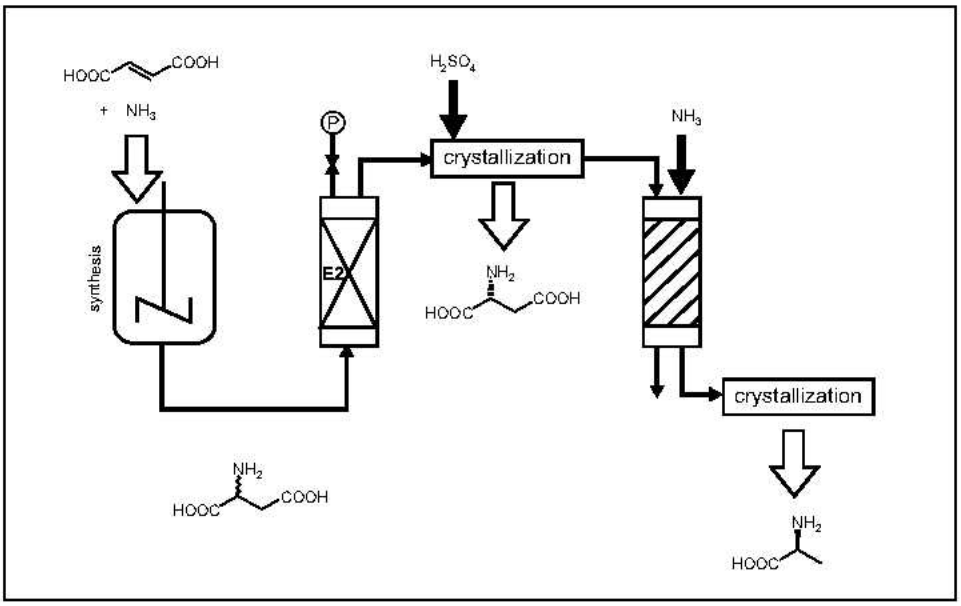


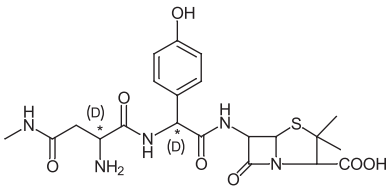
Fig. 4.1.1.12 – 5

4) Process parameters

conversion:	99 %
yield:	86 % after down stream processing
reactor type:	plug flow reactor
reactor volume:	1,000 L (each fixed bed column)
capacity:	114 $t \cdot a^{-1}$ D-aspartic acid; 61 $t \cdot a^{-1}$ L-alanine
residence time:	11 h
space-time-yield:	317 $g \cdot L^{-1} \cdot d^{-1}$ D-aspartic acid; 170 $g \cdot L^{-1} \cdot d^{-1}$ L-alanine
down stream processing:	crystallization
start-up date:	1982
production site:	Japan
company:	Tanabe Seiyaku Co., Ltd., Japan

5) Product application

- The products are used in infusion solutions and as food additives.
- D-Aspartic acid is an intermediate for the synthetic penicillin apoxycillin:



apoxicillin

Fig. 4.1.1.12 – 6

6) Literature

- Chibata, L., Tosa, T., Shibatani, T. (1992) The industrial production of optically active compounds by immobilized biocatalysts, in: Chirality in Industry (Collins, A.N., Sheldrake, G.N., Crosby, J., eds.) pp. 351–370, John Wiley & Sons Ltd, New York
- Furui, M., Yamashita, K. (1983) Pressurized reaction method for continuous production of L-Alanine by immobilized *Pseudomonas dacunhae* cells, J. Ferment. Technol. **61**, 587–591
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- Takamatsu, S., Umemura, J., Yamamoto, K., Sato, T., Tosa, T., Chibata, I. (1982) Production of L-alanine from ammonium fumarate using two immobilized microorganisms, Eur. J. Appl. Biotechnol. **15**, 147–152
- Tanaka, A., Tosa, T., Kobayashi, T. (1993) Industrial Application of Immobilized Biocatalysts, Marcel Dekker Inc., New York

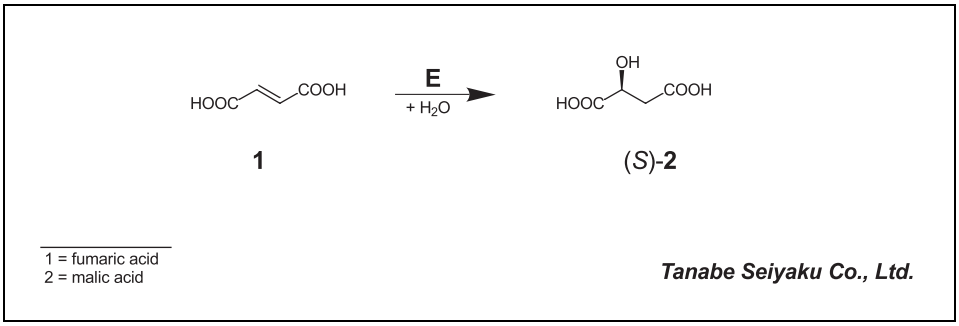

Fig. 4.2.1.2 – 1

1) Reaction conditions

[**1**]:	1.0 M, 116.1 g · L^{-1} [116.1 g · mol^{-1}]
pH:	6.5–8.0
T:	37 °C
medium:	aqueous
reaction type:	C-O bond cleavage (elimination of H_2O)
catalyst:	immobilized whole cells
enzyme:	(*S*)-malate hydro-lyase (fumarate hydratase)
strain:	*Brevibacterium flavum*
CAS (enzyme):	[9032–88–6]

2) Remarks

- The cells are immobilized on κ-carrageenan gel (160 kg wet cells in 1,000 L of 3.5 % gel).
- The side reaction (formation of succinic acid) can be eliminated by treatment of immobilized cells with bile extracts. Additionally, the activity and stability can be improved by immobilization in κ-carregeenan in the presence of Chinese gallotannin.
- The operational temperature of the immobilized cells is 10 °C higher than that of native cells.
- First the strain *Brevibacterium ammoniagenes* was used for the process. During optimization *Brevibacterium flavum* was discovered. The productivity with *B. flavum* is more than 9 times higher than with *B. ammoniagenes*.
- The cultural age of the cells also had a marked effect on the enzyme activity and the operational stability of immobilized cells.
- The same process is also employed by Amino GmbH, Germany, with the difference that they use the non-immobilized, native fumarase (see page 344).

3) Flow scheme

Not published.

4) Process parameters

conversion:	80 % (equilibrium conversion)
yield:	> 70 %
reactor type:	plug-flow reactor
reactor volume:	1,000 L
capacity:	468 $t \cdot a^{-1}$
enzyme activity:	17 $U \cdot mL(gel)^{-1}$ (37 °C); 28 $U \cdot mL(gel)^{-1}$ (50 °C)
enzyme consumption:	$t_{1/2}$ = 243 d (37 °C); $t_{1/2}$ = 128 d (50 °C)
start-up date:	1974
company:	Tanabe Seiyaku Co., Ltd., Japan

5) Product application

- The product is used as an acidulant in fruit and vegetable juices, carbonated soft drinks, jams and candies, in amino acid infusions and for the treatment of hepatic malfunctioning.

6) Literature

- Tosa, T., Shibatani, T. (1995) Industrial applications of immobilized biocatalysts in Japan, Ann. N. Y. Acad. Sci. **750**, 364–375
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- Wiseman, A. (1995) Handbook of Enzyme and Biotechnology, Ellis Horwood, Chichester
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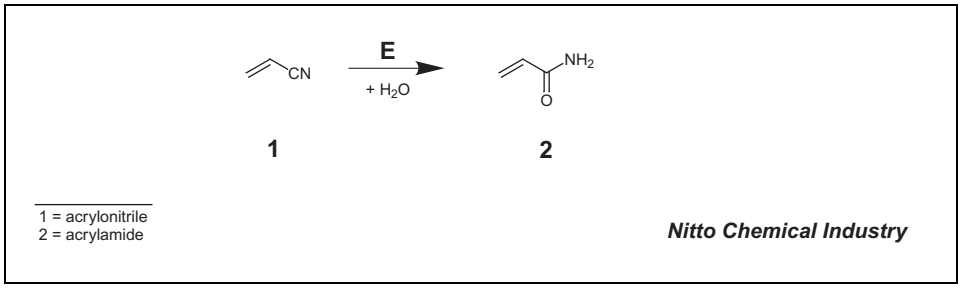


Fig. 4.2.1.84 – 1

1) Reaction conditions

[**1**]:	0.11 M, 6 g · L^{-1} [53.06 g · mol^{-1}] (fed batch)
[**2**]:	5.6 M, 400 g · L^{-1} [71.08 g · mol^{-1}]
pH:	7.0
T:	5 °C
medium:	aqueous
reaction type:	C-O bond cleavage by elimination of water
catalyst:	immobilized whole cells
enzyme:	nitrile hydro-lyase (nitrile hydratase, acrylonitrile hydratase, NHase, L-NHase, H-NHase)
strain:	*Rhodococcus rhodochrous* J1
CAS (enzyme):	[82391–37–5]

2) Remarks

- The chemical synthesis uses copper salt as catalyst for the hydration of acrylonitrile and has several disadvantages:
  1) The rate of acrylamide formation is lower than the acrylic acid formation,
  2) the double bond of educts and products causes by-product formations such as ethylene, cyanohydrin and nitrilotrispropionamide and
  3) at the double bonds polymerization occurs.
- The biotransformation has the advantages that recovering the unreacted nitrile is not necessary since the conversion is 100 % and that no copper catalyst removal is needed.
- This biotransformation is the first example of an application in the petrochemical industry and the successful enzymatic manufacture of a bulk chemical.
- Although nitriles are generally toxic some microorganism can use nitriles as carbon / nitrogen source for growth.
- Since acrylonitrile is the most poisonous one among the nitriles, screening for microorganisms was conducted with low-molecular mass nitriles instead.
- More than 1,000 microbial strains were examined.
- Two degradation ways of nitriles are known:

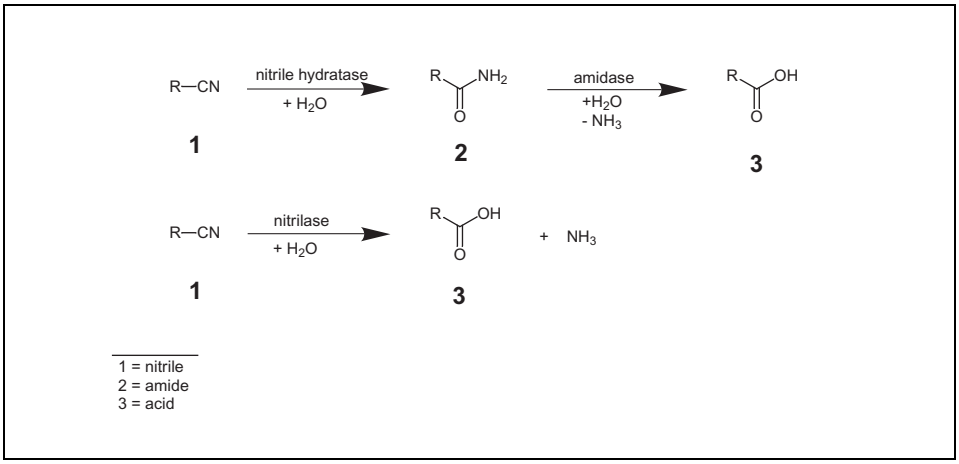


Fig. 4.2.1.84 – 2

- Microorganisms that produce amidases beside the nitrile hydratase are not suitable for the production of acrylamide without adding an amidase inhibitor.
- In the course of improvement of the biocatalyst for the production of acrylamide three main strains were used:

  1) *Rhodococcus* sp. N774

  2) *Pseudomonas chlororaphis*

  3) *Rhodococcus rhodochrous*
- The *Rhodococcus* sp. N774 strain was used for three years before the better *Pseudomonas chlororaphis* strain was found.
- The *Pseudomonas* strain cannot grow on acrylonitrile but grows on isobutyronitrile.
- The optimization of the *Pseudomonas* strain reveals that methacrylamide causes the greatest induction of nitrile hydratase. The addition of ferrous or ferric ions to the culture medium increases enzyme formation, no other ionic addition shows improvements, indicating that the nitrile hydratase contain Fe^{2+} ions as a cofactor.
- The growth medium can be optimized resulting in an amount of nitrile hydratase of 40 % of the total soluble protein formed in the cells.
- A problem during growth of *Pseudomonas chlororaphis* strain in the first optimized sucrose containing medium is the production of mucilaginous polysaccharides. These causes a high viscosity, resulting in difficulties during cell harvest.
- Using chemical mutagenesis methods (*N*-methyl-*N*′-nitro-*N*-nitrosoguanidine = MNNG) mucilage polysaccharide-non-producing mutants could be isolated. The following table shows the improvements (total activity increases 3,000-times) by optimizing the fermentation medium and by mutagenesis:

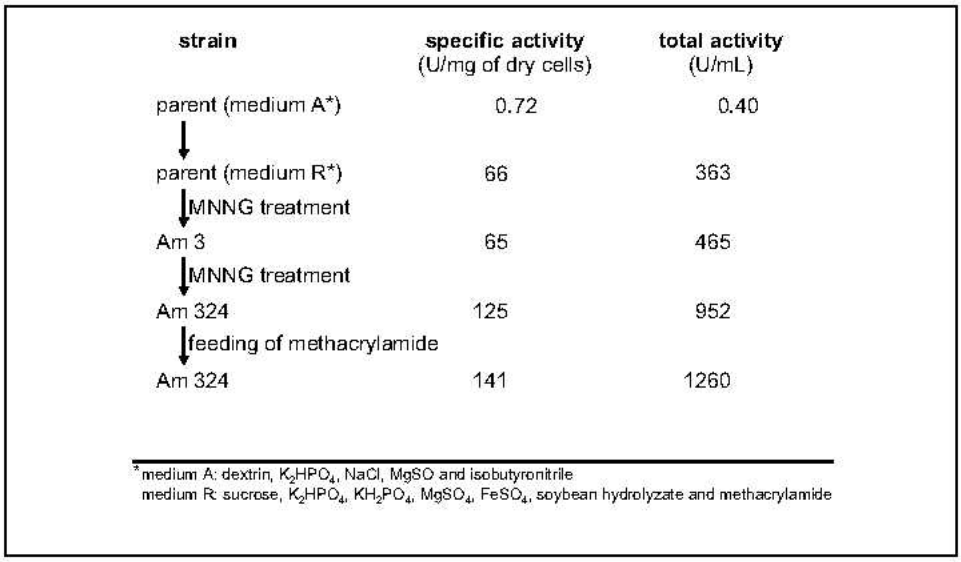


Fig. 4.2.1.84 – 3

- The third generation of industrial strains is the *Rhodococcus rhodochrous* J1 that produces two kinds of nitrile-converting enzymes, the nitrilase and nitrile hydratase. The latter one was found after optimization of fermentation medium.
- Addition of cobalt ions greatly increases nitrilase hydratase activity in comparison to Fe ions for the *Pseudomonas chlororaphis* strain.
- The difference in metal-ion cofactors can be ascribed to a small number of amino acids at their ligand-binding sites, resulting in higher stability of *Rhodococcus rhodochrous* J1 strain against reducing and oxidizing agents. Although the association of 20 subunits depresses the flexibility of the protein, it increases the stability.
- The strains forms two kinds of nitrile hydratases with different molecular weights and characteristics. The following table compares the different enyzmes and shows the advantages of the high-molecular mass hydratase:

	Pseudomonas chloraphis B23	*Rhodococcus rhodochrous* J1	
	(L-NHaseT)	low molecular weigh (L-NHaseT)	high molecular weigh (H-NHaseT)
molecular weight	100,000	130,000	520,000
subunit molecular weight	α25,000	α26,000	α26,000
	β25,000	β29,000	β29,000
number of subunits	4	4-5	18-20
absorption maxima (nm)	280,720	280,415	280,415
(415/280) / (720/280)	0.014	0.031	0.016
optimum temperature (°C)	20	40	35
heat stability	20	30	50
optimum pH	7.5	8.8	6.5
pH stability	6.0-7.5	6.5-8.0	6.0-8.5
V_{max} at 20°C (U·mg^{-1} protein)	1,470		1,760
K_M at 20°C (mM)	34.6		1.89

Fig. 4.2.1.84 – 4

- As inducer urea is used, which is much cheaper than methacrylamide for the *Pseudomonas chlororaphis.* This allows an increase in the amount of L-NHase in the cell free extract to more than 50 % of the total soluble protein.
- The nitrile hydratases act also on other nitriles with yields of 100 %. The most impressive example is the conversion of 3-cyanopyridine to nicotinamide. The product concentration is about 1,465 g · L^{-1}. This conversion (1.17 g · L^{-1} dry cell mass) can be named ‘pseudocrystal enzymation’ since at the start of the reaction the educt is solid and with ongoing reaction it is solubilized. The same is valid for the product which crystallizes at higher conversions so that at the end of the reaction the medium is solid again (see also Lonza, page 361).
- The following table shows some examples and the end concentrations of possible products for *Rhodococcus rhodocrous* J1 induced by crotonamide:

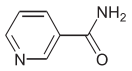
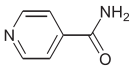
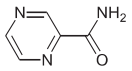
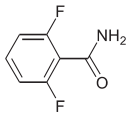
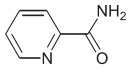
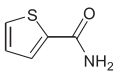
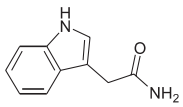
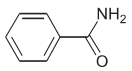
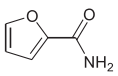
product	concentration
	1,465 g·L^{-1}
	1,099 g·L^{-1}
	985 g·L^{-1}
	306 g·L^{-1}
	977 g·L^{-1}
	210 g·L^{-1}
	1,045 g·L^{-1}
	489 g·L^{-1}
	522 g·L^{-1}

Fig. 4.2.1.84 – 5

- Since acrylamide is unstable and polymerizes easily, the process is carried out at low temperatures (about 5 °C).
- Although the cells, which are immobilized on polyacrylamide gel, and the contained enzyme is very stable towards acrylonitrile, the educt has to be fed continuously to the reaction mixture due to inhibition effects at higher concentrations.
- The following table summarized important production data for the discussed strains:

	Rhodococcus sp. NT774	*Pseudomonas chloraphis* B23	*Rhodococcus rhodochrous* J1
tolarence to acrylamide (%)	27	40	50
arylic acid formation	very little	barely detected	barely detected
cultivation time (h)	48	45	72
activity of culture ($U \cdot mL^{-1}$)	900	1,400	2,100
specific activit ($U \cdot mg^{-1}$ cells)	60	85	76
cell yield ($g \cdot L^{-1}$)	15	17	28
acrylamide productivity ($g \cdot g^{-1}$ cells)	500	850	>7,000
total amount of production ($t \cdot a^{-1}$)	4,000	6,000	>30,000
final concentration of acrylamide (%)	20	27	40
first year of production scale	1985	1988	1991

Fig. 4.2.1.84 – 6

3) Flow scheme

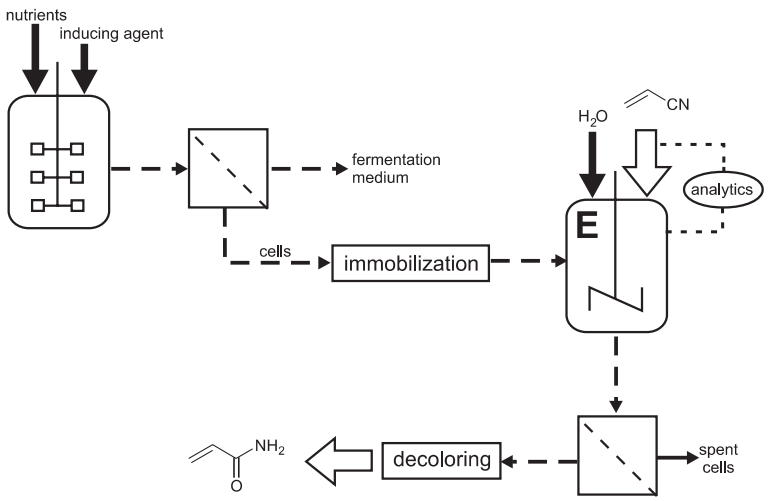


Fig. 4.2.1.84 – 7

4) Process parameters

conversion:	> 99.99 %
yield:	> 99.99 %
selectivity:	> 99.99 %
reactor type:	fed batch
capacity:	> 30,000 $t \cdot a^{-1}$
residence time:	5 h
space-time-yield:	1,920 $g \cdot L^{-1} \cdot d^{-1}$
down stream processing:	filtration and decoloring
enzyme activity:	76,000 $U \cdot g_{cells}$; 2,100,000 $U \cdot L^{-1}$
start-up date:	1991
production site:	Japan
company:	Nitto Chemical Industry Co., Ltd., Japan

5) Product application

- Acrylamide is an important bulk chemical used in coagulators, soil conditioners and stock additives for paper treatment and paper sizing, and for adhesives, paints and petroleum recovering agents.

6) Literature

- Nagasawa, T., Shimizu, H., Yamada, H. (1993) The superiority of the third-generation catalyst, *Rhodococcus rhodochrous* J1 nitrile hydratase, for industrial production of acrylamide, Appl. Microb. Biotechnol. **40**, 189–195
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1 2

1 = glucose
2 = fructose

Novo-Nordisk
Gist-brocardes
Miles Kali-Chemie
Finnsugar
Nagase

Fig. 5.3.1.5 – 1

1) Reaction conditions

[**1**]:	> 95 % dry matter
pH:	7.5 – 8.0
T:	50 – 60 °C
medium:	aqueous
reaction type:	isomerization
catalyst:	immobilized whole cells or isolated enzyme
enzyme:	D-xylose ketol-isomerase (xylose-isomerase, glucose-isomerase)
strain:	several, see remarks
CAS (enzyme):	[9023–82–9]

2) Remarks

- Glucose isomerase is produced by several microorganisms as an intracellular enzyme. The following table shows some examples:

Trade name	Microorganism	Company	Country
Sweetzyme	*Bacillus coagulans*	Novo-Nordisk	Denmark
Maxazyme	*Actinplanes missouriensis*	Gist-Brocades	The Netherlands
Optisweet	*Streptomyces rubiginosus*	Miles Kali-Chemie	Germany
Sweetase	*Streptomyces phaechromogenes*	Nagase	Japan

Fig. 5.3.1.5 – 2

- The commercially important varieties show superior affinity to xylose and are therefore classified as xylose-isomerases.
- Since the isolation of the intracellular enzyme is very expensive, whole cells are used instead. In almost all cases the enzymes or cells are immobilized using different techniques depending on strain and supplier.
- The educt is purified glucose (dextrose) syrup from the saccharification stage.

- Since these isomerases belong to the group of metalloenzymes, Co^{2+} and Mg^{2+} are required.
- The reaction enthalpy is slightly endothermic and reversible. The equilibrium conversion is about 50 % at 55 °C.
- To limit byproduct formation, the reaction time must be minimized. This can be done economically only by using high concentrations of immobilized isomerase.
- Several reactors are operated in parallel or in series, containing enzymes of different ages. The feed to a single reactor is controlled by the conversion of this reactor.
- The educt has to be highly purified (filtration, adsorption on charcoal, ion exchange) to prevent fast deactivation and clogging of the catalyst bed (for first part of process see page 231).
- Plants producing more than 1,000 t of HFCS (high fructose corn syrup) (based on dry matter) per day typically use at least 20 individual reactors.
- The product HFCS contains 42 % fructose (53 % glucose) or 55 % fructose (41 % glucose) (as dry matter).
- Glucose isomerases have half-lives of more than 100 days. To maintain the necessary activity the enzyme is replaced after deactivation of about 12.5 %.
- The reaction temperature is normally above 55°C to prevent microbial infection although enzyme stability is lowered.

3) Flow scheme

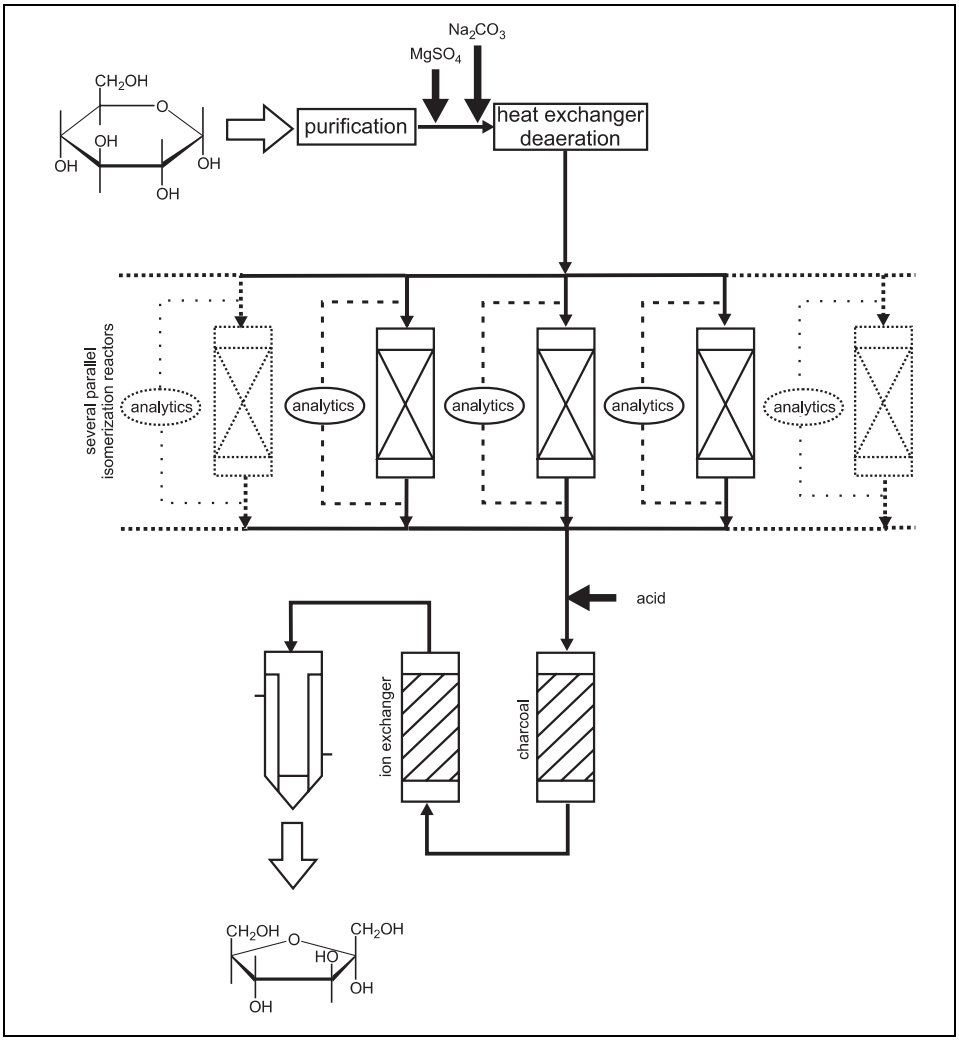

Fig. 5.3.1.5 – 3

4) Process parameters

reactor type:	continuous, fixed bed
reactor volume:	variable
capacity:	$> 7 \cdot 10^6$ t · a^{-1}
residence time:	0.17 – 0.33 h
down stream processing:	55 % fructose: chromatography; 42 % fructose: no down stream processing
enzyme consumption:	see remarks
start-up date:	1967 by Clinton Corn Processing Co. (USA); 1974 with immobilized enzyme

production site:	Denmark, the Netherlands, Germany, Finland, Japan
company:	Novo Nordisk, Gist-Brocades, Miles Kali-Chemie, Finnsugar, Nagase and others

5) Product application

- The product is named high-fructose corn syrup (HFCS) or ISOSIRUP.
- It is an alternative sweetener to sucrose or invert sugar in the food and beverage industries.
- The chromatographically enriched form (55 % fructose) is used for sweetening alcoholic beverages.
- 42 % HFCS obtained directly by enzymatic isomerization is used mainly in the baking and dairy industries.

6) Literature

- Antrim, R.L., Colilla, W., Schnyder, B.J. and Hemmingesen, S.H., in: Applied Biochemistry and Bioengineering **2** (Wingard, L.B., Katchalski-Katzir, E., Goldstein, L., eds.), pp. 97–183, Academic Press, New York
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there are many processes that are not included in our book.
If you know of any processes which you find worth to be shown in our book, please do not hesitate to contact us and to help us to increase the number of interesting processes in the field of biotechnology.

We will add your suggestion in the next edition of this book.

There are several possibilities how you can get in contact with us:

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2) Email

industrial.biotransformations@web.de

3) Please send us the following completed form

On the following pages you will find a blank form with all requested parameters. After completion please fax it to one of the above given fax numbers.

Thank you very much for your help!

Data Sheet for your process

enzyme:
strain:
EC-number:

Reaction scheme (if more complex please enclose an extra page)

1) Reaction conditions

concentration of starting material/product 1:
concentration of starting material/product 2:
concentration of starting material/product 3:
pH:
T:
medium:
catalyst (immobilizes, solubilized etc.):

2) Remarks (if necessary please enclose an extra page)

3) Flow scheme (if necessary please enclose an extra page)

4) Process parameters

conversion:
yield:
selectivity:
optical purity:
chemical purity:
reactor type:
reactor volume:
capacity:
residence time:
space-time-yield:
down stream processing:
enzyme activity:
enzyme consumption:
enzyme supplier:
start-up date:
closing date:
production site:
company:
contact address:

5) Product application (if necessary please enclose an extra page)

6) Literature (if necessary please enclose an extra page)