BIOMOLECULAR ENGINEERING SOLUTIONS FOR RENEWABLE SPECIALTY CHEMICALS

MICROORGANISMS, PRODUCTS, AND PROCESSES

EDITED BY
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WILEY

Biomolecular Engineering Solutions for Renewable Specialty Chemicals

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Preface

Biocommodity Engineering

Microorganisms have been realized as promising sources for production of biocommodities such as biofuels, pharmaceuticals, organic acids, amino acids, vitamins, biopolymers, surfactants, detergents, and enzymes. They offer several advantages over the conventional chemical processes including mild operating conditions, stereospecificity of the products, environmentally benign nature, and ecofriendly. Translating the bioproducts from laboratory to the industry remains a bottleneck. Biocommodity and biomolecular engineering approaches help in overcoming these limitations, developing new products, and improving the processes.

Considering the importance of the field, this book is specifically focused on potential technologies that can help in commercializing the processes. The objective of the book is to provide advanced technologies in producing different products using improved microorganisms/enzymes. This book will also discuss on improving the microbes or enzymes using protein engineering, metabolic engineering, and systems biology approaches for converting the wastes to value-added products.

Overall, this would be an ideal textbook for bioprocess, biorefinery, biomolecular, and biocommodity engineering courses for chemical, biochemical, and environmental engineering students. We have also included glossary and reasoning type questions at the end of each chapter. This book will also help the scientists to understand the advanced concepts in biomanufacturing. This book discusses the promising strategies that will help overcome the current limitations in the biochemical synthesis processes. The book will help the readers working in industry or research to know about the new ways for improving the efficiency of the biochemical synthesis processes.

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Engineered Microorganisms for Production of Biocommodities

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1.1 Introduction

As we are going toward becoming more developed, we tend to see our transition toward more sustainable resources and knowing and understanding the life form more. This leads to the use of the living system and engineer them to produce biocommodities such as fuels, polymers, hormones, therapeutic proteins and peptides, and neurotransmitters, which is termed as biocommodity engineering. It basically deals with the need of society. Biotechnology, genetic engineering, and biocommodity engineering can be combined to meet these needs. The foundation of biocommodity engineering lies in molecular biology, which is also the foundation of genetic engineering or recombinant DNA technology (rDT). Therefore, it can be said that these terms are interrelated to each other. The majority of the biocommodities consumed by humans were earlier isolated from plants and animals, posing the threat of activation of immune reactions in humans. So, the machinery of the synthesis of these biocommodities can be engineered in microorganisms.

Its main aim is to engineer microorganisms to get a high yield of the product, use cheap raw material as a substrate so that cost of the product can be minimized, easy downstream processing, increasing robustness of the microorganism, etc. All this can be achieved by genetically modifying the organisms using genetic toolkits. This chapter deals with the basics of genetic engineering, giving details about the enzymes used, transformation techniques, and how to select a transformant from non-transformants. Further sections compile the comprehensive data of the problems in the production of biopolymers, organic acids, and therapeutic proteins from conventional methods and development of mutant strains for the synthesis of these biocommodities. The last section of the chapter gives an insight about the biofuel production

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from photoautotrophic organisms such as cyanobacteria and microalgae, which utilizes sunlight and carbon dioxide as energy and carbon source, respectively.

1.2 Fundamentals of Genetic Engineering

The advent of genetic engineering, also called rDT, started in 1952 with the discovery of Hershey and Chase, stating DNA as the genetic material (Hershey and Chase, 1952). Cohen and Boyer in the early 1970s were the first to show that the genetic material of one organism can be easily expressed in the other. Genetic engineering (Figure 1.1), in general, is the process in which the DNA is extracted, modified, transformed into a host cell, and a new organism is formed. The DNA from the desired organism is extracted and purified. It is then cleaved using restriction enzymes to get the gene of interest from it. The DNA fragment is then ligated into a vector, which acts as a driving vehicle for the DNA molecule to the host cells. This chimeric DNA molecule is then transformed into the host cells, and selection procedure under suitable stress conditions takes place. Finally, after numerous generations, the organism growing in the stress conditions is said to be recombinant or genetically modified. Genetic engineering has emerged as a crucial step in the development of industrial bioprocesses.

Each and every organism has a different genetic (DNA) makeup, which in turn makes the whole organism different with respect to their carbohydrates, lipids, and proteins. This is due to the fact that DNA transcribes and translates to mRNA and proteins, respectively (central dogma). This makes DNA the choice for manipulation in genetic engineering as manipulating it leads to the generation of a whole new organism. This postulation gives rise to many other disciplines of genetic engineering like recombinant protein production, protein engineering, metabolic engineering, etc.

Every organism being different makes it difficult to use proteins and other biomolecules of one organism to the other. This was the main reason why proteins/enzymes from animals cannot be used by humans. Earlier, insulin was extracted from the pancreas of slaughtered pigs, posing a threat to human health. This leads to the discovery of the first recombinant product, Insulin, approved by the US Food and Drug Administration (FDA) in 1982 (Goeddel et al., 1979). Now synthetic insulin is easily being produced by yeast worldwide as *Escherichia coli* does not perform post-translational modifications required to form functional insulin.

Similarly, genetic engineering is now used to produce several other biocommodities. Modifying DNA and getting it expressed inside the host organism requires several steps, as shown in Figure 1.1 and the number of enzymes. These enzymes are explained in further sections with other requirements for genetic engineering.

1.2.1 DNA-altering Enzymes

The basis of rDT is the manipulation of DNA molecules with the help of molecular biology tools and biocatalysts. The available purified enzymes that can

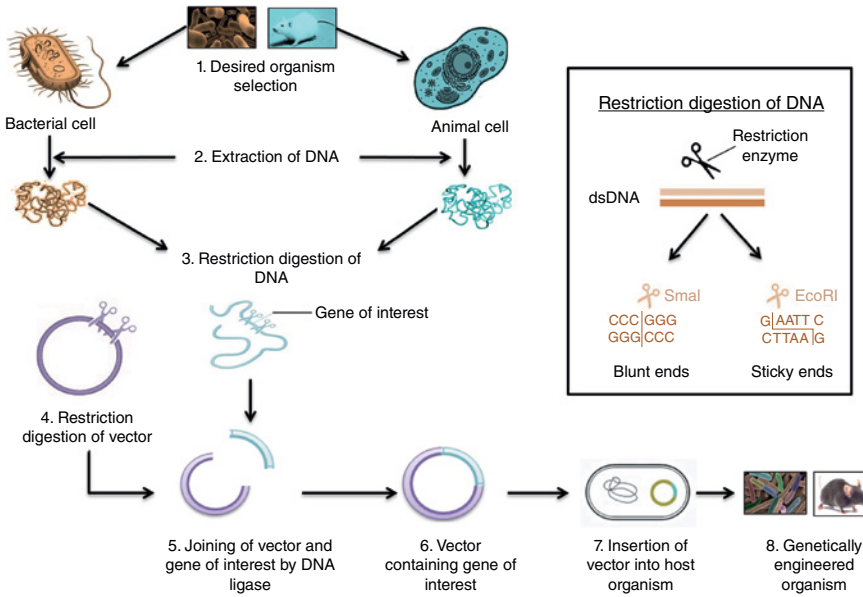

Figure 1.1 Basic steps of gene cloning.

manipulate DNA molecules with specific changes can be categorized in four broad classes: (i) DNA polymerases, (ii) nucleases, (iii) DNA ligases, and (iv) end-modification enzymes.

1.2.1.1 DNA Polymerases

DNA polymerases is the key enzyme in DNA replication driving the synthesis of new DNA strand from the parent DNA or RNA strand acting as a template. DNA polymerases require an oligo nucleotide (primer) for the initiation of DNA strand synthesis. DNA polymerase-I (DNA-dependent DNA polymerase) is widely studied polymerase and has both polymerization and exonuclease activity that can help in synthesizing new strand as well as the degradation for proof reading or repair and primer removal. DNA polymerase I (or Pol I) takes part in the process of prokaryotic DNA replication. It was the first DNA polymerase discovered by Arthur Kornberg in 1956 (Lehman et al., 1958). Pol I has three different enzymatic activities: A $5' \rightarrow 3'$ DNA-dependent DNA polymerase activity, a $3' \rightarrow 5'$ exonuclease activity that helps in proofreading, and a $5' \rightarrow 3'$ exonuclease activity mediating nick translation during DNA repair. Pol I having polymerase but lacking nuclease activity is called klenow fragments (Klenow and Henningsen, 1970; Jacobsen et al., 1974).

Taq DNA polymerase, a thermostable DNA polymerase isolated from *Thermus aquaticus* by Chien et al. (1976). It is frequently used in the polymerase chain reaction (PCR), to amplify small quantities of DNA. It has a functional $5' \rightarrow 3'$ exonuclease domain at the N-terminal, and $3'$–$5'$ exonuclease domain was changed so it is not functional. Optimum temperature for *Taq* pol activity is 75–80 °C, with a half-life of greater than 2 hours at 92.5 °C and minimum 9 minutes at 97.5 °C, and able to replicate a 1000 bp strand of DNA within 10 seconds at 72 °C.

1.2.1.2 Nucleases

Nucleases can cut or digest the DNA molecules either from one end or in middle by acting on phosphodiester bond, which forms the backbone of DNA (Nishino and Morikawa, 2002). Depending on the position of their digestion nucleases are of two types: exonucleases and endonucleases. Phosphodiester bonds present in the ends of the DNA are digested by exonucleases, removing nucleotides one at a time from either end. Whereas phosphodiester bonds present in the middle of the DNA strand are digested by endonucleases. Specificity of nucleases vary from source to source, *Aspergillus oryzae*'*s* endonuclease only cleaves single strands, whereas deoxyribonuclease I (DNase I), extracted from cow pancreas, cuts single as well as double-stranded DNA molecules. DNase I not being sequence specific cuts DNA at random interior phosphodiester bond, leading to production of mononucleotides and very short oligonucleotides mixture. Some of the examples of

nucleases are (i) Mung Bean Nuclease (isolated from mung bean sprouts) – a single-strand-specific DNA and RNA endonuclease which can degrade single strand overhangs from the end of DNA and RNA to make blunt ends. (ii) Nuclease S1 (isolated from *Aspergillus* sp.) – S1 nuclease is a single-strand-specific endonuclease that hydrolyzes single-stranded RNA or DNA into 5′ mononucleotides. The enzyme will hydrolyze single-stranded extensions in duplex DNA such as loops and gaps. *S1 Nuclease* is stable at 65 °C (Balabanova et al., 2012). (iii) Exonuclease III (isolated from *E. coli*) – removes single nucleotides from 3′ termini of the duplex DNA. It is generally used to make a set of nested deletions of the terminal of linear DNA strand. (iv) BAL31 nuclease (isolated from *Alteromonas espejiana*) – it is a 3′-exonuclease and removes nucleotides from both 3′-terminus of the two strands of linear DNA. (v) RNase H – it is an endonuclease that specifically hydrolyzes the phosphodiester bonds of RNA, when the RNA is hybridized to DNA (RNA-DNA) (Cerritelli and Crouch, 2009). (vi) RNase P – the specificity of this enzyme is to cleave other RNA molecules at the junction of single-stranded and the 5′ end of double-stranded regions of RNA (Guerrier-Takada et al., 1983).

Restriction endonuclease (RE) enzymes recognize and cleave the specific phosphodiester bond present in the DNA molecule (Smith and Welcox, 1970). Restriction enzymes are broadly classified into Type-I, Type-II, and Type-III. For their functioning, they require specific temperature, ATP, and divalent magnesium ions. On digestion of the DNA molecule they can produce both blunt and sticky end. Type I REs interact with unmodified target site in dsDNA. They are bifunctional enzymes having methylase and endonuclease in a single protein molecule. They cleave DNA around 1000 bp away from the recognition site. For their function, both ATP and Mg^{2+} are required. Type II REs are highly specific and cleave within or very near to the recognition sequence due to this reason type II are used widely in genetic engineering. They do not require ATP for the restriction digestion, only Mg^{2+} is required. Type III REs cleave dsDNA at defined positions and need ATP, Mg^{2+}. They cleave the DNA 24–26 bp away from the specific site.

1.2.1.3 Ligases

Ligases are considered to be molecular glue in rDT, used to join two DNA segments. It also has role in various aspects of molecular biology such as replication, recombination, and cloning. In the presence of ligase enzyme when two DNA fragments are mixed under a certain condition, base pairing between two fragments occur which results in sealing of two different DNA fragments to make a chimera (Pascal et al., 2004). It occurs due to covalent bonds formation between 2′-PO_4 group and 3′-OH group of adjustment strands.

1.2.1.3.1 Mechanism of Action The DNA ligation reaction has two main steps. First, the DNA ends collide with each other by chance and reside together for

enough time for the ligase to join them. For sticky overhangs, there is an additional reason; lower temperature stabilizes the hydrogen bonding between the complementary nucleotides, which really helps to join easily. DNA ligase catalyzes the joining of the 3′-OH to the 5′-phosphate via a two-step process. First, the AMP nucleotide, which is linked to a lysine residue in the enzyme's active site, is shifted to the 5′-phosphate. Then the AMP-phosphate bond is attacked by the 3′-OH, forming the covalent bond and discharging AMP. To allow the enzyme to conduct further reactions, the AMP in the enzyme's active site must be restocked by ATP. The DNA ligase enzyme has optimal activity at 25 °C so the ligation reaction is carried out at a temperature range of 16–25 °C. Normally, 1 hour at 16 °C is best for ligation but since bringing the DNA ends together is the least efficient part of the reaction favoring this by lowering the temperature to 4 °C can give even more efficiency. Two types of ligases are there that are used in genetic engineering. *E. coli* ligases which is generally used to join two sticky or cohesive ends. It is mainly used to catalyze a phosphodiester bond between duplex DNA-containing cohesive ends. It will not efficiently ligate blunt-ended fragments as much as it efficient for sticky end ligation. NAD^+ is required as a cofactor for the ligation reaction. T4 DNA ligase that is generally used for the ligation of blunt-ended DNA molecules. This enzyme can join blunt-ended termini as well as ends with cohesive overhangs (either 3′ or 5′ complementary overhangs). This enzyme will also function for the repair of single-stranded nicks in duplex DNA, RNA, or DNA/RNA duplexes. ATP is required as a cofactor for the ligation reaction. All the reactions are generally carried out at 16 °C. Sometimes PEG (polyethylene glycol) can be added that helps in fusion and increases the frequency of collision between two DNA molecules.

1.2.1.4 DNA-modifying Enzymes

1.2.1.4.1 Alkaline Phosphatase Alkaline phosphatase prevents self-ligation of DNA molecule (vectors and gene of interest) in genetic engineering experiment by dephosphorylating phosphate group on 3′ end of the DNA molecule. It is extracted from *E. coli* or calf intestine. The enzymes catalyze the hydrolysis of monoesters in phosphoric acid which can moreover catalyze a trans-phosphorylation reaction with large concentrations of phosphate acceptors. It can be used to prevent self-ligation of vectors in the cloning experiments because alkaline phosphate-treated DNA fragments lack the 5′-phosphophate in the terminal, required for the actions of DNA ligases (Tamás et al., 2002).

1.2.1.4.2 T4 Poly Nucleotide Kinase Polynucleotide kinase enzyme is exact reverse of alkaline phosphatase. It adds phosphate group to on the 5′ end of the DNA molecule and is extracted from phage-infected *E. coli*. The reaction with polynucleotides can be made reversible, which consents the exchange of the

γ-phosphate of ATP with the 5′-terminal phosphate of a polynucleotide, thus entangled the need of dephosphorylation the substrate DNA molecule with alkaline phosphatase (Cameron and Uhlenbeck, 1977).

1.2.1.4.3 Terminal Transferase Terminal deoxy-nucleotidyl transferase [Terminal transferase (TdT)] has the capability to transfer or add polynucleotide in 3′ terminus of the DNA molecule. It is extracted from calf thymus. TdT is a template impartial polymerase that catalyzes the addition of deoxynucleotides to the 3′ hydroxyl terminus of DNA molecules with none template strand. In rDT, blunt-ended DNA strands are required every now and then to make it cohesive via the addition of nucleotides at 3′ of one strand (Greider and Blackburn, 1985)

1.2.1.4.4 Topoisomerases Topoisomerases change the affirmation of covalently closed circular DNA molecule, such as plasmids with the aid of removing the supercoils present in the round DNA molecule and alternate the linking number. It performs a major role in replication and transcription when the DNA unwinds, doing away with positive and negative supercoils (Liu, 1989).

1.2.2 Vectors

A vector is a DNA molecule, which act as molecular transporter, that can replicate autonomously in an appropriate host cell and into which the gene of interest (a foreign gene) is inserted. Insertion of a foreign gene into the vector is aiming either to get numerous copies of the gene of interest or obtaining a product from this gene. Vector is basically of two types: cloning vector and expression vector. Characteristics of an ideal cloning vector are mentioned below:

1) It should have origin of replication, able to replicate autonomously.
2) It should be easily isolated and purified.
3) The vector should have suitable selection marker genes that will allow easy selection of the transformed cells from nontransformed cells.
4) For gene transfer, vector should have the ability to integrate either itself or the DNA insert it carries into the genome of the host cell.
5) The cells transformed with the vector containing the DNA insert should be easily identifiable and selectable from those transformed cells having unaltered vector.
6) Unique restriction digestion sites should be present where gene of interest can be inserted.

Expression vectors are different from cloning vectors. They are designed in such a way that they should have a promoter sequence to express the gene. Expression plasmid has all the information regarding transcription which is followed by

translation to synthesize proteins from mRNA. Expression vectors should have the followings gene sequences: A strong promoter for the initiation, termination codon, adaptation of distance between the promoter and cloned gene, incorporated transcription termination sequence, and a compact translation initiation sequence.

Number of cloning vectors are also present which includes plasmid, cosmid, phage vectors, bacterial artificial chromosomes (BACs), and yeast artificial chromosomes (YACs). All the vectors mentioned have different incorporation capacity for foreign DNA. Plasmids are extra chromosomal circular double-stranded DNA replicating elements present in bacterial cells. Plasmids size is ranging from 5.0 to 400 kb. Plasmids are inserted into bacterial calls by a transformation. Plasmids can incorporate an insert size of up to 10 kb DNA fragment. Bacteriophage infects bacteria and has a very unique mechanism for delivering its genome into bacterial cell. Hence, it can be used as a cloning vector for larger DNA segments insertion. Phage vectors can insert DNA fragments of size up to 20 kb. BACs are simple plasmid vectors that are designed to integrate large DNA fragments of size 75–300 kb (Kim et al., 1996b). BACs have antibiotic resistance marker genes and a very stable OriC that promotes the distribution of plasmid after bacterial cell division and maintaining the plasmid copy number to one or two per cell. YACs are yeast expression vectors. A very large DNA fragments sizes ranging from 100 to 3000 kb can be cloned using YACs (Riley et al., 1990). YACs have an extra benefit over BACs in expressing eukaryotic proteins which need posttranslational modifications.

1.2.3 Incorporation of Modified DNA into Host

After engineering the DNA with the help of restriction enzymes, ligase, and getting it attached to the vector, it is ready to go inside the host cells. How this vector that is a recombinant vector now is made to enter inside the host cells is explained in brief.

1.2.3.1 Introducing Recombinants into Prokaryotes

The most frequently used model organism *E. coli* has the potential for becoming a host for cloning purposes. A huge number of *E. coli* vectors are also available. Transformation is the most used and effortless method used to carry the recombinant DNA inside the host cells. There are several other methods some of which are discussed.

1.2.3.1.1 Transformation Transformation is the most commonly used method in cloning. In this simple technique, the DNA from the surrounding is taken up by the competent cells. Some of the host cells like *E. coli*, yeast, and mammalian cells are not naturally competent. These cells are made competent chemically so that they can easily take external DNA. Calcium and magnesium chloride in specific concentrations in cold condition can make *E. coli* cells competent.

1.2.3.1.2 Transduction Using phage particles for transferring foreign DNA into the host cells is called transduction. Therefore, it is also called as phage-mediated gene transfer. It can be classified into generalized and specialized transduction. Generalized transduction involves phage particles attacking host cells and as the assembly of phage particles takes place host DNA gets packed inside them. When this phage particle infects another host cell, the DNA gets integrated into the host genome through recombination. While in specialized transduction, the phage genes get integrated with host genome and lysogenic cycle takes place. When some stimulus is given, the phage particles carry more than one genes from the host. These phage particles can infect a naive host cell by which the gene of interest can be inserted into the host genome.

1.2.3.1.3 Conjugation Conjugation is the procedure of movement of hereditary material from a donor cell to a beneficiary cell when they are in close contact. It was found by Lederberg and Tatum in 1946 who indicated that two distinct strains of microscopic organisms with various development prerequisites could trade qualities. It was found by Lederberg and Tatum in 1946 who indicated that two distinct strains of microscopic organisms with various development prerequisites could trade qualities. They derived that the bacterial cells must cooperate with one another so as to move the genetic material and the procedure is currently known as sexual conjugation by direct contact. A section (not often all) of the donor's chromosome recombines with the recipient chromosome through homologous recombination. For conjugation the key characteristic is the physical contact. The two cells should be in close proximity. Recipients containing donor DNA are called transconjugants. Genetic exchange through conjugation is unidirectional. Fertility plasmid (F plasmid) plays an important role in conjugation. The donor cells are F+ and have F plasmid, while the recipient is not having F plasmid and is F−.

1.2.3.2 Introducing Recombinants into Eukaryotic Hosts

The usage of bacterial hosts for genetic engineering laid the muse of rDT era; however, researchers have additionally had high-quality avocation in genetically engineering eukaryotic cells, especially the ones of plant life and animals.

1.2.3.2.1 Transfection Every other technique to transfer rDNA into host cells includes mixing the foreign DNA with charged substances like calcium phosphate, cationic liposomes, or diethylaminoethyl (DEAE)-dextran is a polycationic derivative of the carbohydrate polymer dextran and covering on recipient host cells. Host cells take in the DNA in a technique called transfection.

1.2.3.2.2 Electroporation An electric current is used to create transient microscopic pores in the recipient host cell membrane allowing rDNA to enter.

1.2.3.2.3 Microinjection Exogenous DNA can also be brought immediately into animal and plant cells without using eukaryotic vectors in the system of microinjection, foreign DNA is directly injected into recipient cells the use of a quality micro syringe under a section contrast microscope to useful resource imaginative and prescient.

1.2.3.2.4 Biolistics An excellent technique that has been developed to introduce foreign DNA into in particular plant cells is by means of using a gene or particle gun. Microscopic particles of gold or tungsten are covered with the DNA of interest and bombarded onto cells with a device just like a particle gun. Subsequently, the time period biolistic is used.

1.2.4 Selection of Transformants

The artwork of cloning is to determine the precise transformed cell that consists of the cloning vector with the gene of interest (referred to as a recombinant cell). It is also very important to find the recombinant cells from the culturing transformed petri plate where nonrecombinant (containing the vector without insert DNA) transformed cells are also present. If the final goal is achieved, that means our desired gene of interest is inserted into the vector then it will be called as clone. Direct selection and selection from gene library are the two basic concepts for selection procedures. Direct selection involves designing of experiment in such a way that transformation takes place only for the clones containing specific gene of interest. Selection happens on the plating-out stage of the transformation where the colonies are grown on agar plates. It is the preferred technique because of the ease and unambiguous in nature. Selection from gene library involves initial "shotgun" cloning test, to produce a clone library representing all or most of the genes present inside the cell, observed through evaluation of each individual clones to identify the correct one.

1.2.4.1 Direct Selection

Majority of cloning vectors are designed in such a way that inserting gene of interest in them inactivates the gene already present in the vector. This leads to insertional inactivation of gene. Two of the common examples of insertional inactivation, i.e., direct antibiotic resistance screening and blue white screening are discussed below.

1.2.4.1.1 Direct Antibiotic Resistance Screening Cloning vectors are planned in such a way that insertion sites are present in between the genes in the vectors, often antibiotic resistance gene. Vector carrying ampicillin resistance gene confers resistance to ampicillin to the cells in which it will be transformed to. Transformed

cells when grown in agar plates containing ampicillin shows resistant to the antibiotic and are capable to grow. But this does not guarantee the presence of gene of interest in the vector or the vector is recombinant. Ampicillin resistance gene become disrupted in vector having gene of interest. Thus, the transformed cells having self-ligated vector grows on ampicillin agar plate, whereas the cells having recombinant vector does not show growth. The recombinant cells can be selected from the non-recombinant one through replica plating method.

1.2.4.1.2 Blue–White Color Screening Blue-white screening is also based on insertional inactivation of a gene giving blue color compound as a visual representation of the result. The gene that plays a key role in this screening is lacZ gene which codes for the enzyme β-galactosidase is under the control of the inducible lac promoter. lacZ gene is repressed by lac repressor and is induced by IPTG (isopropyl-β-D-thiogalactopyranoside). The enzyme formed can breakdown X-gal (five-bromo-4-chlore-3-indolyl-β-D-galactopyranoside) to give a blue color compound.

Insertional inactivation of lacZ gene due to insertion of desired gene prevents the formation of the enzyme, breakdown of X-gal, and hence no blue color is obtained. The cells are plated on agar plates having IPTG, X-gal, and an antibiotic giving combination of blue and white colonies. The transformed cells with religated vector having functional lacZ gene shows blue color colonies. Whereas the cells with gene of interest and disrupted lacZ gene forms colorless colonies. Further the colorless colonies can be picked and plated again.

1.2.4.2 Identification of the Clone from a Gene Library

Screening a positive clone from gene library involves typical techniques such as nucleic acid hybridization, functional screening, and chromosome walking. Nucleic acid hybridization needs preliminary information of both the gene of interest and area of gene to be cloned. Contrary, functional screening entails the information about the vector.

1.2.4.2.1 Nucleic Acid Hybridization Nucleic acid hybridization utilises short synthetic radiolabeled oligonucleotides called as probe. These probes are used for locating complementary sequences in individual cells or phages containing an insert. The success of the hybridization experiment relies upon the probe. Therefore, probe should be designed with care and should have some part complementary to the sequence of the cloned gene. If there is no information about the gene, then the sequences from the related protein can be derived and degenerate probes can be synthesized to be used. So, in this technique first the colonies are transferred to a nylon membrane and then lysis of cells will be done. The released DNA will be denatured through an alkali treatment. The denatured

single-stranded DNA will be heat treated so that they could bind to the membrane. After that the membrane will be submerged into solution containing DNA probes and incubated for a certain time to get hybridized by complementary base pairing. Finally, the hybridized radiolabeled probes will be identified by autoradiography.

1.2.4.2.2 Functional Screening If the gene encodes for a product that has a specific role, then the expression library is by means of cloning DNA (cDNA in case of eukaryotes). These libraries are prepared using unique cloning vectors for expression of cloned genes. The prepared library can be used to detect the product or the clones generated by using antibodies or other ligands. These antibodies or ligands binds with the encoded product and clones.

1.2.4.2.3 Chromosome Walking Usually a genomic clone may not be includ all of the sequences for a specific gene so it is required to isolate overlapping clones that protect the genomic segment of interest. This process is known as chromosome walking.

1.3 Beneficial Biocommodities Produced Through Engineered Microbial Factories

Earlier biocommodities (up to some extent now also) were extracted from plants and animals leading to their overexploitation (Brower, 2008). Plants are the great source of secondary metabolites that have complex structures and used by humans, e.g. medicines, terpenes, flavoring and coloring agents, etc. (Facchini et al., 2012). Their yields are greatly affected by the time, climate, and other factors, which affect circadian rhythm of the plants (Li and Vederas, 2009). Moreover, the yields cannot satiate the current demand of the population. Being structurally complex in nature relying on chemical synthesis of the secondary metabolites is not feasible. Chemical synthesis also leads to toxicity of the end products and environmental pollution (Du et al., 2011). To overcome these challenges, the principles of genetic engineering explained in the earlier section are used to engineer microorganisms to become microbial cell factories to produce essential biocommodities such as bioplastic, biofuels, and antibiotics important to human welfare. Advancement in the knowledge of metabolic pathways for synthesis of secondary metabolites and full genome sequencing made fabrication of microbial cell factories easier (Keasling, 2012). Fabrication generally involves introduction of the gene of interest in the host organism so the heterologous pathway begins in it. It also includes exclusion of native pathways that are not crucial for the growth of the host organism (Tyo et al., 2007). Apart from the development of genetically modified strains, optimum conditions according to the product of interest and

growth of strains is to be provided in a contained environment (bioreactor). This is done to observe the full potential of the strain (Kiss and Stephanopoulos, 1992).

1.3.1 Biopolymers

Any substance that is made up of repeating monomeric units is called as polymer. The polymers that are naturally synthesized by the living organisms are called biopolymers such as proteins, polysaccharides, polyphenols, lipids, polyamides, and polyhydroxyalkanoate (PHA) (spanning from liquid solutions to bioplastics). They are biodegradable hence eco-friendly. Naturally occurring polymers form the basis for life. Polynucleotides (DNA and RNA) and proteins (amino acids) are present in every living form. Starch is the reserve form of carbohydrate in which plants store food and is the main carbohydrate in the human diet. Lipids store energy and its bilayer acts as a barrier in the living cells. Whereas cellulose is the primary component in the cell wall of plant kingdom providing structural rigidity. Microbial synthesis of biopolymers is known from old age. Louis Pasteur and Van Tieghem, in the mid nineteenth century, discovered that *Leuconostoc mesenteriodes* synthesize dextran. 40 years after, and PHA reserves were found in *B. megaterium*, which serves as a basis for bioplastics to date. As stated earlier, understanding in the molecular pathways for synthesis of biopolymers leads to the engineering of microorganism for the production of custom made, altered biopolymers (Rehm, 2010).

Biopolymers have a wide range of application in biomedical, food industry, packaging to cosmetics, electronics, etc. Its biomedical applications are in drug delivery, tissue engineering, wound healing, and medical and dental devices. Porous gelatin, electrospun poly (lactic-co-glycolic acid), etc. are used as a scaffold for this purpose (Van Vlierberghe et al., 2007; Zhao et al., 2016). Each biopolymer has a specific characteristic to be used for particular conditions. Poly (D,L-lactides) scaffolds are used for bone engineering, whereas poly (trimethylene carbonate) scaffolds are used for soft tissue engineering. Poly(L-lactide-*co*-glycolide) scaffolds provides good adhesion and proliferation (Ulery et al., 2011). Some of the commercially available scaffolds are BioFiber™, an orthopedic scaffold made of a leno weave of P4HB monofilaments (Tornier) for tendon repair, BioFiber®-CM, an orthopedic scaffold made of P4HB coated with bovine collagen (Tornier) for tendon repair, etc. Use of plastic in packaging industry is growing day by day leading to their disposal problem. Plastic films production takes one-third portion of the total plastic production. Cellulose and starch are widely used for this purpose after some modifications. The material to be used for packaging should have properties similar to polyethylene terephthalate (PET) i.e. barrier, sealing and thermal properties. Standard cosmetics have nonbiodegradable polymers in them such as polyethylene microparticles in face and body scrubs. These microparticles

eventually enter in the ecosystem through waste water stream. Biodegradable poly lactic acid (PLA) powder can be used in exfoliating agents. Chitin absorbs UV light and effectively used in sunscreen lotions. Also, PLA mixed with titanium dioxide and zinc oxide powder shows photocatalytic activity.

Some of the biopolymers having industrial application are highlighted in the section below with their structures shown in Figure 1.2.

1.3.1.1 Cellulose

Cellulose is the major biopolymer found in nature and commercially extracted from wood (Klemm et al., 2005). It is homopolysaccharide of glucose linked by β-1,4 glycosidic bonds (Cannon and Anderson, 1991). In 2015, the value of cellulose market calculated was US$20.61 billion and is estimated to reach US$48.37 billion by 2025 (Cellulose Fiber Market Size and Share Industry Report, 2014–2025 market [Accessed 20 December 2017]). To meet such high demands overcutting of trees are being done leading to imbalance in nature and global warming. Bacterial cellulose (BC) is the alternative and green approach toward it which is biodegradable, nontoxic, and biocompatible. Apart from this, BC can be used directly in its native form as it is free from contaminants such as lignin, pectin, hemicellulose, and other constituents of lignocellulosic materials (Rahman and Netravali, 2016). There are several bacterial strains that synthesize BC specially the acetic acid bacteria group as they are generally recognized as safe (GRAS) such as *Komagataeibacter xylinus, Komagataeibacter hansenii, Komagataeibacter medellinensis, Komagataeibacter nataicola, Komagataeibacter*

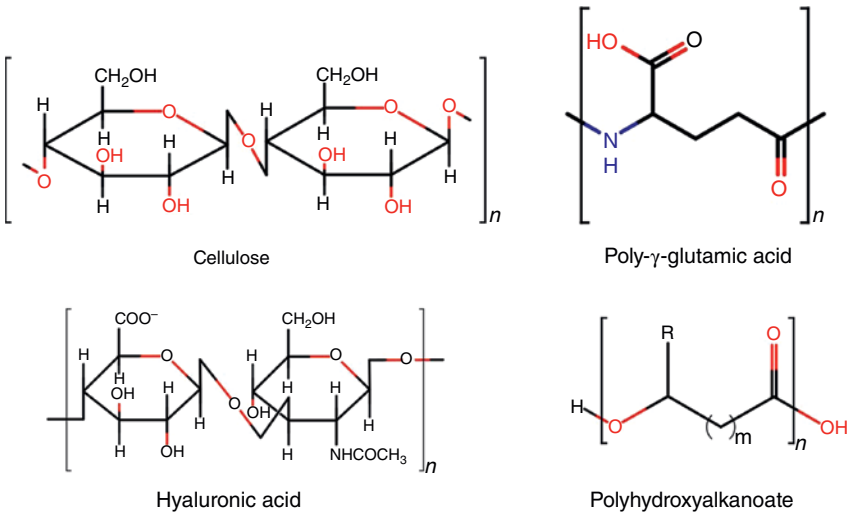

Figure 1.2 Representative chemical structures of biopolymers.

oboediens (Škraban et al., 2018; Castro et al., 2012,2013). Bacterial cellulose synthase (Bcs) is the primary enzyme for cellulose synthesis which adds glucose monomers to the growing chain. There are two subunits BcsA and BcsB necessary for the formation of polysaccharide chain (Römling and Galperin, 2015). Commercial usage of BC and low yields from the strains producing BC lead to the development of microbial cell factories. These cell factories overexpress genes essential for BC like cmc (carboxymethylcellulose), ccp (cellulose complementing factor protein), cesAB, cesC, cesD, bgl, bcsABCD (BC synthase operon), etc. into the host organism to increase the yields and crystallinity of BC.

Gluconacetobacter xylinus six genes *cmc–ccp–cesAB–cesC–cesD–bgl* were overexpressed in *Synechococcus* sp. PCC 7002 and resulted in very high-yield production of extracellular type-I cellulose (Zhao et al., 2015). BC synthase operon (bcsABCD) from *Gluconacetobacter hansenii* was engineered in *E. coli* with its upstream operon (*cmc*ax and *ccpAx*) giving BC of 1000–3000 μm and a diameter of 10–20 μm (Buldum et al., 2018). Cellulose synthase D subunit (bcsD) increases the crystallinity structure of BC, but not the yield. *G. xylinus* bcsD when engineered in *E. coli* synthesizes BC with high crystallinity. FTIR results showed crystallinity index of 0.84, which was 17% more than the wild-type strain (Sajadi et al., 2017). Oxygen plays an important role in the synthesis of BC. *G. xylinus* was engineered with *Vitreoscilla* hemoglobin (VHb)-encoding gene *vgb*. vgb help cells to grow in hypoxic conditions. The mutant strains showed significant increase in BC in oxygen tension also (Liu et al., 2018). Recently, *Komagataeibacter* sp. nov. CGMCC 17276 genome was completely sequenced. BC operon genes were aligned with other BC producers to find sequence similarity. Apart from this growth rate, substrate utilization, BC production, etc. were evaluated. This gives future opportunity to engineer this strain for better BC synthesis (Jang et al., 2019).

1.3.1.2 Poly-ϒ-glutamic Acid

Poly-ϒ-glutamic acid is a naturally occurring, abundant poly amino acid which is water-soluble, anionic, biodegradable, and edible biopolymer produced primarily by *Bacillus subtilis*. Glutamic acid can be L- and D- in nature linked by ϒ-amide bond (Cao et al., 2018). Due to the presence of ϒ-amide linkage, it is resistant to cleavage by proteases (Candela and Fouet, 2006). It is gaining importance because of being the potential candidate for drug delivery, cryoprotectant, thickening agent, biopolymer flocculant, bioabsorption of heavy metals, etc. (Bhattacharyya et al., 1998; Shih and Van, 2001). It is also widely used in skin serums in combination with vitamin C to increase skin elasticity and making it smooth (Tanimoto, 2010; BEN-ZUR and GOLDMAN, 2007). Predominantly, *Bacillus* sp., such as *Bacillus licheniformis, Bacillus subtilis, B. megaterium, Bacillus pumilis, Bacillus mojavensis*, and *Bacillus amyloliquefaciens*, are producer of PGA. It is synthesized in ribosome independent manner. Glutamate is the precursor of PGA,

which is derived from glutamic acid biosynthesis or glutamate transportation. In turn, α-keto glutaric acid serves as a precursor for glutamate in tricarboxylic acid cycle (TCA) also known as Krebs cycle. The reaction is catalyzed by glutamate dehydrogenase in absence of glutamic acid, while 2-oxoglutarate aminotransferase in presence of glutamic acid (Stadtman, 1966; Holzer, 1969). Furthermore, the glutamate units are joined together to produce PGA through racemization, polymerization, and anchoring or releasing. This can be studied in detail in review by Najar and Das (2015). PGA synthetase (Pgs), a membrane associated enzyme polymerizes glutamate to PGA. Genes encoding Pgs are different in different *Bacillus* species. Like Pgs is encoded by four genes (pgsB, C, A, and E) in *B. licheniformis* and *B. amyloliquefaciens*, capB, C, A, and E, in *B. anthraci*, while in *B. subtilis* they are *ywsC*, *ywtABC*. Role of pgsBCA operon was shown in *B. subtilis* for PGA production. Mutants with disrupted pgsBCA did not showed PGA synthesis. While the xylose-induced pgsBCA operon started PGA synthesis (Ashiuchi et al., 2006). Sometimes the overexpression of pgsBCA leads to decrease in PGA production as seen in mutants of *B. amyloliquefaciens* (Feng et al., 2015).

Under nutrient deprivation conditions PGA is sometimes hydrolyzed by PGA hydrolases. Deletion of PGA hydrolases can increase the yield of PGA. Depending on where the PGA hydrolase will cleave PGA, it can be (i) PgdS (the γ-DL-glutamyl hydrolase) cleaves the γ-glutamyl bond between D- and L-glutamic acids residues of γ-PGA; (ii) the D/L-endopeptidase hydrolyzes the peptide bond between the glutamic acid residues; and (iii) the γ-glutamyltransferase (GGT) possesses a powerful exo-γ-glutamyl hydrolase activity. The GGT is different from PgdS and D/L-endopeptidase as it hydrolyzes the γ-PGA from the N-terminal to release both D- and L-glutamic acids (Cao et al., 2018).

1.3.1.3 Hyaluronic Acid

Hyaluronic acid (HA) also called as hyaluronan is a structural biopolymer produced by extracellular matrix of animal and human epithelial, neural, and connective tissues. HA is copolymer disaccharide between N-acetyl-*o*-glucosamine and *o*-glucuronic acid linked by $\beta 1 \rightarrow 3$ and $\beta 1 \rightarrow 4$ glycosidic bonds. Due to the water retention properties of HA, it is widely used in cosmetics as it binds water to collagen and makes skin look plumper and more hydrated. Hyaluronic acid synthase (HAS-a) is the integral enzyme present in the plasma membrane of mammalian cells and bacteria and synthesizes HA (Chen, 2002). It has several isoforms depending on the organism in which it is forming. Traditionally, it was extracted from animals that pose threat of infection from the animal source. Its bacterial production started with using *Streptococcus zooepidemicus* with a production titer value of 5–10 g/l in batch fermentation. Later, *S. zooepidemicus* was considered to be a human pathogen and was not GRAS. HA being viscous in

nature pose problems in fermentation leading to lower yields. This leads to optimization of fermentation conditions and improvement in the strains genetically (Chong et al., 2005).

Hyaluronidase enzyme activity in the strains is not beneficial as it depolymerizes HA. Kim et al. (1996a) selected *Streptococcus equi* chemically derived mutant strain (after nitroglycerine treatment) which is nonhemolytic, hyaluronidase-negative in nature, thus producing high molecular weight HA. Controlled expression of hyaluronidase is beneficial too as it reduces the molecular weight and viscosity of HA. Expressing tuaD, gtaB, glmU, glmM, and glmS genes plus glycolytic pathway genes in *B. subtilis* with controlled expression of hyaluronidase by N-terminal engineering increased production from 5.96 to 19.38 g/l (Jin et al., 2016). HA produced from this strategy was low in molecular weight in the range of 2.20×10^3 to 1.42×10^6 Da. One of the reasons *Streptococcus* sp. were not considered as GRAS is that they produce streptolysin which causes haemolysis. *Streptococcus equisimilis* CVCC55116 mutated by ultraviolet ray combined with $^{60}C_o$-γ ray treatment produces 174.76 mg/L HA without producing streptolysin (Chen et al., 2012).

In an attempt to find the GRAS producers of HA, *B. subtilis*, *Lactococcus lactis*, *E. coli*, *Pichia pastoris, Corynebacterium glutamicum*, etc. were engineered to express HAS-a, b (UDP-glucose 6-dehygrogenase), c (glucose-1-P uridyltransferase), d and e gene, which using precursors *N*-acetyl-*o*-glucosamine and *o*-glucuronic acid forms HA (Jia et al., 2013; Kaur and Jayaraman, 2016; Jeong et al., 2014). *Lactococcus lactis* is not the natural producer of HA due to absence of HAS gene. The attempt to express HAS gene in *L. lactis* was performed by number of research groups by using non-integrative plasmids pRKN, pNZ8148, etc. (Prasad et al., 2010; Chien and Lee, 2007; Prasad et al., 2012). Strains expressing only HAS-a and b genes give a production of 0.097 g/l, while the one expressing HAS-c gene also gives the maximum production of 0.234 g/l (Prasad et al., 2010). This indicates the importance of HAS-c gene expression for HA production. This is due to the fact that HAS-c diverts the flux of glucose-1-phosphate toward UDP-glucose synthesis, which is critical for HA synthesis. Genome integrative approach is also carried in *Lactobacillus* sp. giving twofold increase in the production and molecular weight (MW) of HA in comparison to plasmid bearing strains (Hmar et al., 2014). It was explained by the precursors (*N*-acetyl-*o*-glucosamine/*o*-glucuronic acid) and HAS-a/HAS-b mRNA ratio. In genome-integrated strains HAS-b expression was high compared to HAS-a, which gives greater availability of precursors to bind to HA synthase leading to high MW HA. *E. coli* the most common microbe to be engineered has a wide variety of genetic tools available to be modified. It was the first microorganism in which human HAS was successfully expressed (Hoshi et al., 2004).

1.3.1.4 Polyhydroxyalkoate

Poly-3-hydroxyalkanoates (PHA) are polyesters that have gained economic importance due to their biodegradability and tendency to replace petroleum synthetic polymers. PHAs are produced in the form of intracellular inclusion bodies under nutrient deprivation and carbon source excess conditions. These inclusion bodies or PHA granules serve as carbon reserves for microorganisms during stress conditions (Sudesh et al., 2000). The inclusion bodies have PHA hydrophobic core surrounded by PHA synthase involved in synthesis of PHA (Grage et al., 2009). Depending on the number of monomeric units PHA can be divided into short chain length (SCL), medium chain length (MCL), and combination of both (SCL/MCL). SCL PHA have three to five carbons in the monomeric unit, is thermoplastic nature, brittle, and lacks toughness (3-hydroxypropionate). MCL PHA have monomers consisting of six to fourteen carbons and have elastomeric property (3-hydroxytetradecanoate). SCL/MCL PHA are derived from both short-chain-length and long-chain-length PHA having three to fourteen carbons. It has a wide range of physical and thermal properties. The generalized structure of PHA is shown in Figure 1.2. Till date 150 different PHAs composition have been identified according to the modifications made in existing PHA and genetically engineered organisms to produce PHA (Loos, 2011; Zinn and Hany, 2005). Apart from its application to produce bioplastics PHA has application in the field of tissue engineering, drug carrier, biomedical, etc. The widespread use of PHA is still restricted due to the fact that the cost of production of PHA is 5–10 times the cost of petrochemical-derived plastic (Chen, 2009).

PHA synthase (PHAc) is the primary enzymes which polymerizes R-3-hydroxyacyl-CoA precursors. For this all the carbon metabolism is shifted to form R-3-hydroxyacyl-CoA thioester. PHA synthase is broadly classified into four classes. Class I consists of one type of PHAc, while class II contains two type of synthases PHAc1 and PHAc2. Classes II and IV consist of PHAc-PHAe and PHAc-PHAr units, respectively (Pötter and Steinbüchel, 2005). Classes I, III, IV favor SCL-PHA, while Class II favors MCL-PHA synthesis. *Ralstonia eutropha* is a model organism for PHA production as it accumulates polymer in nutrient-deficient condition. Its wild type only synthesizes SCL-PHA and has to be modified for tailor-made PHAs (Pohlmann et al., 2006). *R. eutropha* is engineered to produce copolymer poly(3-hydroxybutyrate-*co*-3-hydroxyhexanoate) (PHBHHx) from palm oil. Engineered strain contains PHAc from *Rhodococcus aetherivorans*, enoyl-CoA hydratase gene (PHAj) from *Pseudomonas aeruginosa*, and acetoacetyl-CoA reducatase gene (PHAb). PHAj accumulates PHA, while PHAb alters the level of hydroxyhexanoate (Budde et al., 2011). Since *R. eutropha* grows slowly and difficult to lyse engineering *E. coli* is better option. *E. coli* is not a natural producer of PHAs, as it does not have PHA synthase gene. The first

heterologous production of polyhydroxybutyrate (PHB) in *E. coli* was done in 1988 (Schubert et al., 1988). PHB synthase gene from *R. eutropha* was cloned in *E. coli*. The engineered *E. coli* is able to synthesize PHB but lack the ability to accumulate it.

Increasing the number of PHA synthase gene copies in an organism only increases the PHA levels occasionally that too when the cell dry weight increases. This indicates the importance of cell dry weight over additional copies of PHA synthase gene and shown by many researchers (Tombolini et al., 1995; Huisman et al., 1992; Huisman et al., 1991). *E. coli* binary fission pattern is changed to multiple fission, by deleting the genes minC and minD (related to binary fission) and overexpressing the genes ftsQ, ftsL, ftsW, ftsN, and ftsZ (related to cell division) (Wu et al., 2016a). This results in more dry weight and more PHB accumulation. Apart from the cell dry weight, cell shape also matters. Along with the deletion of minC and minD gene, deleting gene envC and nlpD which degrades peptidoglycan layer leads to filamentous shape and accumulated 70% PHB (Wu et al., 2016b). All carbon source getting converted to biomass and no product is not beneficial industrially. Therefore, a glucose-sensing toggle switch is created that automatically senses glucose limitation and activates product formation leading to shorter growth phases and good titers of PH (Bothfeld et al., 2017).

There is no doubt that genetic engineering increases the production of PHAs in many aspects. But the substrate used in PHA production is costly, and unusual substrates (whey, lipid, molasses, glycerol, etc.) are not directly converted to PHA. Whey contains 4.5% of lactose, engineering *Cupriavidus necator* which is the natural producer of PHA so that it can grow on lactose. For this purpose, lac operon consisting of lac I, lac Z, lac O from *E. coli* is inserted into *C. necator* (Povolo et al., 2010). The lac operon is inserted within the depolymerase gene (phaZ1) which reduces the depolymerization of the polymer and increase in its accumulation. *E. coli* which naturally utilizes lactose but do not have PHA synthase gene is engineered with PHA synthase gene from *C. necator*. The recombinant strain was grown in cell recycle fed-batch culture giving 87% PHB content (Ahn et al., 2001). Molasses, which is the predominant waste produced by sugar industry, contains almost 50% sucrose. Microorganisms should be engineered with sucrose utilization genes in order to use molasses as the carbon source for PHA production. One such gene is sucrose permease (csc) of *E. coli* which has been engineered in *C. necator* to produce PHBHHx using sucrose as a sole carbon source (Loo et al., 2005). Glycerol is the major waste of soap industry by fat hydrolysis and is now also being generated from biodiesel manufacturing. Aerobic metabolism gene from *E. coli*, glycerol kinase, aquaglyceroporin, FAD-dependent glycerol 3-phosphate dehydrogenase are transformed in *C. necator* to obtain PHB accumulation (Fukui et al., 2014).

1.3.2 Organic Acids

Organic acids are widely distributed in nature from ages and have been used by us in our daily life be it lactic acid (in curd) or citric acid (in fruits like lemon, orange, etc.), indicating them to be the building block chemicals. Most of the organic acids can be produced microbially or as an intermediate compound. These microorganisms can be wild type or improved genetically to increase the yield. Products produced by microorganisms are of high purity, selectivity, and are cost efficient (Singh et al., 2016). Organic acids have a wide range of applications and its market is expected to grow at a rate of 60% till 2021. Though the direct demand of the particular organic acids in the market is low but can be created by chemical industry (Sauer et al., 2008). For example, maleic anhydride can be replaced by succinic acid if there is no price limitation. There are number of products that can be derived from succinic acid which includes butanediol, butyrolactone, tetrahydrofuran, bionolle, etc. Bionolle is the polymer of succinic acid and has property similar to polyethylene.

Their applications are in food, beverages, pharmaceuticals, textile, detergents, solvents, petrochemicals, dyes and adhesives, rubber, perfumes, and plastics, etc. If we talk about gluconic acid then gluconic acid and its component have a variety of applications. Gluconic acid is used to prevent milk stone formation in dairy industry and to clean aluminium cans. Various salts of gluconic acid like sodium, calcium, and iron are used as additive in cement, supplement in calcium and iron deficiency, respectively (Ramachandran et al., 2006).

Citric acid, succinic acid, lactic acid, and gluconic acid are discussed below with their structure shown in Figure 1.3.

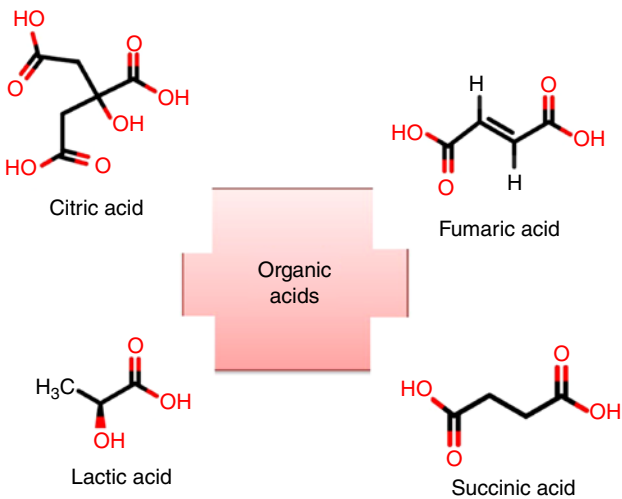

Figure 1.3 Different organic acids with their structures.

1.3.2.1 Citric Acid

Citric acid (2-hydroxy-propane-1,2,3-tricarboxylic acid) is colorless, odorless, in its pure form, and is water soluble. It is widely distributed in plants and animals and is omnipresent in nature as the intermediate compound in krebs cycle or tricarboxylic acid cycle. It is used as flavoring agent in food industry (Soccol et al., 2006). It has a major role in pharmaceutical industry as a blood preservative, in chemical industry as antifoam agent, and in soap industry as stabilization of fats and oils (Max et al., 2010). Its estimated production capacity is approximately 2 million tons per year (Steiger et al., 2017). Citric acid synthesis pathway along with other organic acids synthesis is shown in Figure 1.4.

It was first discovered in lemon (citrus fruit) and now about 99% of citric acid is produced from microbial fermentation. Wehemer (1893) discovered the production of citric acid by *Penicillium glaucum*, but could not scale up the process (Vandenberghe et al., 1999). In 1917, strains of *Aspergillus niger* were found to synthesize citric acid and soon became the method of choice for industrial production of citric acid (Chen and Nielsen, 2016). Highest producing strains of *Aspergillus* can accumulate 200 g/l citric acid under optimum conditions (Steiger et al., 2017). For improved production rate, metabolic and genetic engineering of *Aspergillus* strains are done which can tolerate low pH levels and can secrete excess citric acid in the medium (Ruijter et al., 1997; Steiger et al., 2019). Carbon source utilization plays an important role in industrial production of citric acid. Despite giving higher yields glucose cannot be used industrially as it is not cost efficient. Therefore, liquified corn starch, glycogen, etc. are used. Using liquified corn starch leads to the production of isomaltose with the action of enzyme glucosidase and cannot be used for citric acid production. There is another enzyme glucoamylase that releases glucose from starch and is beneficial. So, Wang et al., (2016) deleted the glucosidase encoding gene and overexpressed glucoamylase gene in *A. niger*. This leads to the decrease in residual sugar to about 88.2% and increase in citric acid production to 16.9%, reaching up to 185.7 g/l.

From Figure 1.4, it can be seen that oxaloacetate and Acetyl-CoA act as a precursor for citric acid production. Oxaloacetate is generated by malate dehydrogenase and fumarase while Acetyl-CoA by pyruvate dehydrogenase, acetyl-CoA synthetase, and ATP-citrate lyase (ACL1, ACL2). ATP-citrate lyase, as the name suggests consumes citrate to synthesize Acetyl-CoA. There was an increase in citric acid production when ACL1 was deleted from *A. niger* (Meijer et al., 2009). But when both the subunits ACL1 and 2 were deleted, there was a decrease in levels of citric acid (Chen et al., 2014). Overexpression of fumarase gave increased citrate levels, while malate dehydrogenase only expedites levels initially. Role of citrate exporter (CexA) is also very important. Citric acid produced inside the cell and not able to get released in the medium could lead to its inhibition. CexA is the

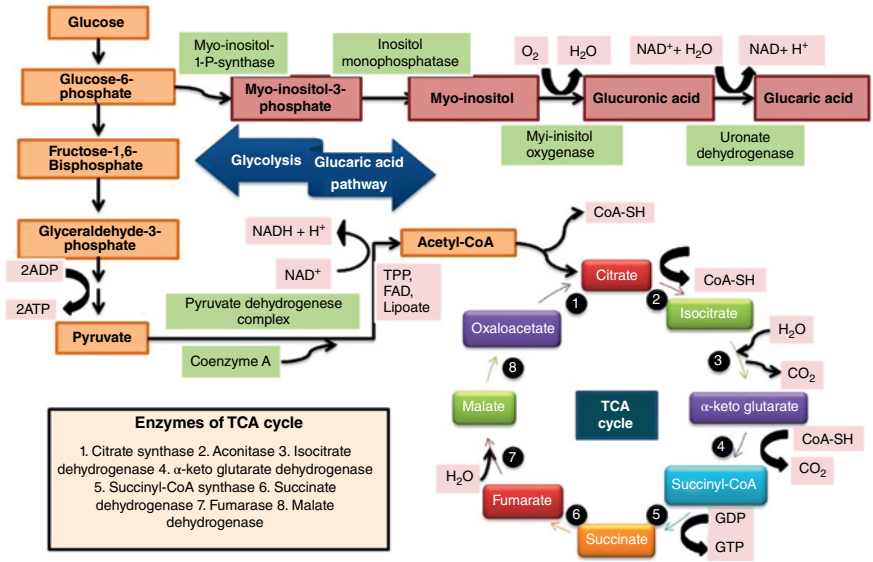

Figure 1.4 Biosynthetic pathway for production of organic acids.

main transporter of citric acid in *A. niger*. Overexpression of this transporter gene leads to increased production of citric acid (Steiger et al., 2019).

Apart from *A. niger*, a well-established producer of citric acid, several strains of bacteria and yeast are also used for citric acid production. Some other producers of citric acid are yeasts which include *Candida oleophils*, *Candida guilliermondi*, *Saccaromicopsis lipolytica*, *Hansenula anamola, Candida parapsilosis, Candida tropicalis, Candida citroformans*, and *Yarrowia lipolytica*. Among bacterial species, i.e. *B. licheniformis, Arthrobacter paraffinens,* and *Corynebacterium* sp., were also used previously for citric acid production by using many raw materials as a substrate with the percentage yield of 27–88% per sugar consumed by the microbial strains.

1.3.2.2 Lactic Acid

Lactic acid ($C_3H_6O_3$) history is dated long back in 1780 when it was first discovered in sour milk by Swedish chemist, Scheele (Figure 1.3). However, in 1847 lactic acid was discovered as a final product of fermentation, and its commercial production from microorganisms is new. It is colorless to light yellow in color available in solid or liquid form. It is widely found in nature among human beings, animals, plants, and microorganisms, in two isomeric forms, i.e. L (+) and D (−) isomers, and as a racemic mixture (DL-lactic acid). Originally, lactic acid was used as a preservative but now has a wide range of applications in food industry as a flavor enhancer in juices, jams, syrup, etc. Recently, polylactide (formed by condensation of lactic acid) a biodegradable thermoplastic that requires pure lactic acid is used for food packaging.

Microbial production of lactic acid utilizes two types of bacteria heterofermentative and homofermentative bacteria. As the name suggests heterofermentative bacteria produces other by products apart from lactic acid, while homofermentative bacteria solely produce lactic acid. A part of lactic acid group bacteria (LAB), *Lactococcus* and *Lactobacillus* are the most important producer of lactic acid. Twenty-two different *Lactobacillus* species are identified utilizing different substrate. *Lactobacillus delbruekii* requires glucose as a carbon source, while *Lactobacillus pentosus* grow on sulfite wastewater (Breed et al., 1957). *Lactobacillus xylans* is homofermentative utilizing xylose. Other genera of LAB include *Streptococcus*, *Pediococcus*, and *Leuconostoc*. Majority of species of the genus *Streptococcus* are pathogenic to humans like *Streptococcus pyogenes*, *Streptococcus pneumoniae*, etc. Out of these, *Streptococcus thermophilus*, a homofermentative facultative anaerobic is nonpathogenic and used to produce curd rich with Gamma-amino-butyric acid (GABA) (Linares et al., 2016). *Leuconostoc mesenteroides* synthesizes D-Lactic acid in pure form. Wild-type *L. mesenteroides* is grown on lactic acid and strains resistant to lactic acid were isolated giving a production of 76.8 g/l as twice as wild-type strain (Ju et al., 2016).

Lactic acid production by LAB is mostly affected by demand to keep pH of the culture acidic which implies that the strain should be acid tolerant. The complete genome sequencing of *Lactobacillus lactis* opens the opportunity for genetic manipulation to increase lactic acid production (Bolotin et al., 2001). After a year of full-genome sequencing Patnaik et al. (2002) identified new lactobacillus species through genome shuffling, which can produce three times the lactic acid compared to the wild type at pH 4.0.

Similarly, mutant strain of *Lactobacillus delbrueckii* was also obtained by genome shuffling giving 40 g/l lactic acid under low pH conditions (John et al., 2008). Apart from pH, high temperature, salts, lactate, and alcohol-tolerant *Lactococcus lactis* strain expressing *E. coli* chaperone DnaK proteins were made (Sugimoto et al., 2010). This shows multiple resistance effect of DnaK protein. Acid-resistant strain of *Lactobacillus casei* under acid stress shows an overexpression of RecO protein as compared to wild-type strain (Wu et al., 2012). This indicates the role of RecO protein in acid stress. Therefore, RecO protein from *L. casei* is engineered in *L. lactis* under the effect of nisin inducible expression system (Wu et al., 2013). The engineered strain grows well in stress condition, and there was an increase in lactate dehydrogenase (LDH) enzyme and hence lactic acid.

LAB cannot utilize starch as a carbon source due to unavailability of an enzyme α-amylase. α-Amylase hydrolyzes complex sugars like starch to release simple sugars like glucose which can be easily used as carbon source by microorganisms. Keeping this in mind high-yielding lactic acid strain *Lactococcus lactis* IL 1403 was engineered with α-amylase from *Streptococcus bovis*. The strain generated can easily be grown on starch and giving a yield of 1.57 g/l/h (Okano et al., 2007). Production of pure lactic acid in a particular isomeric form is of interest. *Lactobacillus plantarum* expressing *S. bovis* α-amylase and LDH deficient produces pure D-lactic acid from corn starch (Okano et al., 2009). As the strain is LDH deficient therefore cannot produce L-lactic acid and can be grown on starch as expresses α-amylase. The strain was able to produce D-lactic acid with the optical purity of 99.6%.

In addition to bacteria, filamentous fungi *Rhizopus oryzae* also accumulates lactic acid when grown on mineral medium and starch or xylose (Koutinas et al., 2007).

1.3.2.3 Succinic Acid

Succinic acid ($C_4H_6O_4$) is the intermediate metabolite of krebs or TCA cycle. In earlier times, it was formed by distillation of amber and therefore called as amber acid or spirit of amber (Song and Lee, 2006). After that it was produced from butane through maleic anhydride. It is used as a flavoring agent in food industry. It has also a range of applications in dyes, perfumes, and in the manufacturing of clothing, paint, fibers, etc. Succinic acid is widely used as precursor for chemicals

such as 1,4-butanediol, γ-butyrolactone, *N*-methyl-2-pyrrolidone, tetrahydrofurane, and 2-pyrrolidone (Pateraki et al., 2016). These chemicals are further derived into biodegradable polyesters such as polybutyrate succinate, polyamides, pesticides, and green solvents (Sun et al., 2015). This leads to the increased demand of succinic acid which was 50 000 metric tons in 2016 to double by 2025 (Chinthapalli et al., 2018).

Like citric acid, succinic acid is also synthesized in almost all plants, animals, and microorganisms. Various microorganisms like *E. coli*, *Actinobacillus succinogenes*, *Mannheimia succiniciproducens*, etc. produce succinic acid. Among them *M. succiniciproducens* gives the highest yield of 1.64 mol/mol glucose and productivity of 6.02 g/l/h (Lee et al., 2016). *M. succiniciproducens* was first isolated from the bovine rumen of Korean cow. Availability of whole genome sequence of *M. succiniciproducens* makes it easier candidate for genetic engineering (Lee et al., 2005). Glucose-6-phosphate 1-dehydrogenase (zwf) gene is upregulated when succinic acid production is increased. Overexpression of zwf gene in *M. succiniciproducens* increases succinic acid synthesis (Kim et al., 2017).

Escherichia coli produce succinic acid in a very scarce amount, but is the model organism for genetic manipulation due to its fast growth and availability of genetic toolboxes. One of the strains NZN111 is generated by knocking out pyruvate formate lyase and LDH (Singh et al., 2009). This leads to the inhibition of formic and lactic acid, increasing the succinic acid production. But the strain generated was not able to thrive anaerobically producing lactic acid. This was solved by further overexpressing the gene for malate dehydrogenase in the same strain producing 31.9 g/l of succinic acid (Wang et al., 2009). Spontaneous chromosomal mutation of glucose phosphotransferase generates strain AFP111, which can anaerobically grow on glucose giving productivity of 0.87 g/l/h (Chatterjee et al., 2001). Prolonged anaerobic conditions can hamper cell growth giving low production rates. To produce succinate under aerobic conditions, five genes were inactivated in *E. coli*, namely, succinate dehydrogenase, pyruvate oxidase, acetate kinase phosphotransacetylase, aceBAK operon repressor, and glucose phosphotransferase (Lin et al., 2005). Apart from inactivation of these genes phosphoenolpyruvate carboxylase gene was overexpressed giving productivity of 1.08 ± 0.06 g/l/h of succinic acid.

Species of yeast like *Y. lipolytica* and *Saccharomyces cerevisiae* are also prospective host for succinic acid production. A robust *Y. lipolytica* PGC01003 strain was developed by deleting the succinate dehydrogenase gene (SDH). This leads to accumulation of succinic acid giving 43 g/l succinic acid from crude glycerol (Gao et al., 2016a). Later acetyl-CoA hydrolase (*Ylach*) gene was deleted from *Y. lipolytica* to inhibit aceitic acid production and giving a yield of 23.8 g/l succinic acid (Yu et al., 2018). Ylach-deficient strain with overexpression of phosphoenolpyruvate carboxykinase (ScPCK) and endogenous succinyl-CoA synthase beta subunit

(YlSCS2) improved succinic acid titer by 4.3-fold (Cui et al., 2017). In a recent study, in spite of deleting SDH gene, SDH promoter was truncated with expressing ScPCK from *A. succinogenes*. It produced 7.8 ± 0.0 g/l succinic acid with a yield of 0.105 g/g glucose (Babaei et al., 2019). *S. cerevisiae*'s SDH have four subunits SDH1, SDH2, SDH3, and SDH4 (Kubo et al., 2000). For SDH to be inactivated SDH1 and SDH2 is to be disrupted. This leads to increase in succinic acid synthesis by further adding malic transporter from *Schizosaccharomyces pombe* (Ito et al., 2014).

1.3.2.4 Fumaric Acid

Fumaric acid ($C_4H_4O_4$) is an intermediate of TCA cycle. It is white crystal solid acid with no odour and is used in plastic industry and in manufacture of sizing resins in paper industry. Being nontoxic in nature it is used in corn tortillas, wheat, sour dough, fruit juice, rye breads, refrigerated biscuit dough, nutraceutical drinks, gelatin desserts, pie fillings gelling aids, wine, and as supplement in animal feed (Khan et al., 2017). In 2014, the global market size of fumaric acid was 245.4 kilo tons and expanding at a compound annual growth rate of 6.1% from 2015 to 2022 (Fumaric acid market analysis by application, https://www.grandviewresearch.com/industry-analysis/fumaric-acid-market).

It was first isolated by a plant *Fumaria*. After that many microorganisms' strains such as *Rhizopus, Mucor, Cunninghamella,* and *Circinella* are used for the production of fumaric acid, and among them *Rhizopus nigricans, Rhiozopus arrhizus, R. oryzae,* and *Rhiozopus formosa* give higher yield (Singh et al., 2017). Yu et al. irradiated *A. oryzae* by femtosecond laser twice, and the mutant strains were selected for fumaric acid production (Yu et al., 2012). There was an increase in yield of 36.6% in comparison to the wild-type strain. Expressing exogenous gene phosphoenolpyruvate carboxylase and overexpressing endogenous gene pyruvate carboxylase increase the carbon flux to oxaloacetate and hence fumarate (Zhang et al., 2012). Fumarase overexpression leads to decrease in fumaric acid as fumarase does hydration of fumarate to malate (Zhang and Yang, 2012). Site-saturation engineering of proline 474 in pyruvate carboxylase from *R. oryzae* was done to improve fumaric acid production in *S. cerevisiae* (Xu et al., 2012). Ethanol yield was lower down by deleting fumarate hydratase gene. The generated strain gives a yield of 314.5 ± 3.8 mg/l.

Similarly, *E. coli* can also be engineered by overexpressing and deleting the above-mentioned genes. The iclR gene encoding a repressor of glyoxylate shunt operon was deleted to direct carbon flux to glyoxylate shunt (Song et al., 2013). All three fumarase genes were also deleted enhancing fumaric acid production to 1.45 g/l. in a strain with all three fumarase genes deleted with the overexpression of phosphoenolpyruvate carboxylase gene gives yield of 3.65 g/l fumaric acid (Li et al., 2014).

1.3.3 Therapeutic Proteins

Proteins are the building block of our body and are essential for proper functioning of body. Any defect in a particular protein can lead to a disease such as hemophilia (lack of various blood-clotting factors), dwarfism (lack of growth hormone), and diabetes (less or no insulin production) (Vajo et al., 2001; Takeda et al., 2010). These diseases caused by the deficiencies of the proteins can be treated by regular administration of them. These are synthesized by the other organisms, isolated, purified, and then given to patients. Proteins synthesis for human use is completely different form the enzymes used for chemical industry as for human's quality constraints are strict and detailed clinical trials are required. More therapeutic proteins are under clinical trial than in the market.

Out of the many recombinant therapeutic proteins human growth hormones (hGH) is at the top. Its market was estimated to be $2850.70 million in 2018 and is anticipated to reach $5653.60 million by 2026 observing a CAGR of 8.2% over the estimated period. Two of the most common hGh currently in the market are Accretropin™ and Sermorelin (Reh and Geffner, 2010). Accretropin™ (recombinant human growth hormone (r-hGH); somatropin) is a protein, which is manufactured by rDT. The production process includes fermentation of *E. coli* that gives a protein product containing 192 amino acids. Later process includes removal of the N-terminal amino acid, methionine that yields a product which is physio-chemically and chemically similar to pituitary-derived hGH, comprising of 191 amino acids. These are arranged in a single polypeptide chain. In general terms, Accretropin vitalize linear growth in those patients whose body lacks sufficient production of the endogenous growth hormones. Other than that, it also shows substantial effect in process such as tissue growth, metabolism of protein, carbohydrate, and lipids along with minerals. The drug got approved by FDA on 23 January 2018. It is manufactured by Cangene Corporation, Canada.

Similarly, Sermorelin or growth hormone releasing factor 1-29 NH_2-acetate is classified as a member of the growth hormone-releasing hormone analogue class drug. It is made up of 29 amino acids out of the 44 that originate in its natural state (Walker, 2006). It can notably increase the release of the growth hormone in the body. It also aims to enhance growth hormone serum concentrations and insulin-like growth factor 1 (IGF-1). The bodies of those children, which does not produce enough growth hormone, can be provided with Sermorelin to assist with the increase in amount of growth hormone processed by the pituitary gland. Other than that, it is also commonly prescribed for the treatment of adult growth hormone deficiency. It is also getting popular with those doctors who use them partly in their medical weight loss plans. The drug was first developed during early 1980s and was approved by the FDA by 1997 for sale via prescription.

Another fine example is IGF-1 (Laron, 2001). They can be related to the family of insulin-related peptides, which comprise of relaxin and various other peptides, which are extracted from lower invertebrates. Being a small peptide and comprising of 70 amino acids and with a molecular weight of 7469 Da, it binds to the insulin receptor with a relatively low affinity. Along with IGF-2, they were first found in 1957 by Salmon and Daughday. The IGF-1 plays major role in proliferation and function of just about every cell, tissue, and organ in the body. Their action mechanism conciliates through IGF/IGFR binding, the activation of kinase, and signaling via AKT pathway.

1.4 Photosynthetic Production of Biofuels

The increase in energy demand globally is affecting environment due to global warming and depletion of nonrenewable energy sources. Conventional fossil fuel sources such as petrol, diesel, natural gas, and coal, which were considered to be the primary sources earlier are getting exhausted due to extensive uses (Kumar et al., 2017). A 37% surge in fuel demand by 2040 has been estimated due to the rapid increase in demand (Joshi et al., 2017). Besides this the petroleum-based fuel upon burning releases greenhouse gases. This requires a renewable substitute of petroleum-based fuels. Biofuels are the renewable energy source produced by photosynthetic organisms utilizing Earth's biggest fuel source, the Sun as the carbon source. Harvesting solar energy via photosynthesis is one of nature's noteworthy achievements that could also be a solution for the future worldwide economy. Earlier biofuels were produced from plants known as the first generation of biofuels. They are mainly generated from wheat, barley, corn, oilseed, sunflower, etc. the plants, which are rich in carbohydrates and oils. Biofuels produced by them compete with agriculture croplands leading to crisis in food production for human beings (Surriya et al., 2015). Additionally, harvesting of plants take full season and then processing the complex sugars from plants to simpler sugars that can be used by microorganisms limits the production of biofuel (Voloshin et al., 2019). Second generation of biofuels solves the problem of utilizing croplands as they are based on agricultural waste, forest dregs, waste wood residues, and organic waste materials. Biofuels from algae are considered to be third-generation biofuels. Microalgae and cellulolytic bacteria are fast growing and can fix CO_2 (Dragone et al., 2010). Fourth-generation biofuels use genetically modified organisms such as algae and cyanobacteria and metabolic engineering to produce biofuels (Dutta et al., 2014). Bioethanol, biodiesel, biogas, biohydrogen, etc. come under biofuel.

1.4.1 Biohydrogen

Biohydrogen is the clean fuel as it burns leaving only water. It is produced biologically and therefore is a promising future fuel (Hosseinpour et al., 2017). Hydrogenase or nitrogenase are the crucial enzyme for biohydrogen regulation in both prokaryotes and eukaryotes. Autotrophic organisms such as microalgae, green algae, cyanobacteria, etc. are most efficient for biohydrogen production. The main principle behind the hydrogen production is the electrons generated during metabolism inside the cells by the action of hydrogenase enzyme are made to form hydrogen. Genetic manipulation and metabolic engineering of autotrophic organisms for production of biohydrogen has received much attention.

In cyanobacteria three alternative pathways (i) photolysis of water using photosystems (PS); (ii) fermentative pathway; and (iii) photofermentative pathway for the production of biohydrogen. In the first pathway, PSI and PSII through light-dependent reaction transfer electron from water to ferredoxin producing NADPH leading to biohydrogen formation (Carrieri et al., 2011). In second, the source of NADPH is degraded polysaccharides or lipids, and the electrons are transferred to plastoquinone pool for hydrogen formation (Ghirardi et al., 2000). Third pathway is the combination of first two. Cyanobacteria having heterocyst use it as the sites for nitrogen fixation. A vegetative cell originates NADPH and transport electrons to the plastoquinone pool inside the heterocyst. Inside this hydrogenase gets inactivated and nitrogenase reaction take place (Kufryk, 2013). Before the hydrogen production by cyanobacteria can become industrially viable improvements in strains are to be done. There are two types of hydrogenase: uptake hydrogenase (Hup) and bidirectional hydrogenase (Hox). *Anabaena* sp. PCC 7120 contains both Hup and Hox (Masukawa et al., 2002). The species were tested for both Hup and Hox inactivation. Hup inactivated strain gave four to seven times increase in H_2 production. In another study, *Nostoc* sp. PCC 7422 was chosen having highest nitrogenase activity and was subjected to Hup gene disruption (Yoshino et al., 2007). The generated mutant was able to produce hydrogen at a rate of threefold than wild type. Electrons play an important role in hydrogen production. Electrons generated by ferredoxin and NADPH are sometimes transferred to other competing pathways (e.g. nitrate assimilation pathway). This can be engineered to redirect electron flow to, for example, Hox (Baebprasert et al., 2011). Disrupting nitrate assimilating pathway in *Synechocystis* sp. strain PCC 6803 results in higher hydrogen production as electron gets redirected to Hox. LDH mutants of *Synechococcus* 7002 lacking have exhibited 5 times greater hydrogen production compared with the wild type (Park et al., 2008). In a different study, *Cyanothece* sp. ATCC 51142, a unicellular diazotrophic cyanobacterium demonstrated the ability to generate high levels of hydrogen (465 μmol H_2/mg of chlorophyll h) using glycerol as a substrate under aerobic conditions (Bandyopadhyay et al., 2010).

Green algae have two light-dependent and one light-independent pathway mediated by [Fe] or [FeFe] hydrogenase for hydrogen synthesis (Meyer, 2007). In it [Fe] or [FeFe] hydrogenase is the key catalyst and ferredoxin is electron donor. In the first pathway, water is used as the electron sink and biophotolysis of water takes place. Here water splitting PSII and ferredoxin-reducing PSI act together. The second pathway is PSII independent where electrons from metabolic pathways like glycolysis or citric acid are transferred to electron transport chain. The third pathway that is light independent or dark fermentation of decarboxylated pyruvate. *Chlorella vulgaris* strain when overexpressed with hydrogenase gene can produce hydrogen even in atmospheric conditions (Hwang et al., 2014). These strains were able to show maximum production at low intensities of light much less than that is received on Earth by Sun. This can be overcome by decreasing the number of light harvesting antenna as done in *Chlamydomonas reinhardtii* tla1 strain (Kosourov et al., 2011). In total, 50% truncated photosynthetic light harvesting antenna increases hydrogen production. Flavodiiron (Flv3B) proteins have crucial and specific roles in photoprotection of photosystems I and II in cyanobacteria. Absence of Flv3B leads to impaired growth of cyanobacteria in the presence of oxygen. Overexpression of Flv3B in *Nostoc* PCC7120 significantly increases the hydrogen production (Roumezi et al., 2020).

1.4.2 Biodiesel

Biodiesel is a renewable liquid transportation fuel consisting of alkyl esters of fatty acids and produced from triacylglycerides (TAGs). It is synthesized by a process called transesterification of fats in the presence of catalyst (alcohol) to form fatty acid methyl esters (Subramani and Gangwal, 2008). Therefore, properties of biodiesel are dependent on the fatty acid from which it was made (Knothe, 2005). Majority of biodiesel currently is being formed by oilseed crops competing with food and cultivable land. Alternative feedstock for the oil includes oleaginous or grease microorganisms, such as microalgae, cyanobacteria, yeast, and bacteria (Hu et al., 2008). Out of these microalgae and cyanobacteria are of importance as they are autotrophic in nature. Some microalgae accumulate 20–50% TAG, which are same as found in oilcrops such as canola and sunflower (Gaurav et al., 2017). Like plants only they utilize sunlight as energy source and CO_2 as carbon source but can be cultivated on little barren land using wastewater (Pittman et al., 2011). Attempts are being made to improve lipid content of microalgae by metabolic engineering and genetic engineering. *C. reinhardtii* is the model organism for this purpose as its genome is well known.

TAG is derived from acylation or diacylglycerol through acyl-CoA dependent and acyl CoA-independent pathway. Rate-limiting steps of these two pathways can be altered to increase or decrease the TAG production. Inactivation of

ADP-glucose pyrophosphorylase in *Chlamydomonas* resulted in 10-fold increase in TAGs (Miller et al., 2010). In another study, *Chlamydomonas* mutant lacked subunit of ADP-glucose pyrophosphorylase and accumulated 46.5% total lipids out of which 32.5% were neutral lipids (Li et al., 2010).

Knockout and overexpression of enzymes important for fatty acid synthesis like acetyl CoA carboxylase (ACCase) and type-II fatty acid synthase (FAS) are well known to increase lipid content in the cell (Majidian et al., 2018). ACCase catalyzes the rate-limiting step of fatty acid synthesis and therefore *C. reinhardtii* was overexpressed with ACCase and showed increase in fatty acid synthesis to 56.15% as compared to wild type (48.39%) (Chen et al., 2019).

In diatom like *Thalassiosira pseudonana*, deletion of multifunctional lipase/phospholipase/acyltransferase does not hamper growth and increases (up to threefold) lipid content (Trentacoste et al., 2013). Heterologous expression of two thioesterases in microalga *Phaeodactylum tricornutum* accumulates shorter chain fatty acids; lauric and myristic acid (Radakovits et al., 2011). These shorter fatty acids are not secreted and gets incorporated in TAG.

1.4.3 Bioethanol

Bioethanol (C_2H_5OH) is the most common liquid biofuel. It is used as an additive to gasoline as 5–10% blend. It burns clean and help in reduction of greenhouse gases and is being commercially produced in Brazil, Canada, and United States for transportation (Sirajunnisa and Surendhiran, 2016). Initially, bioethanol was produced from biorefinery of sugar-rich agricultural biomass (Rastogi et al., 2018). This bioethanol production was limited due to issues with food competition. Photosynthetic biomass production and getting the biomass converted into ethanol is of great interest and is done easily by cyanobacteria. Some strains can naturally convert carbon source to ethanol and some have to be engineered with key enzymes for ethanol production. Simply ethanol production requires two key enzymes pyruvate decarboxylase which converts pyruvate to acetaldehyde and alcohol dehydrogenase converting acetaldehyde to ethanol.

Pyruvate decarboxylase (pdc) enzyme from *Zymomonas mobilis* is well characterized and used to engineer several cyanobacterial strains such as *Synechocystis* sp. PCC6803. Pdc and alcohol dehydrogenase from *Z. mobilis* were integrated into the genome of PCC6803 using double homologous recombination. The genes were under the control of strong light inducible psbAII promoter (Dexter and Fu, 2009). With incorporation of pyruvate decarboxylase, overexpression of endogenous alcohol dehydrogenase and hampering the biosynthetic pathway of poly-β-hydroxybutyrate, PCC6803 gives an ethanol efficiency of 5.50 g/l (Gao et al., 2012). *Synechococcus* sp. PCC7002 is more tolerant to higher temperature as compared with PCC6803. Glycogen synthesis was blocked in PCC7002 by

introducing two glycogen synthase genes. This lead to hamper the growth of the cyanobacteria. This was accommodated by incorporating double copies of ethanolgenic pathways giving 2.2 g/l ethanol in 10 days (Wang et al., 2020). Pdc and Adh with *E. coli* lac and CI-PL temperature inducible promoter increase ethanol productivity in *Synechococcus* sp. PCC 7942 (Dexter et al., 2015). It is said that increased NADPH increases biomass and ethanol yield. This was confirmed by endogenous expression of glucose-6-phosphate dehydrogenase (zwf) gene in PCC6803. zwf gene of pentose phosphate pathway increases NADPH production and hence biomass and ethanol production (Choi and Park, 2016).

1.4.4 Terpenoids

Terpenoids are also called as isoprenoids and are a large class of plant specialized secondary metabolites, derived from 5 carbon (C5) isoprenoid unit. These precursors are produced by two biosynthetic pathways, the methylerythritol phosphate pathway (MEP) in the chloroplast and the classical mevalonate pathway (MVA). MEP pathway is found in prokaryotes whereas the MVA pathway in eukaryotes. Terpene synthases (TPSs) in plants have been classified into seven subfamilies, designated as TPS-a, TPS-b, TPS-c, TPS-d, TPS-e/f, TPS-g, and TPS-h, according to similarities in their amino acid sequences (Chen et al., 2011). Table 1.1 shows the genome-wide analysis of TPSs from different plants. Farnesene, isoprene, etc. are some of the terpenoids which can be used as biofuel.

Farnesene belongs to the Sesquiterpenes class of terpenes. Farnesene (3, 7, 11-trimethyldodeca-1, 3E, 6E, 10-tetraene) is the simplest sesquiterpenes produced by plants. It is a 15-carbon compound ($C_{15}H_{24}$). It was first discovered in

Table 1.1 Genome wide analysis of terpene synthase genes.

Plant species	Description	References
Jatropha curcas	• 59 putative TPS genes were identified. • Among them 26 belongs to TPS-a family.	Xiong et al. (2016)
Ananas comosus	• 21 putative TPS genes were identified. • Divided into five sub families.	Chen et al. (2017)
Citrus sinensis	• 55 putative TPS genes identified out of which 28 are TPS-A, 18 are TPS-b, and 5 are TPS-g. • Only two of them are TPS-e/f each.	Alquezar et al. (2017)
Ocimum sanctum	• 81 putative genes identified. • Further only 47 putative genes were found to be functional.	Kumar et al. (2018)

apple peels and was found to play a role in plant defence. It can be used as a promising replacement for diesel. It has a major role in cosmetics industry as an antiaging and moisturizing lotions. Being less hygroscopic in nature and having high-energy density (cetane numbers of 58), it forms the precursor for jet biofuel (Yang et al., 2016). Its market size was reported to be 8.51-kilotons in 2015 and is expected to reach 180.9 kilotons and up to $485 million by 2023. Amyris Biotechnologies, USA, dominates the global farnesene market (Biofene). Intrexon and chromatin, USA, is in emerging state to reach production level. Farnesene is basically synthesized by plants. So, adding this machinery to the prokaryotes will increase its production. *E. coli*, *S. cerevisiae,* and *Yarrowia lypolytica* are heterotrophic organisms that were engineered by adding farnesene synthase gene to produce farnesene (Wang et al., 2011; Tippmann et al., 2016; Yang et al., 2016). The heterotrophic organisms require continuous supply of carbon sources. Therefore, using cyanobacteria, i.e. autotrophic organisms can be beneficial as they utilize carbon dioxide as the carbon source and also helps in CO_2 sequestration. Table 1.2 shows the list of photosynthetic organisms engineered to produce farnesene. Much research is needed to be done in the field of farnesene synthesis and is a booming biofuel.

Table 1.2 Photoautotrophic production of farnesene.

Microorganism	Promoter used	Description	Max. farnesene concentration (mg/l)	References
Anabaena sp. PCC 7120	Ptrc	• Codon optimized farnesene synthase gene from Norway spruce is taken. • Expression plasmid was used for the production.	0.0691	Halfmann et al., (2014)
Synechococcus elongatus PCC 7942	Ptrc	• Codon optimized farnesene synthase gene from *Malus domestica* and *Picea abies* was taken. • In addition to the above-mentioned genes dxs, idi and ispA gene were also incorporated int the genome. Thus, optimizing Methylerithritol phosphate (MEP) pathway.	4.6 ± 0.4	Lee et al., (2017)

dxs, deoxy xylulose synthase; idi, isopentynyl pyrophosphate isomerase; ispA, farnesyl diphosphate synthase.

Isoprene (C_5H_8) is another industrially important chemical. It is a volatile hydrocarbon produced by plants under stress condition. One million tons of it is produced from petrochemicals to reach the global need (Morais et al., 2015). Isolating such huge amount of isoprene from plants is not feasible. Therefore, microbial production of isoprene is gaining interest recently. Cyanobacteria are genetically modified for isoprene synthase (Isps) gene, which is usually absent in them. Isoprene synthase gene catalyzes the conversion of di-methylallyl diphosphate to isoprene. Lindberg et al., (2010) reported first isoprene synthesis from cyanobacteria. Isps gene from *Pueraria montana* was introduced in PCC6803 under the light-regulated PsbA2 promoter giving yield of isoprene 50 μg/g DCW. Engineering isoprene synthase gene with other MVA pathway genes improves yield of isoprene by 2.5-folds (Bentley et al., 2014). Apart from introduction of isoprene synthase gene, overexpression of IPP isomerase (IDI) gene leads to 40% photosynthetically fixed carbon toward isoprene (Gao et al., 2016b).

1.5 Conclusion

Biocommodities are now being produced by microbial cell factories. These microorganisms are engineered to increase their robustness. Increasing knowledge of the omics technologies as now we have full access to whole genome makes easy to manipulate any organism. Engineering of microbial cell factories depends on the required end product. Number of genetic tools are now available that makes biocommodity engineering easy. Despite of the plethora of literature available on genetic engineering of microorganisms, very few of them are able to perform well industrially. Therefore, focus is to be made on scale up of the existing cell factories, and new engineered strains should also be taken to industrial level for better production.

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2

Microbial Cell Factories for the Biosynthesis of Vanillin and Its Applications

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2.1 Introduction

Vanillin (3-methoxy-4-hydroxybenzaldehyde), a phenolic compound with organoleptic properties (Walton et al., 2003), was isolated first time from vanilla pods by Nicholas Theodore Gobley (1858). The Spanish word "vania" means pod, and "ila" means small; thus, vanilla means vine yielding small pods. The vanilla is the only edible fruit in the orchid family (Ranadive, 1994). Vanilla receives its nutrients and relies on moisture in the air like other plants' roots connected to ground for its growth. The floret of vanilla is pale yellow-green in color, which is 10 cm in diameter, and the bean is 5–10 inches long (Bythrow, 2005). The tropical climbing orchid, *Vanilla planifolia*, is the leading natural source of vanilla and to a lesser extent of *Vanilla tahitiensis* and *Vanilla pompon*. Vanilla is a sweet-smelling and world's second most expensive flavor compound next to the saffron. Vanillin is the foremost compound responsible for the sweet aroma of vanilla, which is mainly used as a fragrance ingredient of food preparations such as ice creams, chocolates, cakes and other milk products, and beverages (Ranadive, 1994). Moreover, vanillin serves as an intermediate in the synthesis of herbicides, antifoaming agents, or drugs such as papaverine, IL-dopa, IL-methyldopa, and the antimicrobial agents (Hocking, 1997). Naturally, vanilla is extracted from bean or pod-like vanilla fruit using aqueous ethanol. Pure vanillin is a white crystalline solid, which is slightly soluble in water. Vanillin from natural sources has huge market demand due to the way of processing, cost, and bioactive properties of the product. Madagascar,

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Figure 2.1 World map showing the leading countries in the production of vanillin.

Indonesia, Mexico, China, India, and Papua New Guinea are the main vanilla-producing countries (Figure 2.1). It is also grown in a lesser extent in France and India (Kerala, Karnataka, Tamil Nadu, Assam, and Andaman Nicobar Island) (Priefert et al., 2001; Walton et al., 2003; Converti et al., 2010; Zamzuri and Abd-Aziz, 2013; Banerjee and Chattopadhyay, 2019). Madagascar and Indonesia together are the leading producers of natural vanillin, and they have produced 5361 metric tons in the year 2018 as per Food and Agriculture Organization of the United Nations. Vanillin production from plants is a labor-intensive process, which requires approximately 40 000 flowers to be pollinated to produce 1 kg of vanillin from 500 kg of pods. Due to low yield of natural vanillin from plants and the need of huge plant sources that grows slow in nature, vanillin extraction from plant sources is very much limited. Alternatively, vanillin is produced chemically from lignin, guaiacol, and 4-hydroxybenzaldehyde at cheaper costs, but the low quality of the product yield and the release of hazardous contaminants in to the ecosystem by chemical synthesis make this process unlikely with regard to cost and quality. In the recent years, green synthesis of wide array of bioactive metabolites has gained much attention as an alternate to chemical synthesis in terms of high reliability, sustainability, and environment friendliness. Vanillin production through biotechnological approaches like microbes enabled biotransformation of substrates like ferulic acid and eugenol holds promise as a viable alternative and economically feasible way of obtaining vanillin. Thus, it has gained much interest

in recent years due to European and US legislation already classifying the product as "natural." This review mainly focuses on recent strategies for the vanillin production from different sources and on various strategies so far evaluated including biotechnological methods for vanillin production like biotransformation of natural precursors to vanillin using microbial cells or enzymes and on genetic manipulation by metabolic engineering. In recent years, many researchers have explained the production of vanillin from different naturally occurring sources and also reported high yield of vanillin production thus review may help in understanding past, present strategies to get better yield of vanillin to meet high demand of this flavoring compound (Vanillin) in future.

2.2 Natural Sources of Vanilla and Its Production

The genus *Vanilla* belongs to the family Orchidaceae, which is one of the largest families of flowering plants in the world comprising 788 genera and 18 500 species. The genus itself contains over 100 species (Mabberley, 1997). *V. planifolia* is the only orchid that is of direct economic importance because it is the main source of the vanilla aroma. This aroma is widely used in the pharma, food, and cosmetic industry. *V. planifolia* has its origin in Mexico, where it was already brought by Spanish to Europe in 1520 and it became very popular. *V. planifolia* is still the only natural source of the vanilla aroma. In some other plants (narcissus, hyacinth, potato) traces of vanillin occur (Havkin-Frenkel et al., 1999). Vanillin normally present in conjugated form as β-D-glucoside at a concentration of 1.0–2.0% of dry matter in cured vanilla pods (Westcott et al., 1993). The vanilla aroma develops in the pods or beans through a quite labor-intensive process called curing. This process for aroma development is carried out to dry the vanilla beans and to allow chemical and enzymatic reactions to occur. Curing process aims to arrest the vegetative growth and induces the changes responsible for aroma formation. Generally, curing comprises three stages such as killing/scalding, sweating/sunning, drying, and conditioning/packaging. During curing process, conjugated glycoside molecules accumulated in the green pods are hydrolyzed enzymatically by induced β-D-glucosidases through drying and heating. Further packaging of processed pods by storing in closed boxes for few months facilitates various biochemical reactions such as esterification, etherification, oxidative degradation, etc. that results in desired aroma and flavor formation (Rao and Ravishankar 2000; Converti et al., 2010). Conventionally by curing process very less (1–2%) vanillin is produced naturally (Sinha et al., 2008). Moreover, natural vanillin production using plant sources is laborious, time consuming, and also expensive. With the increasing interest in producing natural vanillin and the insufficiency of plant-derived natural vanillin production to

meet the demand, alternative processes are developed to produce vanillin from a natural raw resources through many biotechnological approaches including enzyme catalyzed conversions, microbial bioconversions, the development of tissue cultures, and genetic/metabolic engineering.

2.3 Biotechnological Production of Vanillin

The production of natural vanillin using plant sources is laborious, time consuming, and also expensive. With the increasing interest in producing natural vanillin and the insufficiency of plant-derived natural vanillin production to meet the demand, alternative processes are developed to produce vanillin from a natural raw resources through many biotechnological approaches including enzyme-catalyzed conversions, microbial bioconversions, the development of tissue cultures, and finally, genetic engineering.

2.3.1 Enzymatic Synthesis of Vanillin

Odor-emitting compounds are conjugated in between cellulosic structures in the vanilla pods as glucovanillin, which is hydrolyzed enzymatically during curing process. A large number of enzyme preparations from endogenous and exogenous sources from other plants and microorganisms have been employed to hydrolyze glycosylated cellulosic structures for releasing the flavoring compound vanillin. β-D-glucosidases play the key role in the curing process of vanillin harvesting. β--D-glucosidases catalyze the hydrolysis of β-glucosidic linkages of cellulose, a homopolysaccharide of β-D-glucopyranose residues linked by β-(1-4)-glycosidic bonds. Cellulose is the major polysaccharide present in the plant biomass, which is hydrolyzed primarily during microbial composting by the action of cellulases including β-D-glucosidases (Zang et al., 2018). Green vanilla pods are rich in glucovanillin and other minor glycosides of 4-coumaric acid, *p*-hydroxybenzaldehyde, *p*-hydroxybenzoic acid, vanillic acid, and *p*-hydroxybenzylalcohol (Kanisawa, 1993; Odoux, 2006), which are hydrolyzed during curing process by endogenous β-D-glucosidase activity to release the characteristic vanilla flavor emitting aromatic aglycons (Odoux, 2000). Consequently, vanilla pods treated with glucosidases from external sources yielded vanillin in high concentrations than the pods cured normally (Dignum et al., 2001; Ruiz-Teran et al., 2001). Mane and Zucca (1993) have shown that enzymatic treatment with 40–400 units of β-glucosidase per gram of green vanilla pods is effective for better production of vanillin. The traditional curing process provides only 2% of vanillin yield because uncured green beans contain 10–15% of glucovanillin. Thus, exogenous enzyme preparations

containing cellulase, pectinase, and β-glucosidase were added to increase the yield. Nevertheless, with the addition of exogenous enzymes on green pods 4.25–7.00% of vanillin yield had been achieved (Perera and Owen, 2010). Recently, Naidu et al. (2012) has demonstrated that treatment of tea leaf enzyme extracts (TLEE) with vanilla flavor precursors produced higher content of vanillin (4.2%) with superior flavor quality than Viscozyme-treated vanilla extracts (2.4%).

The escalating demand for the natural vanillin in the world due to low yields from natural vanilla pods, biotransformation of other plant-derived materials like ferulic acid, stilbenes, lignin, and eugenol through enzymatic hydrolysis are being developed to produce high-quality vanillin. Ferulic acid, a cheap raw material found abundantly in the plant biomass (Zheng et al., 2007), is one of the most extensively investigated substrate to produce vanillin through biotransformation. The feruloyl-CoA synthetase (*Fcs*) that degrades ferulic acid and enoyl-CoA hydratase/aldolase (*Ech*) that produces vanillin were characterized in many microbial sources. For example, the enzyme preparations from the recombinant *Escherichia coli* expressed with *Ech* and *Fcs* genes from *Amycolatopsis* sp. strain HR167 and *Streptomyces* sp. strain V-1 resulted in successful conversion of ferulic acid to vanillin (Achterholt et al., 2000; Yang et al., 2013). Van den Heuvel et al. (2001) used vanillyl alcohol oxidase (VAO) obtained from *Penicillum simplicissimum*, which is a flavoenzyme catalyzes the conversion of vanillylamine (the active principle of pungency in chili peppers produced as an intermediate during capsaicin biosynthesis) and creosol (a carbonaceous material obtained by the pyrolysis of wood and distillation of coal tar) to vanillin with high yield. Similarly, eugenol oxidase (EUGO) is a flavoenzyme produced by *Rhodococcus* sp. RHA1 that catalyzes conversion of vanillyl alcohol to vanillin (Jin et al., 2007). Garcia-Bofill et al. (2019) immobilized EUGO on different supports such as MANA-agarose, Epoxy-agarose, and Purolite 8204F to improve its stability in oxidizing vanillyl alcohol for enhanced production of vanillin, which resulted in 2.9 g l/1 H of vanillin.

Lipoxygenase (LOX), a class of iron-containing dioxygenase present in high concentrations in soybean, is well known for catalyzing the hydroperoxidation of polyunsaturated fatty acids and esters. LOX has also been reported to catalyze the oxidative cleavage of isoeugenol to vanillin (Li et al., 2005), which holds potential as a promising route for enzymatic synthesis of vanillin. Liu et al. (2020) used soybean LOX as the catalyst for vanillin synthesis from isoeugenol through addition of denaturants (urea and guanidine) and chelators (EDTA), which were effective in improving yield of vanillin by up to 133% and 406%, respectively than control. Overall, enzymatic production of vanillin seems attractive, though their performance in bioreactors or fermenters in terms of stability and activity that remains elusive.

2.3.2 Microbial Biotransformation of Ferulic Acid to Vanillin

Microbial transformation of phenolic compounds into vanillin is the most attractive alternate for natural vanillin. Many microbes are capable of producing vanillin and vanillic acid by utilizing phenolic substrates such as ferulic acid, eugenol, and isoeugenol (Figure 2.2). Among them, ferulic acid has been widely used as a sole carbon source for vanillin production. *Pseudomonas fluorescens* is one of the most predominantly used organisms for such biotransformation. Under optimized environmental conditions, *P. fluorescence* BF13 is capable converting 95% of ferulic acid to vanillic acid in five hours (Barghini et al., 1998). Likewise, a mutant of *P. fluorescens* FE2 is shown to produce 95% of vanillic acid from ferulic acid in 24 hours (Andreoni et al., 1995). Next to *Pseudomonas* sp., *Lactobacillus* sp. is considered to be effective producer of vanillin as it has phenolic transformation properties. A group of *Lactobacillus* strains are found to produce significant amount of vanillin from ferulic acid supplemented rice bran medium (Kaur et al., 2013). *Streptomyces setonii* and *Amycolatopsis* sp. have been reported to produce notable amount of vanillin under optimized pH (Rabenhorst and Hopp, 2000; Gunnarsson and Palmqvist, 2006). Likewise, *Delftia acidivorans* also has been reported as potent bacteria to transform ferulic acid to vanillin efficiently (Plaggenborg et al., 2001). The second most commonly used carbon source in biotransformation of vanillin is eugenol and isoeugenol, which are essential oil components extracted from clove. The degradation pathway of eugenol in certain bacteria like *Corynebacterium* sp. (Tadasa, 1977), *Arthrobacter globiformis* (Rabenhorst, 1996), *Pseudomonas* sp. (Tadasas and Kayahara, 1983) has resulted in producing vanillin, vanillic acid, and other phenolic compounds. *Arthrobacter* sp. TA13, *Pseudomonas putida* JYR-1, and *Bacillus subtilis* B2 are reported to produce vanillin from isoeugenol (Shimoni et al., 2000, 2003; Ryu et al., 2005). *Bacillus fusiformis* has been reported to produce 8 g/l of vanillin from 50 g/l of isoeugenol with the addition of HD-8 resin to prevent inhibition by product (Zhao et al., 2006). Further, *Bacillus pumilus* S-1 is shown to transform isoeugenol to vanillin with higher efficiency with the yield of 3.75 g/l of vanillin from 10 g/l of substrate in 150 hours (Hua et al., 2007). *Bacillus licheniformis* SHL-1 has been reported to produce 494 mg/l of vanillin from 1 g/l of ferulic acid within 45 hours (Ashengroph et al., 2012). Other than bacteria, *Aspergillus niger* is also most frequently used to transform ferulic acid to vanillic acid, which is further converted to vanillin by *Pycnoporus cinnabarinus* or *Phanerochaete chrysosporium*. The yield of vanillin by this two-step process is reported to be 1.1 g/l after 54 hours of fermentation (Stentelaire et al., 2000). *Sporotrichum thermophile*, a thermophilic fungus has also been reported to produce 4798 mg/l within 20 hours of fermentation using ferulic acid as a substrate (Topakas et al., 2003). On the other hand, vanillin production by fungal bioconversion has many disadvantages, such as lysis

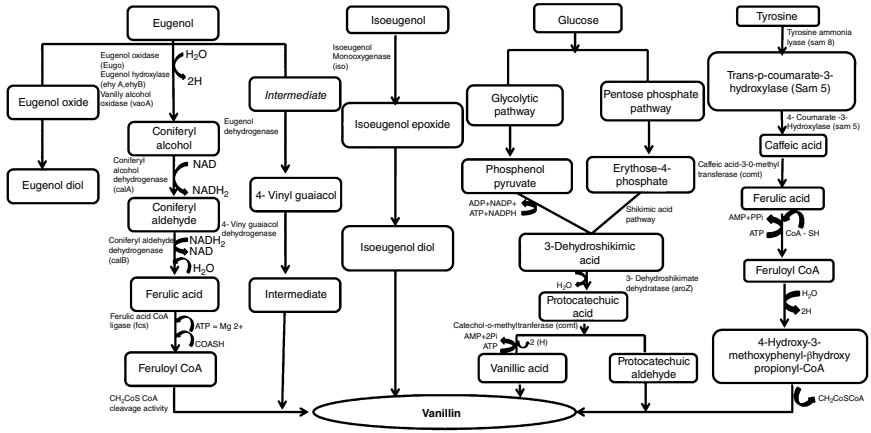

Figure 2.2 Biotransformation pathways of vanillin in microbes using various substrates.

of mycelium, extremely viscous broths, uncontrolled fragmentation, and unfavorable pellet formation, which hinder conventional production processes and increase the price of the downstream process. Apart from these, a list of microbes involved in biotransformation of vanillin is listed in Table 2.1. Besides having good yield, biotransformation process has several disadvantages like formation of undesired by-products, expensive downstream processing, unproductive metabolic flow, cytotoxicity of precursors used (Gallage and Møller, 2015), and the difficulty in process optimization of the microbes used. In order to overcome these problems and to meet the market demand, biotechnological approaches including metabolic and genetic engineering are being employed and these methods have gained much importance nowadays because of higher yield of vanillin.

Table 2.1 List of microorganisms capable of producing vanillin from various substrates.

Organism	Substrate	References
Gram-positive bacteria		
Amycolatopsis sp. HR167	Eugenol	Overhage et al. (2006)
Rhodococcus opacus PD630	Eugenol	Plaggenborg et al. (2006)
Bacillus subtilis B7-S	Ferulic acid	Bomgardner (2016)
Bacillus licheniformis SHL1	Ferulic acid	Ashengroph et al. (2011)
Bacillus aryabhattai BA03	Ferulic acid	Paz et al. (2016)
Streptomyces halstedii GE107678	Ferulic acid	Brunati et al. (2004)
Streptomyces setonii ATCC 39116	Ferulic acid	Achterholt et al. (2000)
Lactic acid bacteria	Ferulic acid	Bloem et al. (2007)
Pediococcus acidilactici	Ferulic acid	Kaur et al. (2013)
Amycolatopsissp.sp. HR167	Ferulic acid	Overhage et al. (2006)
Streptomyces sp. V-1	Ferulic acid	Huaet al. (2007)
Amycolatopsis sp. ATCC 39116	Ferulic acid	Fleige et al. (2013)
Bacillus subtilis MTCC 1427	Ferulic acid, Eugenol and Isoeugenol	Rana et al. (2013)
Bacillus subtilis	Isoeugenol	Shimoni et al. (2000)
Arthrobacter sp. TA13	Isoeugenol	Shimoni et al. (2003)
Bacillus fusiformis SW-B9	Isoeugenol	Zhao et al. (2005)
Bacillus subtilis HS8	Isoeugenol	Zhang et al. (2006)
Bacillus fusiformis CGMCC134	Isoeugenol	Zhao et al. (2005)
Bacillus pumilus S-1	Isoeugenol	Hua et al. (2007)
Bacillus coagulans BK07	Ferulic acid	Karmakar et al. (2000)

Table 2.1 (Continued)

Organism	Substrate	References
Psychrobacter sp. CSW4	Isoeugenol	Ashengroph et al. (2012)
Gram-negative bacteria		
Pseudomonas putida KT2440	Ferulic acid	Plaggenborg et al. (2003)
Pseudomonas sp.	Ferulic acid	Agrawal et al. (2003)
Enterobacter sp. Px6-4	Ferulic acid	Li et al. (2008)
Pseudomonas putida I58	Isoeugenol	Furukawa et al. (2003)
Pseudomonas chlororaphis CDAE5	Isoeugenol	Kasana et al. (2007)
Pseudomonas putida IE27	Isoeugenol	Yamada et al. (2007)
Pseudomonas sp. KOB10	Isoeugenol	Ashengroph et al. (2010)
Pseudomonas aeruginosa ISPC2	Isoeugenol	Ashengroph et al. (2011)
Pseudomonas nitroreducens	Isoeugenol	Unno et al. (2007)
Pycnoporus cinnabarinus MUCL39533	Ferulic acid	Alvarado et al. (2001)
Pseudomonas fluorescens AN103	Ferulic acid	Del Carmen Martínez-Cuesta et al. (2005)
Escherichia coli JM109 (pBB1)	Ferulic acid	Barghini et al. (2007)
Pseudomonas fluorescens	Ferulic acid	Dal Bello (2013)
Pseudomonas sp. HR199	Eugenol	Overhage et al. (2000)
Pseudomonas resinovorans SPR1	Eugenol	Ashengroph et al. (2011)
Fungal strains		
Sporotrichum thermophile LWT	Ferulic acid	Topakas et al. (2003)
Aspergillus niger I-1472	Ferulic acid	Tang et al. (2020)
Aspergillus niger CGMCC0774, *Pycnoporus cinnabarinus* CGMCC1115	Ferulic acid	Zheng et al. (2007)
Pycnoporus cinnabarinus	Ferulic acid	Tilay et al. (2010)
Aspergillus niger K8 and *P. crysosporium* ATCC 24725	Ferulic acid	Motedayen et al. (2013)
Streptomyces sp. V-1	Ferulic acid	Yang et al. (2013)
Aspergillus niger I-1472	Isoeugenol	Tan et al. (2015)
Colletotrichum acutatum and Lasiodioplodia theobromae	Ferulic acid	Numpaque et al. (2016)
Aspergillus niger	Eugenol	Srivastava et al. (2010)
Phanerochaete chrysosporium NCIM 1197	Ferulic acid	Karode et al. (2013)

2.3.3 Agro-wastes as a Source for Biovanillin Production

Agro-wastes are considered useless and include plant stalks, leaves, manures, and any other vegetable matter that generally produced through farming activities. Accumulation of such huge agro-wastes poses serious threat to the environment and human health. These wastes are rich in essential organic components like carbon, nitrogen, and many micronutrients, which can be used as ideal substrates for the microbial fermentation and thus agro-residues are successfully recycled for the production of several metabolites. Agro-wastes are vital renewable natural resources, and they are cheap and readily available. Employing agro-wastes rich in ferulic acid esters as the raw material for cleaner production of vanillin have been a well-supported way to dispose and valorize the waste. Lignin, a complex phenolic polymer rich in biomass, is obtained by sulfite pulping of wood, which was used as a substrate for the production of vanillin (da Silva et al., 2009). However, when using waste from this industry, this method is not recognized as a "green technology," since vanillin is produced together with additional wastes, which needs to be taken into account as unsustainable and not as environmentally friendly. As a result, there is an instant opportunity for the production of vanillin from other agricultural wastes. Potential agro-wastes for the transformation of biovanillin are highly dependent on cereal bran, sugar beet pulp, and rice bran oil (Pandey et al., 2000; Das and Singh, 2004). Ferulic acid is found abundance in rice endosperm cell wall, sugar beet pulp, bamboo shoots, Oat flakes, red beet, soybean, etc. (Table 2.2). Cereal bran is a plentiful by-product of the agricultural process, which contain significant amount of ferulic acid in the form of dimerized or esterified with polysaccharides and proteins (Boz, 2015) and has been delineated as a possible supply of ferulic acid for producing vanillin. Di Gioia et al. (2007) used the hydrolysate of wheat bran for the first time for producing vanillin (0.375 mM/l) through bioconversion of ferulic acid from an agro-waste with a recombinant *E. coli* JM109 (pBB1). *Pediococcus acidilactici* has produced 1.06 g/l of vanillin through bioconversion of the substrate rice bran (Chakraborty et al., 2016). Chattopadhyay et al. (2018) has shown 708 mg/l of vanillin production from wheat bran as a substrate using *Streptomyces sannanensis*. In a two-step bioconversion, *A. niger* CGMCC0774 and *P. cinnabarinus* CGMCC1115 are shown to produce vanillin, where 4 g/l of ferulic acid is converted to 2.2 g/l of vanillic acid by *A. niger*, which is further converted to 2.8 g/l of vanillin by *P. cinnabarinus* (Zheng et al., 2007). Similarly, oil palm is used as a source of ferulic acid in a two-step bioconversion with *A. niger* and *P. cinnabarinus*, which has produced 237 mg/l of vanillin (Lesage-Meessen et al., 1996). Sugar beet pulp has also been utilized as main precursor for biovanillin production in two-step bioconversion, where *A. niger* has transformed 834 mg/l of ferulic acid to 357 mg/l vanillic acid within six days, further vanillic acid is converted to 105 mg/l vanillin using *P. cinnabarinus*

Table 2.2 Amount of ferulic acid in different known natural sources.

Source	Ferulic acid (mg/0.1 kg)
Refined maize bran	2610–3300
Soft and hard wheat bran	1351–1456
Rice endosperm cell wall	900
Sugar-beet pulp	800
Wheat bran	660
Bamboo shoots	243.5
Rye bran	280
Maize, dehulled kernels	174
Barley grain	140
Whole-wheat kernels	64–127
Whole-wheat flour	89
Whole-grain rye flour	86
Whole brown rice	42
Maize flour	38
Whole oats	25–35
Oat bran	33
Red beet	25
Soybean	12
Grapefruit	10.7–11.6
Orange	9.2–9.9
Peanut	8.7
Water dropwort	7.3–34
Eggplant	7.3–35
Spinach	7.4
Banana	5.4
Tomato	0.29–6
Radish	4.6
Broccoli	4.1
Carrot	1.2–2.8
Avocado	1.1
Berries	0.25–2.7

(Lesage-Meessen et al., 1999). On the other hand, *P. chrysosporium* ATCC 24725 has produced 0.5 g/l of vanillin from 0.750 g/l of ferulic acid of lemongrass when used as a substrate (Galadima et al., 2020). Similarly, from the pineapple crown leaves and peel, 34 and 109 μmol of ferulic acid is converted to 2.5 and 5 mg/l of vanillin, respectively (Tang and Hassan, 2020).

2.4 Strain Development for Improved Production of Vanillin

2.4.1 Metabolic and Genetic Engineering

The characteristics property like rapid growth rates and less complexions to molecular genetics, microorganisms are innovative targets for biotechnology research and due to its ability to grow on versatile carbon sources, microorganisms are preferred so far as good candidates for the production of vanillin. Microbes such as bacteria, fungi, and yeast have been used for the small as well as large-scale production of vanillin from various substrates, such as eugenol, isoeugenol, ferulic acid, and other organic compounds. A drawback of microbial production of vanillin is the excess oxidation and reduction of end product to vanillic acid and vanillyl alcohol, respectively as well as inhibition of microbial growth by the product vanillin due to toxicity at high concentrations. Due to these major issues, there is a significant decrease in the vanillin yield (Stentelaire et al., 1998). To prevent these bottlenecks and hence to increase the vanillin yield, metabolic engineering and genetic manipulation were applied so far. Table 2.3 enlists several genetically engineered microorganisms, which were used to enhance the vanillin yield from various substrates. Genetic engineering or genetic modification (GM) is a direct manipulation or modification of the organism's genome using recombinant DNA technology. Due to its high production rate, the vanillin production of GM organisms has gained much attention (Barghini et al., 2007; Ni et al., 2015). The metabolic pathway of eugenol was investigated in many species of bacteria and fungi such as *Pseudomonas*, *Byssochlamys*, *Penicillium*, and *Rhodococcus*. To improve the yield of vanillin, genetic engineering was introduced and in this context many attempts have been made so far. For example, Overhage et al., 1999 constructed a mutant *Pseudomonas* sp. strain HR199 by the insertion of a regulatory element (omega element) into the normal vanillin dehydrogenase gene, which was found to accumulate significantly higher concentrations of vanillin (2.9 mM; 0.44 g/l). *Rhodococci* are well known for their metabolic versatility in utilizing various xenobiotic compounds, and thus they are extensively used in wide range of biotransformation processes. Plaggenborg et al. (2006) investigated the potential of *Rhodococcus* sp. for the biotechnological

Table 2.3 Genetically modified (GM) microorganisms for synthesis of biovanillin.

Substrate	Microorganism	Yield (g/l)	References
Eugenol	*Pseudomonas* sp. HR199	0.44	Overhage et al. (1999)
	Two-step process: *E. coli* XL1-Blue and *E. coli* (pSKechE/Hfcs)	0.3	Overhage et al. (2003)
Isoeugenol	*Pseudomonas putida* IE27	16.1	Yamada et al. (2007)
	E. coli BL21(DE3)	28.3	Yamada et al. (2008)
Ferulic acid	*E. coli* strain JM109/pBB1	0.851 mol/l	Torre et al. (2004)
	E. coli strain JM109/pBB1	2.52	Barghini et al. (2007)
	E. coli XL1-Blue (pSkechE/Hfcs)	Trace amount	Overhage et al. (2000)
	E. coli (pDAHEF)	0.58	Yoon et al. (2005a)
	Recombinant *E. coli*	1.1	Yoon et al. (2005b)
	E. coli DH5α (pTAHEF)	1.0	Yoon et al. (2007)
	E. coli (pTBE-FP)	2.1	Song et al. (2009)
	E. coli NTG-VR1	2.9	Yoon et al. (2007)
	E. coli DH5α (pTAHEF-gltA)	1.98	Lee et al. (2009)
	E. coli BW25113 (pTAHEF)	5.14	Lee et al. (2009)

production of vanillin from eugenol by overexpression of *vaoA* gene corresponding to VAO from *P. simplicissimum* CBS 170.90 for the hydrolysis of eugenol to coniferyl alcohol along with *calA* and *calB* genes corresponding to dehydrogenases of coniferyl alcohol and coniferyl aldehyde from *Pseudomonas* sp. HR199, respectively. The transformant efficiently catalyzed conversion of eugenol to vanillin at a rate of 1.22 mmol/h/l of culture. Likewise, vanillyl alcohol oxidase gene (*vaoA*) from *P. simplicissimum* CBS170.90 has been expressed in *Amycolatopsis* sp. HR167, which efficiently converted eugenol to coniferyl alcohol with a maximum yield of 4.7 g/1 after 16 hour biotransformation (Overhage et al., 2006). Such innovative idea of metabolic engineering for vanillin production has been further evaluated with the recombinant strains of *Ralstonia eutropha* H16 (Overhage et al., 2002), *E. coli* XL1-Blue (Overhage et al., 2003) and it is found to have significant increase in vanillin yield. It has been reported previously that isoeugenol is one of the ideal substrate candidates for metabolized into vanillin through an epoxide-diol pathway involving oxidation of side chains of propenylbenzenes. The biotransformation products of isoeugenol are higher than those obtained from eugenol. Later, it is also evaluated that the production of vanillin using

metabolically engineered *E. coli* cells, where isoeugenol monooxygenase of *P. putida* IE27 is overexpressed that produced 28.3 g/l of vanillin from 230 mM of isoeugenol with a molar conversion efficiency of 81%. It also has been reported that no or less accumulation of unwanted byproducts, such as vanillic acid or acetaldehyde, are observed during this conversion (Yamada et al., 2008). Tang et al. (2018) has generated a recombinant *E. coli* expressing a 9-*cis*-epoxycarotenoid dioxygenase gene from *Serratia* sp. (SeNCED), which is known for catalyzing the cleavage of isoeugenol and 4-vinylguaiacol to vanillin. The recombinant *E. coli* produced 0.53 g/l of vanillin (3.47 mM) in eight-hour reaction by utilizing isoeugenol (4 mM) as substrate.

Ferulic acid is well known as a potential substrate for bioconversion to vanillin, which is available abundantly in the natural sources. Ferulic acid is converted to vanillin by the catalytic activity of feruloyl-CoA synthetase (*fcs*) and enoyl-CoA hydratase/aldolase (*ech*). Yoon et al. (2005a) generated a recombinant *E. coli* expressing with *fcs* gene of *Amycolatopsis* sp. HR104 and *ech* gene of *Delftia acidovorans* under the control of P_{BAD} promoter that produced the highest yield of vanillin (580 mg/l) under the optimized conditions in LB medium, which is 3.6-folds higher than the yield obtained before media optimization. Meantime, another *E. coli* genetically engineered to express *fcs* gene of *Amycolatopsis* sp. HR104 and *ech* gene of *D. acidovorans* under the control of IPTG-inducible *trc* promoter in complex medium (2YT) supplemented with ferulic acid (0.2% w/v) has produced highest amount of vanillin (1.1 g/l) (Yoon et al., 2005b). Improvement of the vanillin production has been investigated further by Yoon et al., 2007 by constructing recombinant *E. coli* carrying plasmid *p*TAHEF cloned with both *fcs* and *ech* genes of *Amycolatopsis* sp. and tested with ferulic acid (2.0 g/l) that produced a maximum of 1.0 g/l vanillin in 48 hours. In due course, bacterial growth and vanillin yield decreased due to vanillin toxicity. In order to avoid vanillin toxicity, generation of NTG mutant and removal of excess vanillin from the culture medium using polystyrene resin XAD-2 has been followed. The vanillin production by NTG mutants has increased three times higher when compared with its wild-type strain. Following the use of polystyrene resin (XAD-2) with NTG mutagenized *E. coli*, the production of vanillin has attained a maximum yield (2.9 g/l), which is twofolds higher than that obtained without using the resin (Yoon et al., 2007). It has been presumed that shortage of CoA for catalyzing conversion of ferulic acid to feruloyl-CoA due to its utilization as acetyl-CoA, could be a reason the scoring less yield of vanillin biotechnologically. To circumvent this impediment, another recombinant *E. coli* carrying plasmid *p*TAHEF is cloned with *gltA* encoding citrate synthase, which produced 1.98 g/l of Vanillin (Lee et al., 2009). For improving the potential of microbial sources for higher production of vanillin, many metabolic optimizations and genetic manipulations have been carried out so far using various organic

compounds as substrates, though more attempts are needed to further enhance yield of biovanillin production to meet higher demand of vanillin.

2.5 Bioactive Properties of Vanillin

2.5.1 Antimicrobial Activity

Resistance of pathogenic bacteria toward majority of antimicrobial drugs has been prominently developed, which warrants the need for new antivirulence drugs for their effective control. Research and development of innovative antimicrobial agents to target various pathogenic bacterial virulence factors is going on since back two decades, but few drugs showing significance in their efficacy. Phytochemicals and microbial transformed metabolites or bioactive metabolites show promising antimicrobial activity by targeting various virulence factors. As we know quorum sensing is a bacterial signaling pathway for communicating to their external environments and in turn quorum sensing facilitate the pathogenic bacteria to enhance their pathogenesis by regulating the expression patterns of various virulence factors gene and associated factors (Deep et al., 2011). Targeting various pathways related to quorum sensing has become a novel and innovative approach for designing antibacterial quorum-sensing agents. Antiquorum-sensing drugs have the ability to affect bacterial pathogenicity because drug affects various factors and pathways involved in quorum sensing pathway in different ways. Vanilla and vanilla-containing foods might promote antimicrobial activity by suppressing quorum sensing and their intermediate pathways particularly colonization and biofilms formation (Jakobsen et al., 2012). For example, *Chromobacterium violaceum* is a Gram-negative bacterium that produces violacein pigment, a molecule that helps this bacterium to communicate with external environment, whose expression is controlled by quorum sensing. Recently, it has been evidenced that vanilla-containing food materials promote inhibition of violacein production and thus help in breaking quorum sensing and in turn also arrest the communication among bacteria and environments around it (Asfour, 2018).

2.5.2 Antioxidant Activity

Reactive oxygen and nitrogen species (ROS and RNS) are generated by normal metabolic pathway in normal body, and the production of ROS and RNS increased day by day, if body is exposed more and persistently to the unwanted higher intake of dietary xenobiotics and as well as unhealthy food. The ROS and RNS create oxidative stress by altering normal cellular functions of our body resulting in

various diseases such as diabetes, hypertension, cancer, and many other complications. Naturally produced antioxidants such as various bioactive phenolic compounds naturally found in dietary sources can be effectively toiled to lower down these oxidative stresses. Certain genes encoding the enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GSHPx) help to neutralize oxidative stress, and findings of naturally derived compounds to enhance expression of these genes are now days prominently explored by researchers (Kurutas, 2016). The antioxidant effect of vanillin has been reported by Deters (2008), where vanillin had moderate free radical scavenging activity (IC_{50} of 2945 ± 247 μM) and also showed mild antioxidant activity in mouse liver microsomes (Yan-Chun and Rong-Liang, 1991). Likewise, antioxidant activity of vanillin is investigated using multiple antioxidant assays, which has shown potent antioxidant activity in the $ABTS^{\cdot+}$ scavenging assay, ORAC assay, and in OxHLI assay, while no activity has been noticed in the DPPH radical scavenging assays (Tai et al., 2011).

2.5.3 Anticancer Activity

2.5.3.1 Apoptosis Pathway

Mitochondrial-dependent apoptosis is an important pathway for the induction of apoptosis, and any disturbance in this pathway could inhibit apoptosis. The apoptosis pathway is regulated by the B-cell lymphoma-2 (Bcl-2) family proteins and this Bcl-2 regulates (elevation or downregulation) the mitochondrial membrane permeability for the release of cytochrome-c and other apoptotic proteins. Furthermore, caspase activation and DNA fragmentation are the main features of induced apoptosis. Xie et al. (2020) have shown that vanilla-containing food-induced apoptosis in HT-29 colon cancer cells by inducing oxidative damage and also modulated the expression of apoptotic markers. Gupta et al. (2010) have reported that vanilla and vanilla-containing food downregulates Bcl-2, which causes upregulation of Bax and thereby increases the permeability of the mitochondria to apoptosis-inducing factor (AIF), which is then released to cytosol from the mitochondria. AIF in the cytosol-activated caspases-3, 7, and 9, which causes DNA fragmentation and finally induced cell death by apoptosis.

2.5.3.2 Tumor Necrosis Factor-induced Apoptosis

Tumor necrosis factor (TNF) also triggers apoptosis, which is a promising anticancer target by which cancer cells are killed selectively with negligible effect on normal cells. In the HeLa cells, pretreatment of vanillin-enhanced tumor necrosis factor-related apoptosis-induced cell death by induced phosphorylation of p65 and transcriptional activity of NF-κB (Lirdprapamongkol et al., 2010).

2.5.3.3 Cell Cycle Arrest

Cells are regulated at different checkpoints by the interactions of various cyclins and cyclin-dependent kinases (CDKs). Before the progression to the next phase of the cell cycle, cell growth is regulated at each checkpoints by cyclin and CDKs. In cell cycle regulation, cyclin, CDKs, and p53 play a key role. Recently, vanilla has shown its potential to arrest cell cycle in colorectal cancer cells at the G1/G0 stage at lower concentration (200 μg/ml), and G2/M arrest occurs at higher concentration of vanillin (1000 μg/ml) (Ho et al., 2009).

2.5.3.4 Nuclear Factor κB (NF-κB) Pathway

NF-κB is one of the transcription factors, which is abnormally regulated in disease like cancer. Activation of NF-κB has been documented in various cancers, including liver, colon, pancreas, breast, prostrate, ovarian, leukemia, and lymphoma cancers and others also. DNA damaging also activates NF-κB, which in turn activates and regulates a number of NF-κB-influenced target genes, including inducible nitric oxide synthase, and COX-2. Furthermore, binding of TNF-α to TNFR leads to homotrimerization of receptors and adaptor proteins resulting in cell proliferation and survival by increasing the expression of NF-κB and activator protein 1 target genes, including vascular cell adhesion molecule-1 (VCAM-1). NF-κB activation triggers the activation of chemokines and its related receptors, including C-X-C chemokine receptor 4 (CXCR4) and CCR7, which play crucial role in cancer cells' migration to target organs. These genes play major roles in antiapoptosis process. Vanilla-containing foods have the potential of therapeutic efficacy against cancer by inhibiting NF-κB pathway activation in cancer cells. In lipopolysaccharide-induced MCF-7 cells, Z138 cells, T24, and THP1 cells, vanilla inhibits proliferation, adhesion, migration, and invasion by regulating the NF-κB pathway. In the NF-κB pathway, vanilla also reported to inhibit the expression of inflammatory factors TNF-α and IL-6 and abnormal NF-κB activation through inhibition of phospho-IκBα, p65 and upregulation of miR16 (Lirdprapamongkol et al., 2010). Vanilla also showed the inhibition of the NF-κB nuclear translocation leading to the inhibition of mRNA expression and COX-2, VCAM, and ICAM proteins.

2.5.4 Anti-sickling Activity

Sickle cell disease (SCD) is a genetic disorder caused by a point mutation in the β-globin gene, where glutamic acid is replaced by valine at the sixth position of the β-chain of hemoglobin (Hb). In 1991, vanillin, a natural flavoring agent, has been reported as anti-sickling agent (Abraham et al., 1991). The results have shown that vanillin covalently binds with HbS and helps in increasing its oxygen carrying affinity and thus vanillin has a moderate anti-sickling property. However,

vanillin showed poor oral bioavailability and thus demands for other mode of administration into the body. To overcome this issue, Zhang et al. (2004) synthesized a vanillin prodrug, MX-1520, which can be biotransformed into vanillin *in vivo*. Oral administration of MX-1520 prior to hypoxia exposure in the transgenic sickle mice exhibited significant reduction in the number of sickled cells, which clearly evidenced the potential of MX1520 as a safe antisickling agent for SCD patients.

2.5.5 Hypolipidemic Activity

Vanillin has exerted significant reduction in the levels of serum triglyceride, VLDL-C, and total cholesterol in high-fat diet-induced hyperlipidemic rats (Belagali et al., 2013). Likewise, vanillin-containing foods showed positive results in reducing obesity in high-fat diet-induced obese mice, wherein vanillin reduced abnormal elevation of inflammatory factors including IL-6 and TNF-α in plasma and liver tissue results from obesity in high-fat-induced mice (Guo et al., 2018).

2.6 Conclusion

The organoleptic compound vanillin has a wide application in number of industries. Among them, vanillin has received much attention by food industry due to its flavoring property. There are three modes of production of vanillin: natural, chemical, and biotechnological synthesis. Only natural and biotechnological vanillin is recommended by food-safety authorities worldwide. The annual production of vanillin in global market is reported to be 5361 metric tons among which the contribution of natural vanillin is much low. In order to meet the world's requirement, many advanced biotechnological methods such as strain development, metabolic engineering, genetic engineering, and enzymatic production have been widely investigated. Yet, only very less biotechnological methods have been employed in industries. Thus, applying metabolically engineered strains in industrial production would greatly increase the annual production rate of vanillin.

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3

Antimicrobials

Targets, Functions, and Resistance

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3.1 Introduction

Antimicrobials are natural, semisynthetic, or synthetic small molecules that are used to kill or prevent the growth of infecting microbes with minimal damage to the host. On the other hand, antibiotics are small molecules that are produced by a microbe and can effectively kill/inhibit other microbes. Therefore, by strict definition, antimicrobials that are synthetic or semisynthetic or plants/animal derived are not antibiotics, but, all antibiotics are antimicrobials. In general, antimicrobials include all antibacterial, antifungal, antiviral, and antiparasitic drugs. They have been proved to be the effective therapeutics in the treatment of infectious diseases, one of the major reasons for human illness and death. Today, antimicrobials are the most widely applied pharmaceutical agents across the globe.

3.2 Classification of Antibiotics

Antibiotics can be categorized based on their range of activity or mode of action (Adzitey, 2015).

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3.2.1 Classification of Antibiotics Based on Mode of Action: Bactericidal and Bacteriostatic

Antibiotics can be classified in two categories based on their mode of action against the microbes: bacteriostatic and bactericidal. Agents that kill microbes by rupturing their cell wall and/or cell membrane are called bactericidal and antibiotics that delay or inhibit the growth of microbes are bacteriostatic agents. The bacteriostatic agents inhibit the fundamental cellular events of microbes like protein synthesis or other metabolic pathways. Some bacteriostatic and bactericidal agents and their mode of actions are represented in Table 3.1 (Nemeth, Oesch, & Kuster, 2015;

Table 3.1 Some bacteriostatic and bactericidal antibiotics.

A. Bacteriostatic antibiotics	**Function**
Sulfonamides	Inhibit folate synthesis
Amphenicols, e.g. chloroamphenicol	Protein synthesis inhibitors
Spectinomycin	Interrupts protein synthesis by binding to the 30S ribosomal subunit
Trimethoprim	Disturbs the tetrahydrofolate synthesis pathway
Tigecycline; it belongs to the glycylcycline class	Inhibits proteins synthesis by reversibly binding to the 30S bacterial ribosomal subunit
Erythromycin, azithromycin and clarithromucin are macrolides	Protein synthesis inhibitors
Linezolid is a member of the oxazolidinone class	Inhibiting the initiation process of protein synthesis
Tetracycline, minocycline, and doxycycline belong to tetracyclines class	Protein synthesis inhibitors
B. Bactericidal antibacterials	**Function**
β-Lactam antibiotic: Penicillins, e.g. penicillin V, penicillin G, benzathine penicillin G, procaine penicillin G, cloxacillin, methicillin, oxacillin, flucloxacillin, and dicloxacillin	
β-Lactam/β-lactamase inhibitors: Carbapenems, e.g. meropenem, imipenem, aztreonam, piperaciintazobactam, and ticaracillinclvulnate; cephalosporins, e.g. ceftazidime, cefepime, ceftriaxone, and cefotaxime	Interfere the synthesis of the bacterial cell wall
Aminoglycosides: Gentamicin, amikacin, and tobramycin	Protein synthesis inhibitors
Quinolones and flouroquinolones: ciprofloxacin, oxifloxacin, and levofloxacin	Block bacterial DNA replication
Glycopeptide: Vancomycin	Inhibit cell wall synthesis
Polymyxins: Polymyxin B and colistin	Disrupt bacterial cell membrane

Pankey & Sabath, 2004). Bactericidal agents are very efficient in eliminating infective microbes and decrease the possibility of resistance development or infection recurrence. However, they also stimulates cytokine production and harmful inflammation due to the surge of endotoxins (lipopolysaccharide in Gram-negative bacteria and peptidoglycans in Gram-positive bacteria) (Finberg et al., 2004). Bacteriostatic agents can be effective in resting slow-growing bacteria, not affected by bactericidal agents but also associated with the risk of infection recurrence or antibiotic resistance (Pankey & Sabath, 2004).

3.2.2 Classification of Antibiotics Based on the Spectrum of Action: Broad- and Narrow-spectrum Antibiotics

Antibiotics can also be classified based on their targets: broad-spectrum and narrow-spectrum. The narrow spectrum antibiotics act against a narrow range of microorganisms and are genus or species specific. The broad-spectrum antibiotics are effective against a wide range of pathogens including Gram-positive and Gram-negative strains. In general, if the causative agent of infection is known, the narrow spectrum antibiotics are preferred since they act specifically with the target bacteria and do not affect the normal commensal microbes in the body. Therefore, they have less ability to cause superinfection as well as antibiotic resistance. Table 3.2 depicts the diverse examples of broad- and narrow-spectrum antibiotics (Acar, 1997). Broad-spectrum antibiotics are very flexible drugs and are advantageous when the infectious microbes are not known or unidentified.

3.3 Antibacterial Agents

Antibacterial agents are used to kill or inhibit the growth of pathogenic bacteria. Penicillins, cephalosporins, macrolides, fluoroquinolones, sulfonamides, tetracyclines, and aminoglycosides are the most effective antibacterial agents (Table 3.3, Figures 3.1–3.3) (Bertino Jr & Fish, 2000; Cheesman, Ilanko, Blonk, & Cock, 2017; Chopra & Roberts, 2001; Kołaczek, Fusiarz, Ławecka, & Branowska, 2014; Lima, da Silva, Barbosa, & Barreiro, 2020).

3.3.1 Penicillins

Revolution of antimicrobials started with the discovery of penicillin by in 1928 from *Penicillium notatum* (Gaynes, 2017). For this, Sir Alexander Fleming was awarded a share of the 1945 Nobel Prize for Physiology or Medicine. The natural or "first-generation" penicillins are bactericidal in nature and naturally obtained from *Penicillium chrysogenum* (Kardos & Demain, 2011). The basic structures of penicillins consist of a thiazolidine ring linked to a β-lactam ring with a variable side chain (Figure 3.1a). Semisynthetic modifications of the side chains of natural penicillins give rise to second, third, and fourth generations of penicillins

Table 3.2 Examples of broad- and narrow-spectrum antibiotics.

Group	Broad-spectrum antibiotics	Narrow-spectrum antibiotics
Peniclins	Ampicillin and its derivative amoxicillin. Amoxicillin/clavulanic acid and temocillin	β-Lactamase-sensitive: penicillin G, benzathine penicillin G, procaine penicillin, penicillin V, propicillin, pheneticillin, azidocillin, penamecillin, and clometocillin β-Lactamase-resistant: Cloxacillin (dicloxacillin flucloxacillin), oxacillin, methicillin, nafcillin
Cephalosporins	Cephalosporins (third, fourth, and fifth generations)	Cephalosporins (first generation and second generation)
Quinolones	Ofloxacin, norfloxacin, lomefloxacin, ciprofloxacin, gatifloxacin, levofloxacin, moxifloxacin, gemifloxacin, cinoxacin, gemifloxacin, trovafloxacin, sparfloxacin, and nalidixic acid	
Aminoglycosides	Gentamicin, kanamycin A, amikacin, dibekacin, sisomicin, tobramycin, neomycins B, C, and neomycin E (paromomycin), netilmicin	Streptomycin
Glycopeptide	Vancomycin	
Macrolides	Erythromycin, clarithromycin, azithromycin, dirithromycin, and roxithromycin	
Tetracyclins	Tetracycline, demeclocycline, chlortetracycline, lymecycline, tigecycline, methacycline, minocycline, meclocycline, and oxytetracycline	Sarecycline
Amphenicol	Chloramphenicol	
Carboxypenicillin	Ticarcillin	
Rifamycin	Rifamycins	Rifampin
Carbepenems	Imipenems	
Others	Sulfonamides, nitroimidazoles	Clindamycin, isoniazid, pyrazinamide, ethambutol, bacitracin, polymixins

Table 3.3 Overview of antibacterial agents.

Group	Drug	Administration route	Adult dose	Mechanism of action
Penicillins	Penicillin	Oral or intravenous or intramuscular	250–500 mg every 6–8 hours for 7–20 days (oral) 3–4 million units/kg per 6–8 hours (IV) 2.4 million units/kg (IM)	Inhibition of cell wall synthesis
	Amoxicillin		250–500 mg three times a day	
Cephalosporins	Cephalexin	Oral	1–4 g daily in four divided doses	
Macrolides	Erythromycin	Oral	1–4 g daily in divided doses for 7–21 days	Inhibition of protein synthesis by binding to 50S ribosomal subunit
	Clarithromycin		250–500 mg twice daily for 7–14 days	
	Azithromycin		250–500 mg once daily for 5–7 days	
Fluoroquinolones	Ciprofolxacin	Oral and Intravenous	250–500 mg every 12 hours for 7–10 days (oral) 200–400 mg every 8 hours (intravenous)	Inhibits bacterial replication and transcription by inhibiting DNA gyrase
	Levofloxacin		250–750 mg once daily for 7–14 days (oral) 500 mg daily (intravenous)	
	Ofloxacin		200–400 mg every 12 hours for 3–10 days (oral)	
Sulfonamides	Sulfamethoxazole–Trimethoprim	Oral	100 mg twice daily for 10 days (oral)	Inhibition of bacterial biosynthesis of folic acid
Tetracyclines	Tetracycline	Oral	250–500 mg three to four times daily for 7–30 days (oral)	Inhibit the 30S ribosomal subunit
	Doxycycline			
Aminoglycosides	Gentamicin	Intravenous or intramuscular	3–5 mg/kg/day IV or IM	Inhibit protein synthesis by binding to the A-site on the 16S ribosomal RNA of the 30S ribosome
	Tobramycin			

(Figure 3.1b). Later generation of penicillins differ among each other with respect to β-lactamase susceptibility, pharmacokinetic properties, and antibacterial spectrum. Several first-generation penicillins are available such as penicillin G, penicillin V, penicillin G procaine, penicillin G benzathine, and aqueous crystalline penicillin G. The addition of an extra amino group to natural penicillin generates aminopenicillins with the advantage of broader spectrum of activity against pathogenic bacteria. One of the aminopenicillins or "third-generation" penicillin is amoxicillin (Weber, Tolkoff-Rubin, & Rubin, 1984) (Figure 3.1b).

3.3.1.1 Mechanism of Action

Most of the bacteria are surrounded by peptidoglycan cell wall that provides structural integrity and protection from osmotic lysis. The peptidoglycan cell wall is built up of repeating units of *N*-acetyl glucosamine (NAG) and *N*-acetylmuramic acid (NAM) connected by a β-(1-4) glycosidic linkage (Silhavy, Kahne, & Walker, 2010).

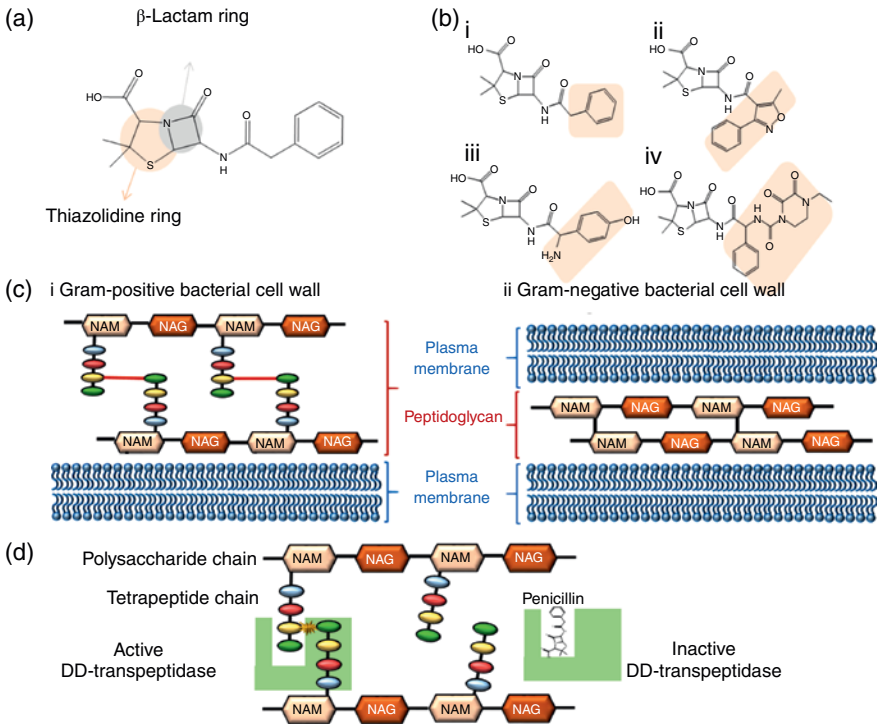

Figure 3.1 (a) Basic structure of penicillin; (b) structures of i. natural penicillin, ii. first generation, iii. second generation, and iv. third generation of penicillin; (c) peptidoglycan cell wall of i. Gram-positive bacterial and ii. Gram-negative bacterial; (d) mechanism of action of penicillin.

The cross-linkage process is catalyzed by the enzyme DD-transpeptidase, a penicillin-binding protein (PBP) (Lobanovska & Pilla, 2017). Compared to Gram-positive bacteria, Gram-negative bacteria consist of a thinner peptidoglycan cell wall, instead they contain a thick outer membrane made up of lipopolysaccharide (Costerton, Ingram, & Cheng, 1974) (Figure 3.1c). The β-lactam ring of penicillin irreversibly inactivates DD-transpeptidase enzyme and inhibits the cross-linkage of the peptidoglycan structure (Figure 3.1d). The fragments of peptidoglycan also activate autolysins and hydrolases. The weak cell wall structure causes the entry of water into the cell due to osmotic pressure which lyse the bacterial cell (Gordon, Mouz, Duee, & Dideberg, 2000) (Figure 3.3).

3.3.1.2 Clinical Implications

3.3.1.2.1 Penicillin Penicillin is effective against Gram-positive Cocci *(e.g., Staphylococcus)*, Gram-negative Cocci (e.g., *Neisseria*), Gram-positive rods (e.g., *Listeria*), Gram-negative rods, and most anaerobes (Friedland & McCracken Jr, 1994). Later generation of penicillins are active against *Escherichia coli*, *Shigella*, *Salmonella*, *Proteus mirabilis*, *Haemophilus influenzae*, *Klebsiella*, *Enterococci*, *Pseudomonas aeruginosa,* and *Bacteroides fragilis* (Perry & Markham, 1999).

3.3.1.2.2 Amoxicillin Amoxicillin is Food and Drug Administration (FDA) approved for the treatment of genitourinary tract infections, respiratory tract infections (such as otitis media, sinusitis, and bronchitis), ear, nose, throat infections, tonsillitis, pharyngitis, and skin infections (Chow et al., 2012). Amoxicillin is active against Gram-positive strains like *Streptococcus* species, *Listeriamonocytogenes* and *Enterococcus*. It also covers some Gram-negative strains like *Actinomyces*, *H. influenzae*, *E. coli, Clostridium* spp., *Shigella, Salmonella,* and *Corynebacterium*.

3.3.2 Cephalosporins

Cephalosporins are another family of bactericidal antibiotic which were first obtained *Cephalosporum acremonium* (a fungus). Similar to penicillin, their basic structure includes a thiazolidine, β-lactam ring, and a variable side chain (Figure 3.2a). Five generations of cephalosporins have been found with a diverse range of antibacterial activities. Cephalexin (Keflex) is a first-generation semisynthetic derivative of the cephalosporin, effective against both Gram-positive and Gram-negative bacteria (Speight, Brogden, & Avery, 1972).

3.3.2.1 Mechanism of Action

Cephalosporins binds and inhibits PBPs present in the bacterial cell wall and cause bacterial cell lysis. Therefore, cephalosporins are also bactericidal antibiotics.

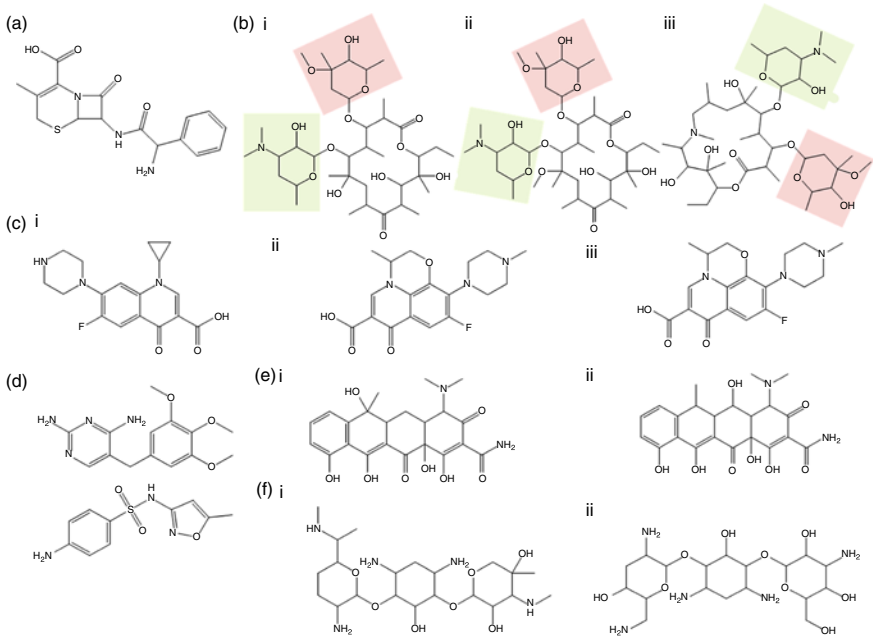

Figure 3.2 Chemical structures of antibiotics. (a) Cephalexin; (b) i. Erythromycin, ii. Clarithromycin, iii. Azithromycin; (c) i. Ciprofloxacin, ii. Levofloxacin, iii. Ofloxacin; (d) Sulfamethoxazole–Trimethoprim; (e) i. Tetracycline ii Doxycycline; and (f) i. Gentamicin, ii. Tobramycin.

However, the degree of antibacterial activity of cephalosporins depends on the range of PBPs of the bacterial cell wall that they inhibit (Speight et al., 1972) (Figure 3.3).

3.3.2.2 Clinical Indications

3.3.2.2.1 Cephalexin (Keflex) Cephalexin (Keflex) is effective against many Gram-positive Cocci like penicillinase producing *Staphylococcus aureus*. It is recommended for the infections of the genitourinary system, upper and lower respiratory tract, soft tissue, skin, bones, and joints (Speight et al., 1972). Second-generation cephalosporins are effective against *Enterobacter, Citrobacter, Neisseria, H. influenzae,* and *Serratia* species. Third-generation cephalosporins are applied against Gram-negative bacteria. Fourth- and fifth-generation cephalosporins are the drug of choice for varied range of both Gram-positive and Gram-negative bacteria.

3.3.3 Macrolides

The macrolides (MLs) show bacteriostatic activity against broad-spectrum of Gram-positive bacteria (Ozumchelouei, Hamidian, Zhang, & Yang, 2020). The chemical structure of macrolides includes a large macrocyclic lactone ring that are attached to one or more deoxy sugars, usually cladinose (red) and desosamine (green) (Figure 3.2b). Macrolides are mild acids, poorly water-soluble, and lipophilic antibiotics (Carvalho & Santos, 2016).

3.3.3.1 Mechanism of Action

Macrolides binds 50S ribosomal subunit and inhibit bacterial protein synthesis (Shaeer, Chahine, Varghese Gupta, & Cho, 2019) (Figure 3.3).

3.3.3.2 Clinical Indications

Macrolides are active against many strains of *Staphylococci, Streptococci, Listeria, Clostridia, Corynebacteria, Neisseria meningitides,* and *Haemophilus, moxicella* (Shaeer et al., 2019). Clinical applications of some important macrolides are described below.

3.3.3.2.1 Erythromycin (E-Mycin) Erythromycin is a first-generation macrolide and bacteriostatic against many Gram-positive bacteria. Erythromycin is advocated in the treatment of mild-to-moderate upper or lower respiratory tract infections, urethritis, pelvic inflammatory disease, urogenital chlamydia infections, Legionnaires' disease, and intestinal amebiasis (Shaeer et al., 2019).

3.3.3.2.2 Clarithromycin (Biaxin) Clarithromycin is a second-generation macrolide and a bacteriostatic agent against Gram-positive bacterial strains. In

addition, it is also active against *Chlamydia pneumoniae*, *Helicobacter pylori*, and several atypical mycobacteria. Clarithromycin is used for bronchitis, upper respiratory infections, community-acquired pneumonia, sinusitis, skin and tissue infections (Shaeer et al., 2019).

3.3.3.2.3 Azithromycin (Zithromax) Azithromycin, a second-generation macrolide, is used against Gram-negative *Enterobacteriaceae* and many Gram-positive bacteria (Girard et al., 1987). Azithromycin is found to be effective against many "atypical" bacteria including legionella (i.e., *Legionella pneumophila*), chlamydiae (e.g., *Chlamydia trachomatis* and *Chlamydophila psittaci*), mycobacteria (e.g., *Mycobacterium avium*), and mycoplasma (e.g., *Mycoplasma pneumoniae*). This antibiotic has FDA approval for the treatment of *Streptococcus pyogenes*-mediated pharyngitis, community-acquired pneumonia (CAP), other upper respiratory infections, including acute otitis media and acute exacerbation of chronic obstructive pulmonary disease (Goldman, Fesik, & Doran, 1990).

3.3.4 Fluoroquinolones

The fluoroquinolones are a family of broad-spectrum bactericidal agents (Bertino Jr & Fish, 2000). Some common second-generation fluoroquinolones are ofloxacin (Floxin), ciprofloxacin (Cipro), and levofloxacin (Levaquin) (Figure 3.2c).

3.3.4.1 Mechanism of Action

The fluoroquinolones inhibits bacterial transcription and DNA replication by targeting bacterial DNA-gyrase or topoisomerase II and topoisomerase IV (Bertino Jr & Fish, 2000; Sharma, Jain, Jain, Pahwa, & Yar, 2010) (Figure 3.3).

3.3.4.2 Clinical Indication

3.3.4.2.1 Ciprofloxacin Ciprofloxacin is the FDA-approved potent antibiotic agent has been applied against a spectrum of Gram-negative bacteria (for instance *Salmonella* spp., *Escherichia coli*, *Neisseria,* and *Shigella* spp.) (Sharma et al., 2010). It is a treatment choice for urinary tract infections, lower respiratory tract infections, skin, bone and joint infections, gonorrhea, typhoid fever, anthrax, plague, gastrointestinal infections, and salmonellosis (Sharma et al., 2010).

3.3.4.2.2 Levofloxacin Levofloxacin is mostly effective against Gram-positive strains (Bush, Chaparro-Rojas, Okeh, & Etienne, 2011; Huang, Li, Kong, Wang, & Zhu, 2014). It is used for mild-to-moderate infections including bronchitis, sinusitis, skin infections, community-acquired pneumonia, prostatitis, pyelonephritis, urinary tract infections, anthrax, and plague (Bush et al., 2011; McGregor, Allen, & Bearden, 2008). It is also effective against other common

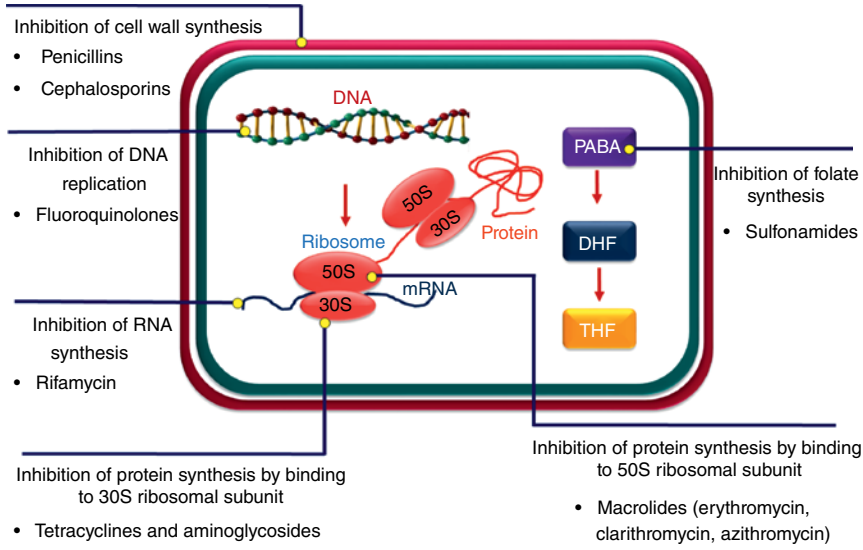

Figure 3.3 Mechanism of actions of antibacterial agents.

respiratory microorganisms such as *H. influenzae*, *Moraxella catarrhalis*, *Legionella* spp, *Mycoplasma* spp, and *Chlamydia pneumonia* (Bush et al., 2011; McGregor et al., 2008).

3.3.4.2.3 Ofloxacin Ofloxacin is a broad-spectrum antibiotic that exhibits a bactericidal effect (Bertino Jr & Fish, 2000). Ofloxacin can be used to treat infections like chronic suppurative otitis, conjunctivitis, corneal ulcers, chronic bronchitis, pneumonia, skin infection, gonorrhea, and urinary tract infections (Kumar, Girdhar, & Girdhar, 2015).

3.3.5 Sulfonamides

Sulfonamides (sulfa drugs) are a type of synthetic antimicrobials with sulfonamide group. Sulfonamides are generally used in formulation like trimethoprim-sulfamethazole (Kołaczek et al., 2014; Smith & Powell, 2000) (Figure 3.2d).

3.3.5.1 Mechanism of Action

Sulfonamides are bacteriostatic antibiotics and inhibit the folic acid synthesis, which is essential for bacterial nucleic acid (DNA and RNA) synthesis (Kołaczek et al., 2014; Smith & Powell, 2000). Sulfonamides can be combined with trimethoprim to make them bactericidal. Trimethoprim inhibits different enzyme of folic acid biosynthesis pathway (Kołaczek et al., 2014; Smith & Powell, 2000). Therefore,

together, they are synergistic in inhibition of folate synthesis (King, Muggleton, Lewis, & Sternberg, 1992; Smith & Powell, 2000) (Figure 3.3).

3.3.5.2 Clinical Indication

3.3.5.2.1 Sulfamethoxazole-Trimethoprim Trimethoprim-sulfamethoxazole is widely used against urinary tract infections and parasitic (toxoplasmosis, *Pneumocystosis jiroveci*) and malarial infections (Kołaczek et al., 2014; Smith & Powell, 2000). It has a varied range of activity against both Gram-positive and -negative microbes (Kołaczek et al., 2014).

3.3.6 Tetracyclines

Tetracycline is a broad-spectrum antibiotic (Chopra & Roberts, 2001; Sapadin & Fleischmajer, 2006). Three generations of tetracyclines are available. First-generation tetracyclines include oxytetracycline, chlortetracycline, demethylchlortetracycline, and tetracycline (Chopra & Roberts, 2001; Sapadin & Fleischmajer, 2006). Second-generation tetracyclines include lymecycline, rolitetracycline, minocycline, and doxycycline (Chopra & Roberts, 2001; Sapadin & Fleischmajer, 2006). A third-generation tetracycline is tigecycline (Chopra & Roberts, 2001; Sapadin & Fleischmajer, 2006). More general forms of tetracycline include tetracycline (Sumycin, Panmycin) and doxycycline (Vibramycin) (Figure 3.2e).

3.3.6.1 Mechanism of Action

Tetracyclines are bacteriostatic agent that inhibit bacterial protein synthesis. It binds 30S ribosomal subunit and hinder the aminoacyl-tRNA binding to the acceptor (A) site on the mRNA-ribosome complex (Chopra & Roberts, 2001; Sapadin & Fleischmajer, 2006) (Figure 3.3).

3.3.6.2 Clinical Indication

3.3.6.2.1 Tetracyclines Tetracyclines a reactive against a broad spectrum of Gram-negative and Gram-positive bacteria (Sánchez, Rogers III, & Sheridan, 2004). It is used in the treatment of anaplasmosis, rickettsial infections, amebiasis, leptospirosis, actinomycosis, brucellosis, nocardiosis, melioidosis, tularemia, pelvic inflammatory disease, chlamydial infections, syphilis, early lyme disease, acne, traveler's diarrhea, legionnaire's disease, and whipple disease (Sánchez et al., 2004).

3.3.6.2.2 Doxycycline Doxycycline is active against a wide range of Gram-positive and Gram-negative organisms. They are also potent drug against spirochetes, rickettsia, mycoplasma, and chlamydia (Tan, Magill, Parise, & Arguin, 2011). Indications include skin, or soft tissue infections, upper respiratory,

gonorrhea, and syphilis with penicillin allergy, acute pelvic inflammatory disease, non-gonococcal urethritis, oorchitis, lyme disease, epididymitis, and traveler's diarrhea (Cross, Ling, Day, McGready, & Paris, 2016; Tan et al., 2011).

3.3.7 Aminoglycosides

The aminoglycosides are natural products or semisynthetic derivatives of natural products synthesized by many actinomycetes (K. M. Krause, Serio, Kane, & Connolly, 2016). The common structure of aminoglycosides includes two or more amino sugars linked together by glycosidic bonds to a hexose nucleus (Kotra, Haddad, & Mobashery, 2000; K. M. Krause et al., 2016). Common aminoglycosides are gentamicin (Garamycin) and tobramycin (Tobrex) (Figure 3.2f).

3.3.7.1 Mechanism of Action

Aminoglycosides binds the 30S subunit of ribosomes and leads to the error prone protein synthesis causing damage to the cell membrane (Kotra et al., 2000; K. M. Krause et al., 2016). Some aminoglycosides also inhibit initiation and elongation stages of protein synthesis (Kotra et al., 2000; K. M. Krause et al., 2016) (Figure 3.3).

3.3.7.2 Clinical Indication

3.3.7.2.1 Gentamicin Gentamicin has a broad bactericidal activity against aerobic Gram-negative bacteria (Nagabhushan, Daniels, Jaret, & Morton, 1975). It acts against *Enterobacteriaceae*, *P. aeruginosa* and some strains of *Neisseria*, *Moraxella*, and *Haemophilus* (Hathorn, Dhasmana, Duley, & Ross, 2014; Nagabhushan et al., 1975). Gentamicin is applied for pneumonia, septicemia, meningitis, peritonitis, bacterial endocarditis, and pelvic inflammatory disease (Hathorn et al., 2014; Nagabhushan et al., 1975).

3.3.7.2.2 Tobramycin Tobramycinis effective for *S. aureus* infection and mainly the infections caused by Gram-negative bacteria like *Klebsiella*, *P. aeruginosa*, *Escherichia coli*, *Enterobacter,* and *Citrobacter* species (Banerjee, Jagannath, Hunter, & Dasgupta, 2000). Tobramycin is mostly given to the patients with pneumonia, septicemia, meningitis, peritonitis, pelvic inflammatory disease, and bacterial endocarditis (Vázquez-Espinosa et al., 2015).

3.4 Antifungal Agents

Fungi mainly includes moulds and yeasts (Garber, 2001). Only a few fungal species are pathogenic to humans causing systemic, subcutaneous, or superficial infections (Garber, 2001). Antifungal agents destroy or block the fungal growth

with minimal damage to the host. Polyenes, azoles, echinocandins, and flucytosine are the four major classes of antifungal agents (Table 3.4) (Lewis, 2011; Nett & Andes, 2016; Perfect, 2017).

3.4.1 Polyenes

Polyenes are derived from a soil actinomycete (*Streptomyces nodosus*) (Nett & Andes, 2016). Polyenes alter cell permeability to leak out the cell components, leading to fungal cell death (Gallis, Drew, & Pickard, 1990). The chemical structure of polyenes comprises of multiple conjugated double bonds within a hydroxylated chromophore (Lakhani, Patil, & Majumdar, 2019). The most potent polyene is amphotericin B which was developed in the 1950s (Gallis et al., 1990). In amphotericin B, conjugated double bond lactone chromophore has an all-trans conformation which is essential for its antifungal activity and stability (Figure 3.4a) (Lakhani et al., 2019).

3.4.1.1 Mechanism of Action

Ergosterol is the most abundant sterol of the fungal cell membrane, regulates permeability, fluidity and integrity of the membrane (Ghannoum & Rice, 1999). Amphotericin B acts through its hydrophobic interaction with the ergosterol and cause the formation of aqueous pores on the fungal cell membrane alters cell permeability and leakage then ultimate cell death (Figure 3.5a) (Ghannoum & Rice, 1999).

3.4.1.2 Clinical Indication

Amphotericin B was the first approved drug for the invasive antifungal diseases. It acts against wide range of fungal infections caused by *Candida*, *Aspergillus*, *Blastomyces*, *Histoplasma*, *Cryptococcus*, *Zygomyces, posadasii*, and *Paracoccidioides* (Nett & Andes, 2016).

3.4.2 Azoles

Azoles are the most commonly used systemic antifungal agents with limited side effects. Most effective antifungal azoles are fluconazole, itraconazole, voriconazole, posaconazole, and isavuconazole (Figure 3.4b). Among them fluconazole and itraconazole are considered as the first-generation azole drugs that are significantly effective against *Candida,* whereas voriconazole, posaconazole, and isavuconazole are the second-generation azoles with broad-spectrum antifungal activities (Nett & Andes, 2016).

3.4.2.1 Mechanism of Action

The azoles interferes the synthesis of ergosterol by directly inhibiting the P-450-dependent enzyme lanosterol C-14-α-demethylase. Loss of ergosterol production

Table 3.4 Overview of antifungal drugs.

Group	Drug	Administration route	Adult dose	Mechanism of action
Polyenes	Amphotericin B	Intravenous	0.6–1.0 mg/kg/day	Interacts with ergosterol
Azoles	Fluconazole	Oral or intravenous	6–12 mg/kg/day	Inhibits lanosterol C-14-α-demethylase
	Itraconazole	Oral	200 mg twice daily	
	Voriconazole	Oral or intravenous	6 mg/kg every 12 hours for 2 doses, then 4 mg/kg every 12 hours	
	Posaconazole	Oral	600–800 mg/day in divided doses	
	Isavuconazole	Oral	372 mg for 48 hours (8 hours interval) then once in daily	
Echinocandins	Caspofungin	Intravenous	70-mg (loading dose), then 50 mg/day	Inhibits β (1,3)-D-glucan synthase
	Micafungin	Intravenous	100–150 mg/day	
	Anidulafungin	Intravenous	200 mg (loading dose), then 100 mg/day	
Nucleoside analogues	Flucytosine	Oral and intravenous	25 mg/kg/dose every 6 hours for 4 weeks	Interferes DNA/RNA synthesis

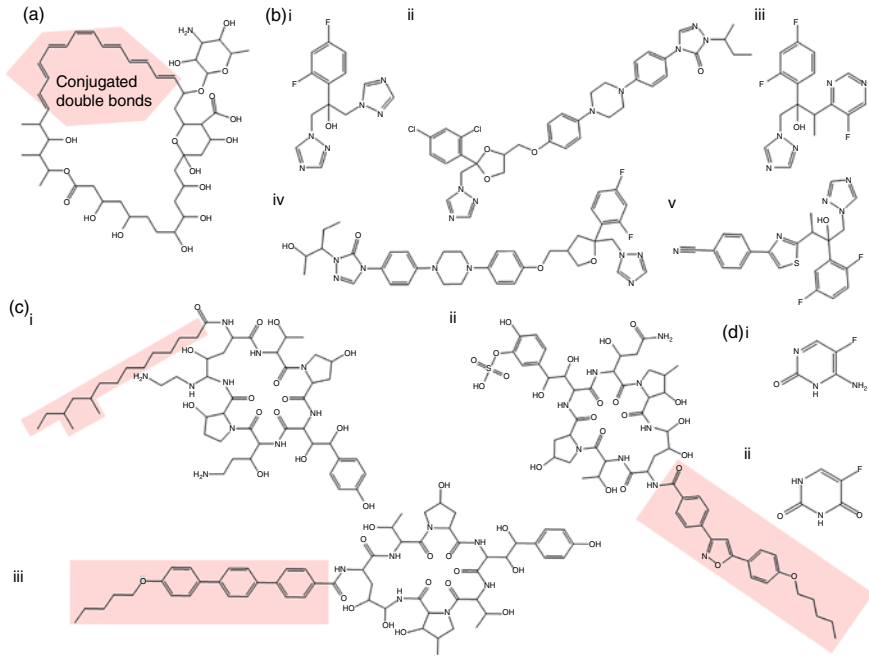

Figure 3.4 (a) Chemical structure of Amphotericin B; (b) i. fluconazole, ii. itraconazole, iii. voriconazole, iv. posaconazole, v. isavuconazole; (c) echinocandins: i. caspofungin, ii. micafungin, iii. anidulafungin; (d) i. flucytosine, ii. 5-fluorouracil.

increases the fungal cell permeability, leading to lysis and cell-death (Figure 3.5b) (Arnold, Dotson, Sarosi, & Hage, 2010).

3.4.2.2 Clinical Indication

3.4.2.2.1 Fluconazole Fluconazole is effective mostly against *Candida* spp. (Nett & Andes, 2016).

3.4.2.2.2 Itraconazole Itrazole is effective against dermatophytes, yeasts, moulds, *Aspergillus* spp., *Candida* spp., as well as fluconazole-resistant strains of *Candida Glabrata* (Pierard, Arrese, & Pierard-Franchimont, 2000).

3.4.2.2.3 Voriconazole Voriconazole is effective against *Fusarium* and *Scedospo rium*/*Pseudallescheria* infections as well as invasive *Aspergillosis* (Saravolatz, Johnson, & Kauffman, 2003). However, voriconazole works best against infections caused by *Candida* spp., *Fusarium* spp, and *Scedosporium apiospermum*.

3.4.2.2.4 Posaconazole Posaconazole has both fungistatic and fungicidal activity caused by *Candida* species (Herbrecht, 2004). Compared to itraconazole and fluconazole, posaconazole is a better choice for all *Candida* spp. However, for infection caused by *Candida glabrata* and *Candida pelliculosa,* voriconazole works best. Posaconazole has broad spectrum of invasive antifungal activities including aspergillosis, zygomycosis, fusariosis, coccidioidomycosis, histoplasmosis, and phaeoyphomycoses and other rare molds (Arnold et al., 2010).

3.4.2.2.5 Isavuconazole Isavuconazole is a newly developed triazole with antifungal activity against yeasts, molds, and dimorphic fungal species like *Candida* spp., *Cryptococcus* spp., and *Coccidioides* (Nett & Andes, 2016). It is approved for the treatment of mucormycosis and invasive aspergillosis (Shirley & Scott, 2016).

3.4.3 Echinocandins

Micafungin, caspofungin, and anidulafungin are the common echinocandins (Figure 3.4c). Caspofungin consists of an aliphatic side chain, whereas micafungin has a large aromatic side chain with a phenylisoxazol-phenyl substitution (Estes, Penzak, Calis, & Walsh, 2009). Both the ring structure and the covalently bound acyl group of echinocandinsare are essential for optimum biological activity (Debono et al., 1995). The terphenyl group of anidulafungin ensures its lipophilicity property and essential size to optimize antifungal efficacy (Estes et al., 2009; D. S. Krause et al., 2004).

3.4.3.1 Mechanism of Action

β(1,3)-D-glucan is an important component of various pathogenic fungi. Echinocandins inhibit the synthesis of β(1,3)-D-glucan and show fungistatic and fungicidal effects (Figure 3.5c) (Zaas & Alexander, 2005).

3.4.3.2 Clinical Indication

3.4.3.2.1 Caspofungin Caspofungin is used against *Candida* spp. Even for the resistant species like *Candida krusei* and less susceptible species like *Candida dubliniensis and Candida glabrata,* caspofungin is the drug of choice (Letscher-Bru & Herbrecht, 2003; Vazquez, Lynch, Boikov, & Sobel, 1997).

3.4.3.2.2 Micafungin Micafungins are effective against *Candida* spp. and Aspergillus species but not *Zygomycetes* or *Cryptococcus* species and emerging molds including *Fusarium* and *Scedosporium* species (Chandrasekar & Sobel, 2006).

3.4.3.2.3 Anidulafungin Anidulafungin shows broad spectrum of activity against *Candida* (including their resistant strains to azoles and polyenes) and *Aspergillus* species (Estes et al., 2009; D. S. Krause et al., 2004).

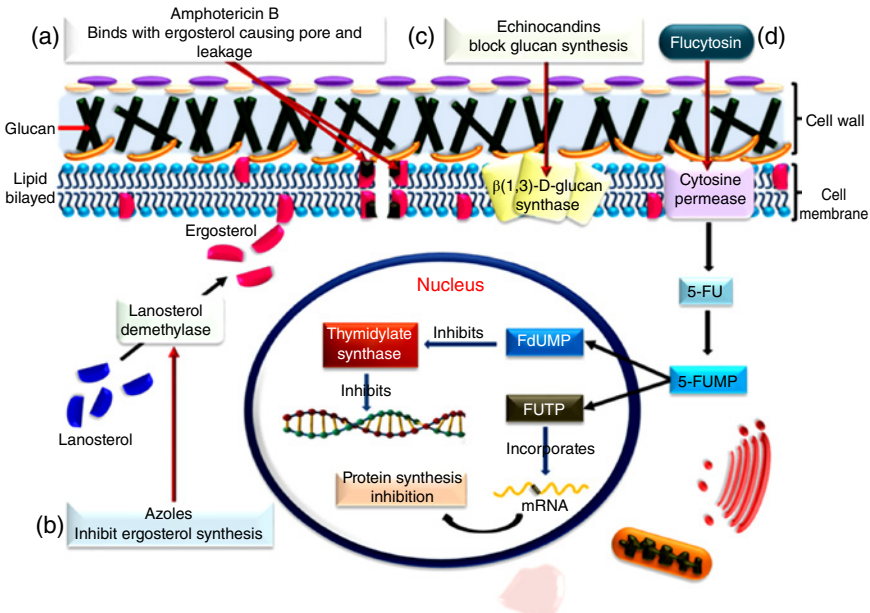

Figure 3.5 Mechanisms of action of antifungal agents.

3.4.4 Flucytosine

In 1990, flucytosine also known as 5-fluorocytosine has been approved as antifungal drug (Figure 3.4d). Flucytosine is a pyrimidine analogue uses mainly in combination with polyenes as drug resistance occurs rapidly when it is used alone (Perfect, 2017). The standard combination of flucytosine is with amphotericin B, which exerts synergistic effect for systemic infections (Day et al., 2013). High risk of drug resistance makes its application limited as antifungal monotherapy (Nett & Andes, 2016).

3.4.4.1 Mechanism of Action

Flucytosine penetrates fungal cell with the help of cytosine permease enzyme and converted into 5-fluorouracil (5-FU) via cytosine deaminase (Figure 3.5d) (Edlind & Katiyar, 2010). 5-FU exerts antifungal activity via two different mechanisms:

1) 5-FU converts into 5-fluorouridine triphosphate (FUTP) which competes with uracil and incorporates into the fungal RNA strand. This impairs aminoacylation of tRNA and inhibits protein synthesis (Houšť, Spížek, & Havlíček, 2020; Vermes, Guchelaar, & Dankert, 2000).
2) Within fungal cells, flucytosine can be converted into several active metabolites like fluorodeoxyuridine monophosphate (FdUMP) which inhibits thymidylate synthase. For DNA replication, thymidylate synthase is essential for synthesizing thymidine. Therefore, inhibition of thymidylate synthase hinders fungal DNA replication (Houšť et al., 2020; Vermes et al., 2000).

3.4.4.2 Clinical Implication

Flucytosine is the drug of choice for the infection caused by *Candida* spp., *Torulopsis, Phialophora, Cladosporium, Cryptococcus* spp., and *Aspergillus* spp. (Vermes et al., 2000).

3.5 Antiviral agents

More than 3600 viruses are known and hundreds can infect human and cause diseases (Dimitrov, 2004). Numerous antiviral drugs and vaccines have been approved for the treatment of various infectious viruses (Erik De Clercq & Li, 2016). Approved antiviral drugs could be randomly divided in 13 functional groups (Table 3.5) (E. De Clercq, 2004; Erik De Clercq & Li, 2016): (i) 5-substituted 2'-deoxyuridine analogues (idoxuridine, trifluridine, and brivudine), (ii) nucleoside analogues (vidarabine, entecavir, and telbivudine), (iii) (nonnucleoside) pyrophosphate analogues (foscarnet), (iv) nucleoside reverse transcriptase (RT) inhibitors (NRTIs) (zidovudine, didanosine, zalcitabine, stavudine, lamivudine,

Table 3.5 Antiviral drugs and their respective clinical uses with mechanism of actions.

Group	Drug	Approved clinical use	Mechanism of action
5-Substituted 2′-deoxyuridine Analogues	Idoxuridine, trifluridine, and brivudine	HSV-1, HSV, VZV	Inhibits viral DNA synthesis
Nucleoside analogues	Vidarabine, entecavir, and telbivudine	HSV, VZV, and HBV	Inhibits the activity of viral DNA polymerase
Pyrophosphate analogues	Foscarnet	HCMV, HSV (acyclovir resistant)	Inhibits the activity of viral DNA polymerase
NRTIs	Zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir, and emtricitabine	HIV and HBV	Targets viral RT and competes with dNTPs to inhibit viral DNA synthesis
NNRTIs	Nevirapine, delavirdine, efavirenz, etravirine, and rilpivirine	HIV-1	Binds directly to HIV RT and inhibits DNA synthesis
Protease inhibitors	saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, and darunavir	HIV, HIV-1	Blocks the active site of HIV protease to prevent cleavage of viral precursor proteins
Integrase inhibitors	Raltegravir, elvitegravir, and dolutegravir	HIV	Targets HIV integrase to inhibit the integration of viral DNA into human chromosomes
Entry inhibitors	RSV-IGIV, palivizumab, docosanol, enfuvirtide, maraviroc, VZIG, and VariZIG	RSV, HSV, HIV, HIV-1, and VZV	May interfere with binding of viral envelope proteins to cell membrane receptors, inhibits viral entry

Acyclic guanosine analogues	Acyclovir, ganciclovir, famciclovir, valacyclovir, penciclovir, and valganciclovir	HSV, VZV, and HCMV	Competes with dGTP to inhibit viral DNA polymerase activity
Acyclic nucleoside Phosphonate analogues	Cidofovir, tenofovir disoproxil fumarate, adefovir dipivoxil, and combination drugs	HCMV retinitis (AIDS patients), HIV, HBV	Inhibits DNA polymerase and DNA replication
HCV NS5A and NS5B inhibitors	Combination drugs	HCV	Inhibits viral replication
Influenza virus inhibitors	Amantadine, ribavirin, rimantadine, zanamivir, oseltamivir, laninamivir octanoate, peramivir, and favipiravir	Influenza virus A/B/C, HCV, RSV, hemorrhagic fever	Inhibits viral uncoating, mRNA synthesis, and virus release from host cells
Interferons, immunostimulators, oligonucleotide, and antimitotic inhibitors	Pegylated interferon alfa 2b, interferon alfacon 1, pegylated interferon alfa 2a, fomivirsen, podofilox, imiquimod, and sinecatechins	HPV, HBV, HCV, HCMV	Treatment of external genital warts

abacavir, and emtricitabine), (v) nonnucleoside reverse transcriptase inhibitors (NNRTIs) (nevirapine, delavirdine, efavirenz, etravirine, and rilpivirine), (vi) protease inhibitors (PIs) (saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, and darunavir), (vii) integrase inhibitors (raltegravir, elvitegravir, and dolutegravir), (viii) entry inhibitors (RSV-IGIV, palivizumab, docosanol, enfuvirtide, maraviroc, VZIG, and variZIG), (ix) acyclic guanosine analogues (acyclovir, ganciclovir, famciclovir, valacyclovir, penciclovir, and valganciclovir), (x) acyclic nucleoside phosphonate (ANP) analogues (cidofovir, tenofovir disoproxil fumarate, adefovir dipivoxil, and combination drugs), (xi) hepatitis C virus (HCV), NS5A, and NS5B inhibitors (combination drugs), (xii) influenza virus inhibitors (amantadine, ribavirin, rimantadine, zanamivir, oseltamivir, laninamivir octanoate, peramivir, and favipiravir), and (xiii) immunostimulators, interferons, oligonucleotides, and antimitotic inhibitors (pegylated interferon alfa 2b, interferon alfacon 1, pegylated interferon alfa 2a, fomivirsen, podofilox, imiquimod, and sinecatechins) (Erik De Clercq & Li, 2016).

Many antiviral drugs and vaccines have been discovered for the treatment of several viral diseases but still more than 200 viral infectious diseases remain to discover their respective drugs or vaccines (Erik De Clercq & Li, 2016).

3.6 Antiparasitic Agents

Parasites are microorganisms that live either inside or on the host organism. There are many parasites that are responsible for many human infections. Parasites include diverse microbes, including fungi and bacteria, protozoa, helminthes, and ectoparasites (Liu & Weller, 1996).

3.6.1 Antiprotozoan Agents

Protozoa are eukaryotic unicellular microrganisms among them *Entamoeba* (dysenteric liver abscess), *Dientamoeba* (colitis), *Giardia* (diarrhea), *Trypanosoma* (sleeping sickness), *Leishmania* (leishmaniasis) (Sundar & Singh, 2018), *Trichomonas* (trichomoniasis), *Plasmodium* (malaria), and *Balanidium* (dysentery) are generally responsible for protozoan parasitic infections (Gupta, Gupta, Aneja, & Kohli, 2004; Liu & Weller, 1996; Polak & Richle, 1978). Several antiprotozoan agents are available including antimalarial (chloroquine), antiamoebic (iodoquinol, diloxanide furoate, metronidazole, dihydroemetine), trypanocidal (benznidazole and pentamidine), antileishmanial (sodium stibogluconate), and antibabesial (clindamycin) against protozoan diseases (Table 3.6) (Liu & Weller, 1996).

Table 3.6 Overview of antiparasitic drugs.

Group	Sub-group	Drugs	Route of administration	Dosage	Mechanism of action
Antiprotozoal agents	Antimalarials	Chloroquine	Oral	500 mg tablets at 6, 24, and 48 hours	Inhibits heme detoxification and nucleic acid biosynthesis
	Antiamoebic agents	Iodoquinol	Oral and IM	650 mg tablets three times a day for 20 days	Reduces ferrous ions necessary for protozoal metabolism
		Diloxanide furoate		500 mg three times a day for 10 days	Split into diloxanide and furoic acid. Unabsorbed diloxanide acts as the amoebicide
		Metronidazole		750 mg for 10 days	DNA disruption and protein synthesis inhibition
		Dihydroemetine		1 mg/kg for 8–10 days	Inhibit protein synthesis by arresting intra ribosomal translocation of tRNA-amino acid complex
	Trypanocidal agents	Benznidazole	Oral, IV and IM	5–8 mg/kg for a duration of 60 days	Nitro-reduction intermediates that are toxic to the parasites
		Pentamidine		4 mg/kg IM or IV once a day for 14–21 days	Disrupt the membrane potential of the protozoan mitochondria
	Antileishmanial agents	Sodium stibogluconate	IV and IM	20 mg/kg/day is for 28 days	Inhibit glycolysis and the citric acid cycle by Inhibiting phosphorylation of ADP and GDP to ATP and GTP
	Antibabesial agents	Clindamycin	Oral	600 mg three times daily for 7 days	Target the parasite apicoplast (a novel organelle)

(*Continued*)

Table 3.6 (Continued)

Group	Sub-group	Drugs	Route of administration	Dosage	Mechanism of action
Antihelminthic Agents	Anticestodal drugs	Praziquantel	Oral	40 mg/kg/day (Schistosomiasis)	Disrupt ion transport by increasing cell membrane permeability to calcium
		Albendazole		400 mg twice daily for 8–30 days (Neurocysticercosi)	Inhibit microtubule synthesis
		Ivermectin		200 mcg/kg for 1–2 days on an empty stomach (Strongyloidiasis)	Cause hyperpolarization which leads to muscle paralysis
	Antinematodal drugs	Diethylcarbamazine		6 mg/kg/day three times daily for up to 14 days (lymphatic filariasis)	Reduces the number of microfilariae by sensitizing them to phagocytosis
		Pyrantel pamoate		11 mg/kg for 3 days (enterobiasis)	Acts on nicotinic acetylcholine receptors of nematodes, causing paralysis, leading to expulsion
Ectoparasiticides	Antiscabietic agents	Permethrin	—	1% cream rinse applied to the infection site; leave on for 10 minutes then rinse off with warm water (Pediculosis)	Causes sodium channel depolarization on nerve axons
		Lindane		30 ml of shampoo should be applied to hair left on for 4 minutes, then rinsed off (Pediculosis capitis)	Inhibits chloride channels on the gamma-aminobutyric acid (GABA)

3.6.2 Antihelminthic Agents

Helminths are mainly parasites that transmit via skin penetration, accidental ingestion, a vector bite, or consumption of the host as food. Trematodes (paragonimiasis, schistosomiasis, clonorchiasis), cestodes (cysticercosis, diphyllobothriasis, and hymenolepiasis) (Solaymani-Mohammadi, Genkinger, Loffredo, & Singer, 2010), and nematodes (enterobiasis, strongyloidiasis, and trichinosis) are common helminths causing parasitic diseases (Liu & Weller, 1996; Martin & Robertson, 2007). Efficient anticestodal (praziquantel, albendazole, and ivermectin) and antinematodal (diethylcarbamazine and pyrantel pamoate) drugs are developed for antihelminthic treatments purpose (Table 3.6) (Liu & Weller, 1996).

3.6.3 Ectoparasiticides

Organisms that live on the skin of hosts such as fleas, mites, lice, ticks, and bedbugs are called ectoparasites and they cause myiasis, pediculosis, scabies, and trombiculosis-like diseases (Drago, Shah, & Shah, 2014; Islam & Lynch, 2012; Liu & Weller, 1996). Antiscabietic agents such as permethrin and lindane are used against parasitic diseases caused by ectoparasites (Table 3.6) (Liu & Weller, 1996).

3.7 Antimicrobial Resistance

Antimicrobial resistance (AMR) occurs when microbes are able to grow in the presence of the certain concentration of antibiotic that is usually sufficient to abolish them (Sabtu, Enoch, & Brown, 2015). WHO has recognized AMR as one of the major public health concern of the twenty-first century (World Health Organization, 2014). Therefore, deeper understanding and tackling AMR should be carefully monitored.

Most antibiotics are naturally produced molecules, so in order to survive in the same environment, the coresident bacteria can develop mechanisms to overcome their action and become resistant. Microorganisms can also develop resistance following exposure to specific antibiotic which is termed as acquired resistance (Munita, Arias, Unit, & Santiago, 2016). The evolution of acquired resistance mechanism can take place by several mechanisms that are discussed below:

3.7.1 Genetic Basis of AMR

Mutational Resistance: A subset of susceptible bacterial cells develop resistance by genetic mutations. Once the resistance is emerged, the antibiotic diminishes the remaining susceptible population and eventually the resistant ones predominate. There are few mechanisms by which such mutations resulting in AMR alter the antibiotic action: (i) modifications of the antibiotic target (decreasing the drug-affinity), (ii) reduction of antibiotic uptake, (iii) stimulation of efflux mechanisms to extrude the harmful molecule, or (iv) global changes in important metabolic pathways via modulation of regulatory networks (Munita et al., 2016).

Horizontal Gene Transfer (HGT): One of the most important drivers for acquiring AMR by the bacterial cells is direct transfer of foreign DNA, encoding a resistance mechanism by horizontal gene transfer (HGT). Transfer of resistance genes by HGT can occur mainly by three mechanisms: (i) conjugation (transfer of genes carried on plasmids), (ii) transformation (direct transfer of naked DNA), and (iii) transduction (transfer of similar DNA by bacteriophage). Thus, bacteria of even unrelated species acquire antibiotic resistance genes and can spread very effectively between the microbial community (Munita et al., 2016).

3.7.2 Mechanistic Basis of Antimicrobial Resistance

Modifications of the Antibiotic Molecule: Bacteria achieve resistance against antibiotics by either producing enzymes that inactivate (or modify) the drug or destroy the molecule itself. Enzymatic modification includes addition of functional groups by (i) acetylation (chloramphenicol, aminoglycosides), (ii) phosphorylation (chloramphenicol, aminoglycosides), and (iii) adenylation (lincosamides, aminoglycosides). This is a common strategy in both Gram-positive and Gram-negative strains. Bacteria like penicillin-resistant *S. aureus* produces β-lactamase enzymes that destroy the β-lactam-containing antibiotics (D'Costa et al., 2011; Wilson, 2014) (Figure 3.6a i and ii).

Decreased Antibiotic Penetration and Efflux: Another efficient strategy of acquisition of AMR is by restricting the entry of the antibiotics in the intracellular target space. Bacterial strains achieve this by mainly two mechanisms: decreased permeability and efflux pump. Many bacterial species reduce the uptake of antibacterials through several porins. Thus, they prevent the agents to penetrate the outer membranes or reach their target in periplasmic or cytoplasmic space. For instance, vancomycin is inactive against Gram-negative bacteria. In other strategy, bacteria can also extrude a toxic compound out of the cell by efflux pumps and achieve antibacterial resistance. *E. coli* has such efflux system to pump tetracycline antibiotic out of the cytoplasm (McMurry, Petrucci, & Levy, 1980) (Figure 3.6b i and ii).

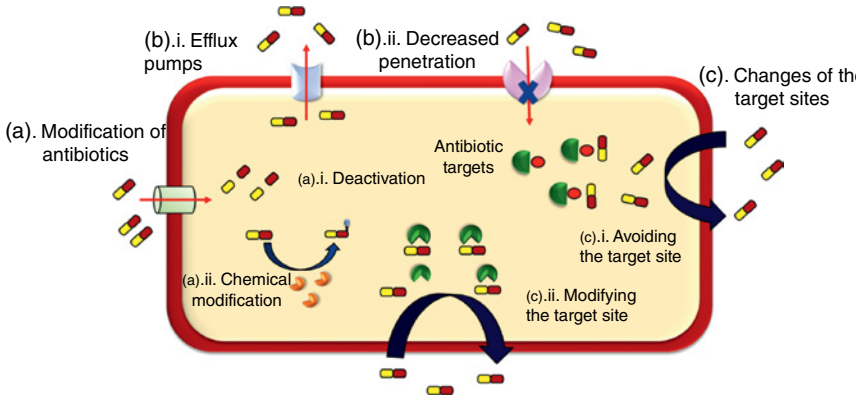

Figure 3.6 Strategies of antibiotic resistance in bacteria.

Changes in Target Sites: A common strategy for the microbe to develop AMR is to deviate the action of the antibiotic by interfering with their target site. Bacteria can modify the target site by mutation or enzymatic alteration that result in decreased affinity for the antibiotic molecule. One of the best examples is tetracycline resistance in *Streptococcus* spp. (Connell, Tracz, Nierhaus, & Taylor, 2003) (Figure 3.6c i and ii).

Resistance Due to Global Cell Adaptations: Bacteria have evolved complex machineries to adapt in presence of various environmental stressors and to avoid the disruption of pivotal cellular process such as cell wall synthesis and membrane homeostasis. Development of resistance to infectious diseases arises due to global cell adaptive response to the antibacterial attack.

3.8 Conclusion

Antibiotic resistance is a natural process. However, misuse of antibiotics accelerates the process. With the emergence of new resistance, "a growing number of infections – such as pneumonia, tuberculosis, gonorrhoea, and salmonellosis – are becoming harder to treat as the antibiotics used to treat them become less effective" (WHO Report). "In 2016, 490 000 people developed multi-drug resistant TB globally, and drug resistance is starting to complicate the fight against HIV and malaria, as well" (WHO Report). "Without effective antimicrobials for prevention and treatment of infections, medical procedures such as organ transplantation, cancer chemotherapy, diabetes management and major surgery (for example, caesarean sections or hip replacements) become very high risk" (WHO Report).

Therefore, antimicrobial resistance is a global concern. The discovery of new and effective antimicrobials together with the awareness about the rational usage of antibiotics could combat the growing threat.

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4

Trends in Antimicrobial Therapy

Current Approaches and Future Prospects

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4.1 Introduction

Infectious diseases were the most common cause of death in the nineteenth century. The introduction of the antibiotics was boon to the society, not only to treat infectious diseases but also to reduce mortality and morbidity. In contrast, the increasing antimicrobial resistance (AMR) is one of the serious threats to global health, leading to health crises, high medical cost, longer hospital stay, and increased mortality, and it reduces the ability to avoid and alleviate a broad range of communicable diseases (Shallcross et al., 2015). The occurrence of AMR is a natural phenomenon, but greatly misuse and overuse of antimicrobials have accelerated spread and increase of AMR resistance in microbes (Mladenovic et al., 2016). Generally, the AMR microbes are found in humans, animals, food, and other environments like soil, water, and air. The AMR microbes can be spread from humans to humans and animals to humans either by direct or indirect contact (Woolhouse et al., 2015). Furthermore, inadequate infection control, poor sanitary conditions, and inapt food management may lead to the spread of AMR within populations (Fletcher, 2015).

According to the World Health Organization (WHO), the center for diseases control and prevention (CDC) and many other international organizations listed AMR as one of the world's most urgent challenges to public health. It is estimated that around two million people are getting diseased due to antimicrobial-resistant bacteria and more than 23 000 thousand lives lost per year (Aryee and Price, 2015). The infectious diseases are running after humans throughout the ages, as in the year 1347–1350 thousands of people died in Europe due to bubonic plague, and it

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was controlled with advances in hygienic and sanitary conditions. However, these infectious diseases had been the foremost cause of deaths in the early 1900s, as responsible for 25% mortality rate in England and soon after the commercialization of antibiotics mortality due to infectious diseases reduced to 1%, that is considered as a medical miracle (Smith et al., 2012).

In the current scenario, around two million North American people getting infectious diseases every year allied with drug resistance resulting in approximately 23 000 deaths (CDC, 2017). Similarly, due to antibiotic resistance infections, more than 33 000 people died per year causing over €1.5 billion extra burden to society in Europe (Cassini et al., 2019). Although there is a 36% increase in antibiotic consumption from 2000 to 2010, still nearly 20% of deaths are due to infectious diseases occurred worldwide (Laxminarayan et al., 2016). It has been reported that this situation is becoming progressively worse and nosocomial infections are the main cause for morbidity and mortality (Akova, 2016), and putting these conditions had created higher health care costs. During the last decade, more than 15% of the infections are due to multidrug-resistance pathogens (Theuretzbacher, 2012), and no effective drug was available. According to a report, there may be around 10 million deaths would be reported per year worldwide from antibiotic-resistant infections by 2050 (O'Neil, 2016).

The increase of multiple drug resistance may be the case of various factors such as taking up the self-medication without prescriptions, high scale use of antibiotics as growth promoters in livestock farming, and high dose medical prescription by the physician (Rather et al., 2017). As an example, more than 60% of the antibiotics were also used for human treatment that is used by the food industry in the United States (Dodds, 2017). Also, global usage of antibiotics has crossed more than 70 billion/annum, and the most commonly used antibiotics in 2010 were penicillin, macrolid, tetracyclins, fluoroquinolones, cephalosporins, and trimethoprime.

Again the matter is being more complicated, as to develop a new antibiotic and its commercialization around $200 million required and that too it would be effective enough for the treatment, is questionable and may help in increasing the antibiotic resistance (Renwick, Brogan, and Mossialos, 2016). There was just one drug approval per year from 2004 to 2009, compelling us to discover novel drugs (Boucher et al., 2009).

4.2 Antibiotics: A Brief History

Antibiotics are defined as the substance produced by a microorganism or a similar product produced synthetically or semi-synthetically by chemical synthesis that is capable of inhibiting the growth of or killing other microorganisms in a lower concentration. The antibiotic means "antibacterial" and it means "against life," any substance or molecule that may be bactericidal or bacteriostatic in the body.

4.2.1 Classification of Antibiotics

Based upon their working mechanism, antibiotics can be divided as synthesis inhibitors of the cell wall, protein, nucleic acid (DNA/RNA), ribosomes, folic acid, and mycolic acid. (i) Cell wall synthesis inhibitors: penicillin, bacitracin, carbapenems, and beta-lactamase inhibitors; (ii) Protein synthesis inhibitors: (a) 30s subunit inhibitors: gentamycin and tetracycline, (b) 50S subunit inhibitors: linezolid, chloramphenicol, and macrolids; (iii) DNA synthesis inhibitors: fluoroquinolones; (iv) RNA synthesis inhibitors: rifampin; (v) Folic acid synthesis inhibitors: sulfonamide and trimethoprim; and (vi) Mycolic acid synthesis inhibitors: isoniazid.

4.2.2 Evolution of Antibiotics

The discoveries of antibiotics are a miracle in medical science. Starting from penicillin approval in 1955 now we have lots of new narrow and broad-spectrum antibiotics to fight against pathogenic bacteria. Novel drugs were discovered not only for the bacteria but also for fungi, viruses, parasites, etc. **(**Figure 4.1).

4.2.3 Mechanism of Action of Antibiotics

The antibiotics must have three properties to work against pathogenic bacteria: (i) should not be inactivated or modified by bacteria, (ii) antibiotic target site must be in the bacterial cell, and (iii) antibiotic must be in ample quantity at the target site (Sutcliffe, 1999). To understand the antibiotic resistance mechanism, it is mandatory to know how and where antibiotics show their effects. Some of the major antibiotic resistance mechanisms are given in Table 4.1 and Figure 4.2.

4.3 AMR: A Global Burden

Infectious diseases have become a serious cause of diseases in humans. The time when the antibiotics were introduced, it was understood that the infectious diseases would be no longer and it is found to be the biggest mistake of humankind, as the bacteria got evolved with antibiotics resistance (Livermore, 2003), and it is relevant enough to study the emergence of antibiotic resistance in pathogens.

The increased antibiotic resistance was the consequence of the continuous pressure of antibiotics. Additionally, the overuse of nutritive and therapeutic antibiotics in animal farms resulted in antibiotic-resistant bacteria. There is an evidence that antibiotic-resistant bacteria from poultry, cattle, and pig farms enter into the food chain and get accumulated and colonized in the human digestive

Figure 4.1 Evolution of antibiotics.

tract and in intestinal parts (Wegener, 2003). As the resistance bacteria were residing in the human colon, they would establish an interaction to healthy microflora and might be transferring resistance determinants via horizontal gene transfer (HGT) (Ochman et al., 2000). The multidrug resistance has been confirmed in *Salmonella enteric*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii*, *Escherichia coli*, *Mycobacterium tuberculosis*, *Staphylococcus aureus*, *Enterococcus faecium*, *Shigella dysenteriae*, *Klebsiella pneumoniae*, and *Haemophilus influenza* (Vladimír, 1994).

4.3.1 Global Scenario

According to WHO thousands of people are dying due to climate change each year, and these deaths are attributed to often occurring epidemics such as dengue

Table 4.1 Mechanism action of antibiotics.

Mode of action	Antibiotics	Mechanism
Cell wall synthesis inhibition	Penicillins/ Cephalosporins	Obstruct the enzymes needed for the biosynthesis of peptidoglycan layer
	Glycopeptides (Vancomycin, Teicoplan)	Interfering with the D-alanyl-D-alanine termini of the peptidoglycan chain, and stopping the cross-linkage
	Telavancin	Inhibit peptidoglycan synthesis via favored targeting of trans-glycosylation (Benton et al., 2007)
Protein synthesis inhibition	Macrolides	Stop the elongation of polypeptide chains by binding with 50S ribosomal subunit
	Aminoglycosides	Inhibition of protein synthesis by binding to the 30S ribosomal subunit
	Chloramphenicol	Inhibits peptidyltransferase reaction by binding to the 50S ribosomal subunit
	Tetracyclines	It binds to 30S subunit of ribosome and inhibit protein synthesis via deteriorating the ribosome-tRNA interaction
	Glycylglycines (semisynthetic tetracycline)	It binds ribosome more strongly than older tetracyclines
	Oxazolidinones	Interacting with the A site of the bacterial ribosome and interfere with the aminoacyl-tRNA (Leach et al., 2007)
Nucleic acid synthesis inhibition	Rifampicin	Interferes with RNA polymerase.
	Quinolones	Interrupt DNA synthesis by interference with type II topoisomerases DNA gyrase and topoisomerase IV during replication and by causing double strand breaks
Metabolic pathway inhibition	Sulfonamides (e.g. Sulfamethoxazole) and Trimethoprim	Stop folate synthesis, a cofactor for the synthesis of nucleotides
Cell membrane inhibition	Polymyxins	Increase the bacterial membrane permeability, and causing cell death
	Lipopeptide Daptomycin	Binds to cytoplasmic membrane and exhibit bactericidal activity (Straus and Hancock, 2006)

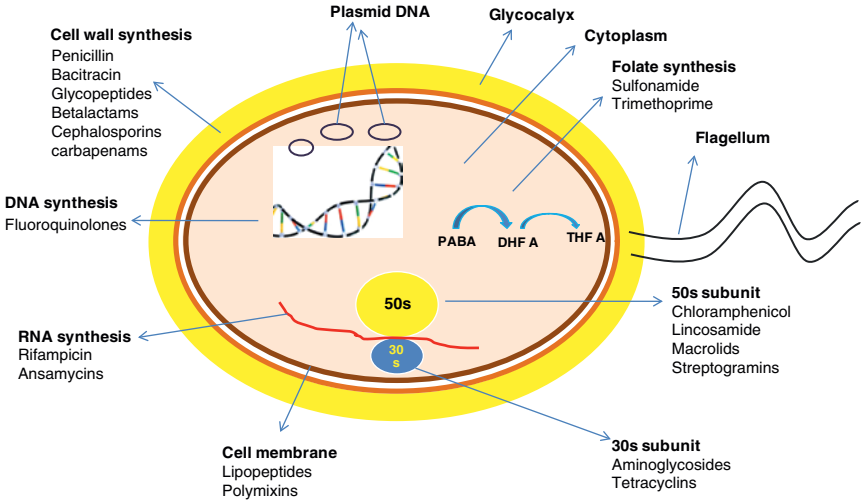

Figure 4.2 Schematic diagram of mechanism action of antibiotics.

and cholera, and by 2030 the climate change may be causing 250 000 extra deaths due to malaria and diarrheal diseases. If the AMR is continuing to rise or it is not controlled sooner, there might be 10 million deaths by the year 2050, and the world economy may have to face about US $100 trillion loss and it would be 2–3.5% of the world total GDP (O'Neill, 2018). The deaths due to AMR might be on top (10 million), followed by cancer (8.2 million), diabetes (1.5 million), diarrheal diseases (1.4 million), measles (130 000), cholera (100 000–120 000) by the end of the year 2050.

4.3.2 Origin of SUPERBUGS and the "END of Antibiotics"

M. tuberculosis is the most serious pathogen in both developed and developing countries. It is mainly a human pathogen that causes tuberculosis (TB) diseases. It mainly affects the lungs, lymphatic system, circulatory system, and central nervous system (CDC, 2005). In a survey, 10 million people were affected with TB and about 1.5 million died in 2018 (WHO, 2019). Some other nosocomial infections causing pathogens are *Acinetobacter baumannii*, *Citrobacter freundii*, *Burkholderia cepacia*, *Enterococcus faecalis*, *Campylobacter jejuni*, *Klebsiella pneumoniae*, *Enterococcus faecium*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia* spp., *Staphylococcus aureus*, *Enterobacter* spp., *Escherichia coli*, *Staphylococcus epidermidis*, *Clostridium difficile*, *Haemophilus influenzae*, *Stenotrophomonas maltophilia*, *Salmonella* spp., and *Streptococcus pneumoniae*.

The most widespread gram-negative bacteria, such as *Kebsiella pneumoniae*, *E. coli*, and *Salmonella enteric*, cause numerous diseases in humans and animals, and increased antibiotic resistance was noticed in the past few decades. The *P. aeruginosa* and *A. baumannii* are gram-negative nosocomial pathogens and are responsible for the hiked rate of mortality and morbidity (Peleg et al., 2008). In the current situation, one of the most legendary, superbug is *S. aureus*, which has emerged as multidrug-resistance bacteria as a major nosocomial infection (Enright et al., 2002). Additionally, *Clostridium difficile* is a gram-positive, spore-forming bacteria. It causes severe intestine infections and produces hyper-virulent toxins. It can spread through aerosols, upon equipment, or by hospital personnel (Kelly and LaMont, 2008).

The superbugs are all-pervading in our surrounding areas, and they are not just microbial threats but responsible for most morbidity and mortality worldwide. The *Vibrio cholerae* might be on the top as superbugs in developing countries, and it is an endemic in Asia and South America. Irrespective of this, it is not frequent in developed nations (Lipp et al., 2002).

4.4 Antimicrobial Resistance and Virulence

Antibiotic resistance has been provoked due to the massive use of antimicrobials. It has developed various mechanism especially HGTs (Horizontal Gene Transfer) of AMR genes, which is frequently enhanced by the biofilm formation. The microbes are always protected and sheltered within the biofilm from the external environment. Microbes connect to each other via signaling pathways (two-component system or quorum sensing system) and produce to enhance virulence and gaining antibiotic resistance. Unfolding the genetic connections between virulence and antibiotic resistance in biofilm may provide new antibiotic targets, leading to new drug discovery (Tay and Yew, 2013).

4.4.1 Molecular Insights and Mechanism of AMR

(i) Efflux pump: Increase in efflux pump expression (fluoroquinolones, β-lactam, aminoglycosides, chloramphenicols, tetracyclines, and trimethoprim), (ii) Porins: reduced porins expression (chloramphenicols and β-lactam), (iii) Target over productions, (iv) Decreased drug uptake, (v) Modification in lipopolysaccharides: decreased antibiotic binding (colistin), (vi) Drug modification: e.g. aminoglycosides, (vii) Antibiotic inactivation: enzymatic inactivation of antibiotics (β-lactam), (viii) Methylation in 30S rRNA: reduced antibiotic binding (aminoglycosides), and (ix) Mutation in DNA gyrase or topoisomerase IV: decreased antibiotic binding (fluoroquinolones) (Figure 4.3).

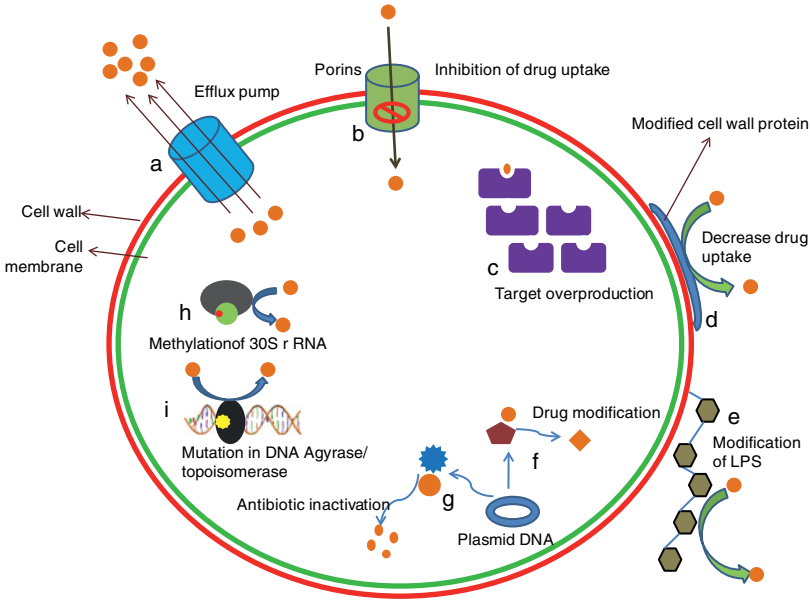

Figure 4.3 Mechanism action of antibiotic resistance in bacteria.

4.4.2 Antibiotic Resistance in Bacteria

4.4.2.1 Horizontal Gene Transfer

Most of the resistance genes are exchanged via horizontal/lateral gene transfer among bacteria (Rodríguez-Rojas et al., 2013). The HGT can occur in three different ways: (i) Bacteriophages infection (Chen and Novick, 2009), (ii) Plasmid exchange (Cirz et al., 2005), and (iii) environmental DNA uptake (Von Wintersdorff et al., 2016).

4.4.2.2 Increased Mutation Rate

The elevated mutation rates are generally associated with mismatch repair (MMR) system (mutL, mutY, mutH, and mutM) (Oliver et al., 2002). Increased mutation in MMR system also enhanced the genetic recombination that led to a range of antibiotic resistance mechanism (Rodríguez-Rojas et al., 2013).

4.4.2.3 Antibiotic Inactivation

One of the common ways that bacteria overcome the resistance is antibiotic inactivation, and the bacteria do so by modifying the antibiotic target or by degrading the antibiotics. Four different types of enzymes modify the antibiotics targets such as lyases, redox enzymes, group transferase, and hydrolase (Wright, 2005).

The lyases enzymes hew the carbon–oxygen, carbon–carbon, carbon–sulfur, and carbon–nitrogen bonds by the oxidative or hydrolytic mechanism. The streptogramin resistance is an example of lyases enzyme caused by Vgb protein. The redox enzymes are not common in bacteria for antibiotic modification. The only studied example is TetX enzyme that induces tetracycline resistance. The group transferases enzymes cause covalent bond modification and inhibit target binding for the antibiotic. There is a list of group transferases enzymes such as phosphotransferases, nucleotidetransferases, acyltransferases, thioltransferases, ADP-ribosyltransferases, and glycosyltransferases. The aminoglycoside resistance is the result of group transferase enzymes. The hydrolases enzymes are water-soluble and responsible for cleaving the beta-lactam ring in beta-lactam antibiotics. The example is amidases that break amide and ester bonds (Tillotson and Theriault, 2013).

4.4.2.4 Alteration of the Antibiotic Targets

The alteration of the drug target site is another resistance mechanism. The penicillin-binding protein 2 is the best example of altering the target site. In this modification beta-lactam antibiotics find low affinity to bind with the target site and the bacteria can biosynthesize the cell wall.

4.4.2.5 Changes in Cell Permeability and Efflux

To pump out the drugs, the bacteria have developed an efflux mechanism, found in both gram-positive and mostly in gram-negative bacteria. The efflux pumps sustain the cell homeostasis and communicate with others by propelling out the metabolites, solutes, toxins, and quorum-sensing molecules (Soto, 2013). Five different efflux pumps families are found such as (i) The major facilitator superfamily, (ii) The ATP-binding cassette (ABC) superfamily, (iii) The multidrug and toxic compound extrusion family, (iv) The resistance–nodulation–division (RND) superfamily, and (v) The small multidrug-resistance family. Generally, bacteria exhibit drug resistance by up-regulating a single efflux pump (Alekshun and Levy, 2007).

4.4.2.6 The Major Facilitator Superfamily

These are the prime group of antibiotics/solutes transporters and found mostly in gram-positive bacteria (Poole, 2005) and also in gram-negative bacteria (Saidijam et al., 2006). The substrates are efflux by proton motive force.

4.4.2.7 The ATP-Binding Cassette Superfamily

These ABC transporters are omnipresent, present in all living kingdoms. These transporters are primary efflux pumps and the transport is accomplished by ATP hydrolysis.

4.4.2.8 The Multidrug and Toxic Compound Extrusion Family

Most commonly present in gram-negative bacteria and execute the sodium antiport mechanism or proton motive force to efflux the drugs. The examples are PmpM from *P. aeruginosa*, MepA from *S. aureus*, and VcrM from *Vibrio cholerae*, transporting various drugs such as benzalkonium chloride and fluoroquinolones (Kaatz et al., 2005).

4.4.2.9 The Resistance–Nodulation–Division (RND) Superfamily

These are tripartite transporters present in gram-negative bacteria, and the drugs are pumped out by proton motive force and the studied example is AcrAB-TolC transporter, discovered first time in *E. coli* (Tikhonova and Zgurskaya, 2004).

4.4.2.10 The Small Multidrug-Resistance Family

These transporters are small in size (110–120 amino acids long) and pump out the antibiotics by using the proton motive force. The example of this efflux pump includes EmrA from *E. coli* and QacC from *S. aureus*, transporting harmful organic cations as methyl viologen (Paulsen et al., 1995).

4.4.3 Development of Antibiotic Resistance

Sometime bacteria are nonpathogenic or nonresistant to antibiotics, but under the high pressure of antibiotics, some of the bacteria get mutated in their genome and acquired resistant toward one antibiotic or multiple antibiotics. The bacteria showing resistance to many antibiotics properly are known as multidrug resistance. Based upon that drug-resistant bacteria can be categorized as (i) Multidrug resistance (MDR): when bacteria show resistant to more than two class of antibiotics, (ii) Extensive drug resistance: when bacteria show resistant against all antibiotics except one or two class of antibiotics, and (iii) Pan drug resistance: when bacteria show resistant to all known class of antibiotics.

4.4.4 Prioritization of Antibiotic Resistant Bacteria

The scope of the current work is to identify the most important resistant bacteria at a global level for which there is an urgent need for new treatments. WHO reported in February 2017 its first directory of antibiotic-resistant "priority pathogens" consisting of 12 families of bacteria that pose the greatest threat to human health. The list highlights in particular the threat of gram-negative bacteria that are resistant to multiple antibiotics. The WHO list is divided into three categories according to the urgency of the need for new antibiotics: critical, high, and medium priorities (Table 4.2).

Table 4.2 Prioritized antibiotic resistance bacteria.

Recognition	Bacteria name	General features
Urgent pathogen	*Acinetobacter baumannii*, carbapenem-resistant	Gram-negative, short round to rod shaped, opportunistic bacterium
	Pseudomonas aeruginosa, carbapenem-resistant	Gram-negative, encapsulated, rod-shaped bacterium
	Enterobacteriaceae[a], carbapenem-resistant, 3rd generation cephalosporin-resistant	Gram negative
	Klebsiella pneumonia, 3rd generation cephalosporin-resistant	Gram-negative, rod-shaped, non-motile, lactose-fermenting, encapsulated, facultative anaerobic bacterium
Serious pathogens	*Enterococcus faecium*, vancomycin-resistant	Gram-positive, alpha-hemolytic or non-hemolytic bacterium
	Staphylococcus aureus, methicillin-resistant, vancomycin intermediate and resistant	Gram-positive, round-shaped bacterium, mostly found in the upper respiratory tract and on the skin
	Helicobacter pylori, clarithromycin-resistant	Gram-negative, helically shaped, microaerophilic bacterium, found in the upper part of small intestine
	Salmonella spp., fluoroquinolone-resistant	Gram-negative, rod shaped bacterium
	Campylobacter, fluoroquinolone-resistant	Gram-negative, comma- or s-shaped bacterium
	Neisseria gonorrhoeae, 3rd generation cephalosporin-resistant, fluoroquinolone-resistant	Gram-negative, diplococci bacteria
Concerning pathogen	*Streptococcus pneumoniae*, penicillin-non-susceptible	Gram-positive, spherical bacteria, alpha or beta-hemolytic, facultative anaerobic, Found in human respiratory tract
	Haemophilus influenzae, ampicillin-resistant	Gram-negative, coccobacillary, facultative anaerobic, capnophilic pathogenic bacterium
	Shigella spp., fluoroquinolone-resistant	Gram-negative, facultative anaerobic, non-spore-forming, nonmotile, rod-shaped

[a] *Enterobacteriaceae*: Group of bacteria under large single family.

4.4.5 Understanding Biofilm Resistance

Most of the bacteria under antibiotic stress produce extracellular substances and resides protective under the biofilm matrix (Bayramov and Neff, 2017). The biofilm is formed due to various causative parameters such as cell to cell communications (i.e., quorum sensing) by secreted autoinducers such as peptides in gram-positive bacteria and small molecules in the case of gram-negative bacteria (Tay and Yew, 2013).

The biofilm formation starts when autoinducers concentration reaches a certain level and then the bacteria may be embedded in the biofilm matrix, change their metabolic activity, produce a shielding gylocalyx, upregulate virulence genes, and reduce the antibiotic susceptibility (Khardori and Yassien, 1995). The secreted extracellular proteins provide a sheltered environment where antibiotic-resistance plasmid genes are exchanged and sometimes mutations in genetic elements exhibited antibiotic resistance over time (Thallinger et al., 2013). There are several evidences that show that biofilm plays a major role in antibiotic resistance development. Sometime biofilm forming bacteria secret such enzymes that can degrade antibiotics and some bacteria show, upregulation of efflux pumps (Kester and Fortune, 2014).

4.5 Alternatives to Antibiotics

4.5.1 Peptide Antibiotics

Peptide antibiotics are also called antimicrobial peptides (AMP) that are synthesized by the living system (animals, plants, and humans) and have a broad-spectrum antimicrobial activity thus playing a vital role in innate immunity. AMPs are produced by all forms of life and are highly conserved in their genome (Daphny et al., 2015). AMPs are part of the immune system in almost all multicellular organisms (Mahlapuu et al., 2016). AMPs are is considered as a potential alternative for antibiotics because of the low probability of the pathogens to develop resistance against these compounds (Faccone et al., 2014). The first isolated antimicrobial peptide is thionins from plants in the 1970s. The most widely studied AMPs are cationic antimicrobial peptide (CAMP) and marine antimicrobial peptide.

4.5.1.1 Cationic Antimicrobial Peptides (CAMPs)

Naturally occurring AMPs are cationic, ranging from 10 to 80 amino acid residues. Usually, these peptides are liner in structure but undergo subsequent folding when it gets exposed to an amphiphilic environment that help in attacking the membrane of microbes (Zanetti, 2004). Cationic peptides normally function as

innate immune modulators and as an antimicrobial agent. CAMPs are divided into two classes based on their structure α-helix and β-sheet. All α-helix AMPs are liner in structure and attain helix structure only when it comes in contact with cell membranes e.g. LL-37 and melittin. β-sheet AMPs are diverse and have a well-defined sheet structure e.g. protegrin and gramicidin S (Yount et al., 2006).

CAMPs usually attach to the bacterial cell wall through an electrostatic interaction after which the AMP gets integrated into the membrane; the entry of AMP to the outer membrane drives a large number of AMPs to get inside the cell thus it reaches the inner membrane of the cell (Melo et al., 2009). Most of the antibiotics cause endotoxinaemia by releasing LPS during lysis of the cells, in contrast, cationic peptides are reported to neutralize the endotoxinaemia in the blood (Gough et al., 1996). In contrast with conventional antibiotics, CAMPs produce several bacterial targets and cause the immediate death of the microbes (Kang et al., 2017). CAMPs can act on both antibiotic susceptible and MDR strains (Zasloff, 2002). Some studies prove the synergistic relation between antibiotics and CAMPs, where CAMP augment the activity of antibiotics (Zhang et al., 2014). Some of the CAMPs such as gramicidin-S and polymyxin-B are used in skin creams.

Polycationic trisaccharides such as gentamicin and polycationic lipopeptides are used in aerosol treatment in *P. aeruginosa* patients. Nisin is provided as an oral supplement for patients infected with *Helicobacter pylori* (Ahmad et al., 1995). The VG-16, an AMP (16 amino acid residue) has been used as an active fragment of dengue virus fusion peptide, antibacterial activity against various gram-negative bacteria, and also has antifungal property (Datta et al., 2015). Two different CAMPs including "human cathelicidin" (LL-37) and Cecropin (1–7)-melittin A(2–9) amide (CAMA) showed antibacterial activity against *S. aureus* and *P. aeruginosa* (Geitani et al., 2019). Many CAMPs have been discovered but their working mechanism is still unclear, and thus an extensive study is needed to explore their mechanism of action as an antimicrobial agent.

4.5.1.2 Marine Antimicrobial Peptides

Around 70% of the planet's surface constitutes the marine environment, which consists of the diverse microbial population (Egan et al., 2008). All marine species including bacteria, fungi, protozoa, vertebrates, invertebrates, plants, and sponges consist of AMPs (Semreen et al., 2018). Marine AMPs are large in number and contain a complex structure that makes it difficult to synthesize chemically, thus recombinant technology is employed to synthesize these compounds. Marine AMPs are classified based on their structure as (i) liner α-helix e.g.: clavanin A-E and styelin, (ii) helical or nonlinear: arasin and hyastatin, (iii) peptide forming hairpin like β-sheet e.g.: hepcidin and tachyplesin, and (iv) cyclic peptides e.g. antillatoxin and aplidine. AMPs produced by marine bacteria are also classified based on the synthesis process ribosomal and nonribosomal pathway.

AMPs from actinomycetes, cyanobacteria, and proteobacteria species are the most widely studied (Bertrand and Munoz-Garay, 2019). Some of the proteobacteria produced AMP include holomycin, indigoidine, kahalaides, solonamides, etc. (Desriac et al., 2013). From marine invertebrates (sponges) 40 different AMP families, 5300 different compounds and 200 novel metabolites have been studied (Kang et al., 2015). AMPs are also isolated from sea urchins like *Strongylocentrotus droebachiensis*, *Cucumaria* sp., etc. An AMP from *Rapanavenosa*, a mollusca was reported to have antibacterial activity against *S. aureus* and *K. pneumoniae*. AMPs from oyster species like *Mytilus galloprovincialis* and *Mytilus edulis* showed antibacterial and antifungal activity against *Fusarium oxysporum*, *Candida albicans*, and *Micrococcus luteus* (Daphny et al., 2015). Fish AMPs also shows extensive antimicrobial activity in killing fish pathogens e.g. pleurocidins, misgurins, chrysophrys, piscidins, and hepcidin (Kang et al., 2015).

4.5.2 Nano Drugs

Conventional antibiotics have several disadvantages that have led to the development of alternatives for antibiotics. Nanodrug delivery systems have gained much attention in recent years because of their unique properties like small size, high surface to volume ratio, and easy surface modification. These unique properties make it a potential vehicle for targeted drug delivery, cell labeling, and gene therapy (Blecher et al., 2011). To make the antibiotic more definite, some recent research studies have focused on combing both antibiotics and nanomaterial that help to increase the specificity and amount of antibiotics delivered at the target site (Sharma et al., 2012).

Some of the highly efficient nanoparticles such as chitosan nanoparticle, a natural biopolymer, have a larger surface area and so pose high surface charge density thus shows higher affinity toward bacteria. Chitosan coated silver nanoparticle showed significant antibacterial activity against *S. aureus* and *E. coli* (Potara et al., 2011). Liposomes are spherical vesicles, surrounded by phospholipids bilayers and lipoproteins, and antibiotics encapsulated in liposome showed good antibacterial activity against the pathogens.

Small-sized liposomes without antibiotics exhibited antibacterial activity against *K. pneumoniae*. Lipid-complexed amphotericin B is being currently in use for treating AIDS patients with complex *Mycobacterium avium* infection. Inorganic nanomaterials include the use of semiconductive material and magnetic materials as an important material for developing an antibiotic delivery system (Veerapandian and Yun, 2011). For centuries silver has been in use to prevent bacterial infections. The extremely small size of this particle (<10 nm) helps them to easily penetrate the microbial cell wall and disrupt the molecular mechanism of the microbe (Sanpui et al., 2008).

Antibiotics like erythromycin and penicillin G encapsulated in silver nanoparticle showed antibacterial activity against *S. aureus* and *E. coli* (Shahverdi et al., 2007). Recent studies revealed that copper nanoparticles are more effective than silver, CuO-NP showed antibacterial activity against *S. aureus* and *E. coli* that was higher than that of silver (Ren et al., 2009). Apart from these, carbon quantum dots (CQDs) are said to have an efficient drug delivery system because of its biocompatible property. CQD is said to exhibit antibacterial activity against *E. coli* and *S. aureus* with a MIC value of 64 and 16 μg/ml (Bing et al., 2016). Metronidazole-derived CQD was reported to have higher activity against *Porphyromonas gingivalis* (Liu et al., 2017). At present many nano encapsulated antibiotics are in use such as Monoolein LLC NPs with rifampicin is used against *S. aureus* (Tran et al., 2018), a pH-sensitive lipid with vancomycin against MARS (Jadhav et al., 2018), chitosan with amoxicillin against *H. pylori* (Jing et al., 2016), PLGA NPs with amoxicillin against *H. pylori* (Luo et al., 2018), lipase-sensitive polyurethane micelles with Ag NPs against *E. coli* and *S. aureus*, lipid and peptide-coated mesoporous silica NP with gentamicin against *S. aureus* (Su et al., 2017), amoxicillin complexed with gold against *S. aureus*, AGS-NPs with clarithromycin against *H. pylori* (Pei et al., 2017), etc. Though the nanoparticle drug delivery system has many advantages, it is essential to develop a cost-effective method for the development of nanoparticles so that it can be encapsulated by antibiotics for better drug delivery.

4.5.3 Probiotics

Probiotics are beneficial bacteria associated with human and animal health. The term probiotics were coined by Lily and Stillwell in the year 1965. Probiotics can also be termed as a modified microbial community from the human gut that replaces the harmful microflora. Besides, probiotics are considered as a consortium of bacteria that benefit human health by improving the indigenous flora (Havenaar and Huis, 1992). Bacteria comprise half of the wet weight of the human colon and the human stomach consists of 103 different groups of bacteria (Slavin, 2013). Bacterial colonization in the gut begins when newborns are exposed to a nonsterile environment, which further evolves and transforms depending upon the host life style (Lloyd-Price et al., 2016). These gut bacteria act as a barrier to protect against various ingested harmful microorganisms and thus these are also said as beneficial bacteria that have an impact on our immune system and normal metabolic process. Any change in these microflorae may result in severe immune compromising status. To overcome this targeted drug delivery approach, nano encapsulation supplements have been used but these methods are not cost-effective thus cost-effective probiotic supplements have been developed. Microbial strains of human origin are usually considered as probiotics (Ouwehand et al., 2002).

A good probiotic should include the following criteria: (i) isolated from the same host, (ii) nonpathogenic, (iii) can survive in the gastrointestinal tract of the host, and (iv) can survive for a longer period in the host system (Harish and Varghese, 2006). Initially, *Lactobacillus* and *Bifidobacterium* have been extensively used as probiotics (Holzapfel et al., 2001). Recently, many other microbes that are currently used include *Saccharomyces*, *Propionibacterium*, *Akkermansia*, *Peptostreptococcus*, *Bacteroides*, *Bacillus*, *Lactococcus*, *Sterptococcus*, *Enterococcus*, and *Pediococcus* (Kerry et al., 2018). Among these bacteria, *Lactobacillus* (LAB) bacteria are extensively studied and are the most effective probiotic because of its ability to improve health, prevent diarrhea, cure lactose intolerance, prevent food allergies, etc. (Andersson et al., 2001). Consumption of probiotics has many benefits like maintaining and promoting the GIT (Gastrointestinal effect) homeostasis, stimulating the growth of beneficial microbes, inhibiting the growth of harmful microbes (Ohashi and Ushida, 2009), and reducing the risk of diarrhea (Ouwehand et al., 2002). It improves irritable bowel syndrome symptoms, cures constipation, and prevents allergic reactions in newborns and infants with allergic rhinitis.

At present probiotics are used for the angiogenic activity, antipathogenic activity, antiobesity activity, antidiabetic activity, antiallergic activity, anticancer activity, anti-inflammatory activity, for cholesterol normalization, lactose metabolism, and in proper digestion of food protection from parasitic infection. Several probiotics are been in use commercially, *Lactobacillus* sp. is been supplemented for bacterial vaginosis (BV) and urinary tract infection (UTI) (Waigankar and Patel, 2011). Probiotics like arabinoxylan and arabinoxylan oligosaccharides have been recently used to treat type 2 diabetic patients (Kerry et al., 2018). *Lactobacillus*, *Bifidobacterium*, *Enterobacter*, and *E. coli* in food have been used as an effective probiotic having anti-inflammatory activity; moreover, genetically engineered probiotic bacteria are also been in use e.g. gram-positive bacteria that produces immunomodulators to boost up commensal bacterial species thus protecting against inflammation.

Lactobacillus plantarum 06CC2 is currently been used as an effective antiallergic probiotic (Song et al., 2016). *L. plantarum* WCFS1 is used as a daily supplement for children having autism spectrum disorder. Apart from this, several probiotics have been commercially available and were found to have several beneficial activities in humans thus owing to the above cost-effective and beneficial properties of probiotics; it may be considered as an effective alternative to antibiotics.

4.5.4 Bacteriocins

Bacteriocins are AMPs, shielding bacteria from being attacked by other bacteria and harmful compounds. The first bacteriocin colicin-V was discovered by Andre

Gratia in the year 1925. It is an antibacterial compound, produced by *Enterobacteriaceae* and *E. coli* strain inhibiting the growth of virulent *E. coli*-V. The bacteriocins are found in both gram-positive and gram-negative bacteria, but bacteriocins from gram-positive bacteria are more beneficial as they are commonly found in commercially used LAB bacteria like *Lactococci*, *Lactobacilli*, etc., which are used frequently in food and fermented products. Initially, these peptides were reported to have specific inhibition within its ecological niches but shortly various peptide compounds were identified that can act upon a wide variety of microorganisms apart from its ecological niches (Blay et al., 2007). These peptides are consisted of mostly less than 100 amino acids and have both hydrophobic and hydrophilic residues (Giuliani et al., 2007). Besides bacteriocin used as an effective antibacterial product, these peptides are also posing antibiotic activity against fungi and protozoans (Aley et al., 1994). The mode of action varies for each peptide-like changing the transmembrane potential, electrostatic interaction, inhibiting biosynthetic pathway (replication, translation, and transcription), inhibiting spectrum formation, creating voltage gradient, and membrane channels. Large numbers of bacteriocins are produced by the LAB group of bacteria. The bacteriocins contain either glycoproteins (Van Belkum et al., 1991) or lipoproteins e.g. Lactocin 27 and Lacstrepcins.

The recombinant bacteriocins namely BMP32 produced by *E. coli* expression system are reported as broad-spectrum activity on MDR strains with MIC value of 9.2–36.8 mg/l (Qiao et al., 2020). Capidermicin, a novel bacteriocin that is produced by *Staphylococcus capitis* was found to have antimicrobial activity against a wide range of gram-positive bacteria like the number of *Staphylococcus* sp., *Lactococcus* sp., *Micrococcus* sp., and *Streptococcus* sp. (Lynch et al., 2019).

4.5.5 *Bdellovibrio*

The MDR microbes can combat with antimicrobial drugs and leads to cause ineffective treatment that resulting in the persistence and spreading of serious infections. Therefore, it is essential to develop new strategies to control such infection caused by MDR strains. *Bdellovibrio* sp. are small, motile, gram-negative predatory bacteria discovered by Stolp and Petzold in the year 1962; they are said to depend on other gram-negative bacteria for its survival.

These organisms showed intraperiplasmic growth and epibiotic predation. During the intraperiplasmic growth, the predators attach to the outer membrane of the prey that is mediated by chemotaxis, aero taxis, or by random collision to its prey organism. After attachment, the predator enters the prey by producing several hydrolytic enzymes that help them to cleave the cell wall of the prey. Once it enters the prey, the predator resides inside the periplasmic space and forms a somatically stable structure called bdelloplast that further elongates by feeding on

the cytoplasmic content of the prey organism, after complete utilization of the prey organism the elongated bdelloplast get separated to produce many progenies and finally the young progenies are released by lysing the prey cell (Stolp and Starr, 1963). On the other hand, *Bdellovibrio exovorus* sp. feed upon its prey in an epibiotic fashion where the predator remains attached to the outer membrane of the pre organism and sucks its internal contents (Koval et al., 2013).

4.5.6 *Bdellovibrio* as Live Antimicrobial Agent

The lifecycle of *Bdellovibrio* sp. leads to complete degradation of its prey (Figure 4.4), and several studies have shown that *Bdellovibrio* sp. feeds on a variety of human pathogens including MDR (Im et al., 2019). Further, the inability of these organisms to attach and grow on animal cells and its low immunogenic nature (Schwudke et al., 2003) has made it a promising tool to be used as a live antibiotic. To check its safety in the living system, the first animal trial was done in 1973 by Verklova by injecting *Bdellovibrio bacteriovorus* with some lab animals that showed a completely nonpathogenic effect on these animals. Further, chicks

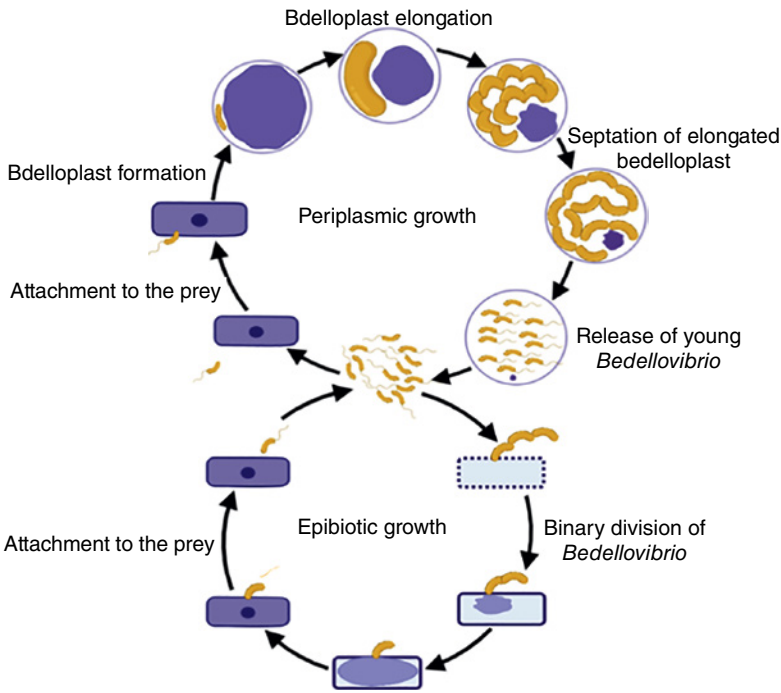

Figure 4.4 Life cycle of *Bdellovibrio*.

induced with *Salmonella* infection were treated with 9.8×107 PFU/mlof *B. bacteriovorus* HD 100 for 10 days that showed a significant reduction in *Salmonella* count (Atterbury et al., 2011). About 5 µl of *B. bacteriovorus* 109J containing 5×1010 PFU/ml was capable of reducing *K. pneumoniae* in rat liver (Shatzkes et al., 2016).

In another study, Willis et al. (2016) reported that $1–2 \times 105$ PFU/ml is capable of reducing *Shigella* count in Zebrafish larvae. *Bdellovibrio* sp. are also found in the human gut microflora, and according to Lebba et al. (2014), healthy individuals have a higher amount of *Bdellovibrio* in their gut than patients affected with inflammatory bowel disease, celiac disease, and cystic fibrosis. *B. bacteriovorus* 109J is capable of predating upon oral pathogens that contributes to periodontal disease. Sinha et al. (2014) have suggested that *Bdellovibrio* can be used along with β-lactam antibiotics, which is usually used to treat dental diseases; the activity of *Bdellovibrio* remains unaffected by the antibiotics. Further, *B. bacteriovorus* of 1×108 CFU/ml concentration is capable of predating upon periodontal pathogens like *Eikenella corrodens*, *Aggregatibacter actinomycetemcomitans*, *P. gingivalis*, and *Fusobacterium nucleatum* in aerobic conditions (Patini et al., 2019). Ottaviani et al. (2018) showed the predatory effect of *B. bacteriovorus* against eight different MDR *E. coli* strains. Very few reports are available to support that *Bdellovibrio* sp. can also affect gram-positive bacteria. The first report was published in 2014 by Lebba et al. showed epibiotic predation of *Bdellovibrio* sp. on a gram-positive *staphylococcus* sp., and in the second report, *Bdellovibrio* showed dispersion of biofilm of *S. aureus* without a decrease in the concentration of predator. Also, *Bdellovibrio* sp. is capable of feeding on a wide verity of gram-negative bacteria like *Pseudomonas*, *Salmonella*, *Shigella*, *Erwinia*, *Helicobacter*, etc. (Markelova, 2010); thus, *Bdellovibrio* and like organism (BALOs) can be considered as a promising alternative for antibiotics (Socket and Lambert, 2004).

4.6 Antibiotics: Global Action Plan on Antimicrobial Resistance

The most problematic issue of overuse of antibiotics is the development of bacterial resistance, which was first observed in the case of penicillin and later in erythromycin to treat *S. aureus* infections in the early 1950s. Soon it was completely introverted as *S. aureus* isolates found to be resistant to erythromycin. Similarly, chloramphenicol, chlorotetracyclin, and other antibiotics also become ineffective (Finland, 1979). Some international expertise health groups (e.g. WHO and the CDC) have strictly suggested are the following: (i) control the use of antibiotics, (ii) take the correct prescriptions, (iii) stop self-medication, (iv) complete the drug

dose, and (v) minimize the use of therapeutics in agricultural and in animal husbandry (DeVincent and Viola, 2006). In contrast to this, the situation is much more critical, as in many developing countries, the antibiotic usage is uncontrolled. Overcoming the issue of increasing AMR is a serious question of all the countries. To fight back against AMR, a global action plan was primed, having five different strategic tasks: (i) Make awareness through surveillance and research, (ii) increase the awareness and understanding of AM, (iii) Slow down the occurrence of infections, (iv) Limited and required use of antimicrobial agents, (v) To create a sustainable asset to cut down the hardship of the needs of all countries and to create investments for new drugs/medicines/vaccines/diagnostic tools and other discoveries.

4.7 Conclusion

Antibiotics are the antibacterial agent used for the treatment of various infectious diseases. The antimicrobial susceptibility testing is an important step in the formulation of effective antibiotic policy for the treatment of infectious diseases and diminishing the morbidity and mortality. Avoidance of standard protocols, extensive use of drugs, and lack of proper information are the main reasons for the emergence of AMR. The development of resistant strains has led to the inventions of several beneficial compounds that can be used as an alternative to antibiotics such as antimicrobial peptides that were isolated from various microbes and plant bodies, reported having antibacterial property against various bacteria by disrupting the cell wall integrity of bacteria. Bacteriocins act as a natural bacterial immune weapon system that are capable of inhibiting the growth of various gram-positive and gram-negative bacteria. Probiotics are live microbes having antimicrobial and antagonistic activity. Besides, the live antibiotics include predatory bacteria that feed on other bacteria for its survival like *Bdellovibrio*. These alternatives have gained much attention as they benefit the living system without causing any side effects. Thus, apart from antibiotics, probiotics, live therapeutic agents, and cationic peptides, bacteriocins must be implemented to control the AMR in microbes.

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5

Fermentation Strategies in the Food and Beverage Industry

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5.1 Introduction

Food and beverage industry comprise of food, alcoholic, and nonalcoholic drinks, which a consumer spends minimum effort to prepare them in a consumable form. It aims to transform raw foods to create more value-added food products such as ready-to-eat meals, fruits, soft drinks, wines, beers, etc. It is one of the highest earning industries that is set to earn US $108 402 million in 2019, and the revenues are expected to increase to US $159 000 million by 2023 (https://www.statista.com/outlook/253/100/food-beverages/worldwide). Figure 5.1 shows the share of the different countries in the food and beverage industry worldwide. The food and beverage industry is divided into two sections: (i) production and (ii) distribution of food and beverages. The production involves preparation and processing of meat, cheese, soft drinks, alcoholic beverages, packaged foods, and other modified foods excluding direct agricultural products. On the other hand, distribution involves transporting the finished food product to the consumers involving companies that ship food to retail outlets, restaurants, or directly to consumers. The chapter will focus on the production aspect of the food and beverage industry.

As the food and beverage industry is growing efforts are being made to introduce new flavors and increase the shelf life of the items to reduce the losses. Food processing is one of the most commonly used techniques to turn fresh foods into

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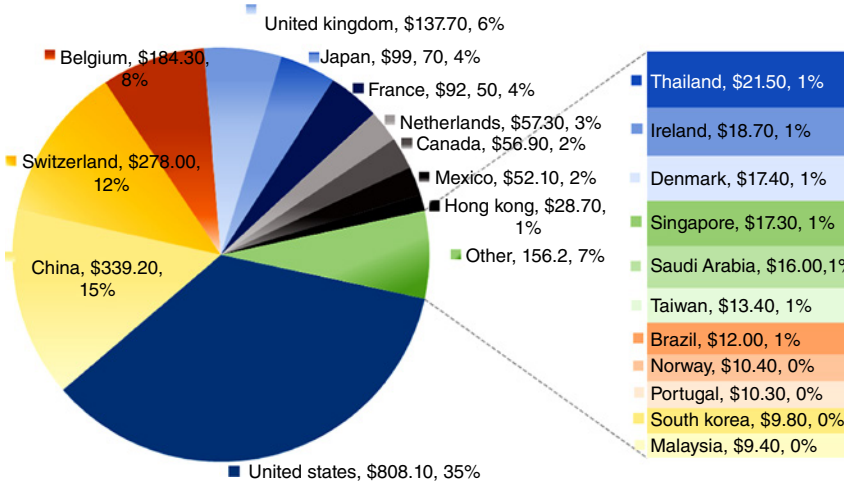

Figure 5.1 Market share of different countries in the food and beverage industry in billion USD. *Source:* Data from Global Edge, Food and Beverage Corporations. https://globaledge.msu.edu/industries/food-and-beverage/corporations.

food products. It can comprise of washing, salting, chopping, pasteurizing, freezing, fermenting, packaging, etc., either alone or in combination (https://www.eufic.org/en/food-production/category/food-processing). Fermentation is a biochemical process where a carbohydrate is oxidized by an organic molecule that is converted to alcohols, acids, or gases (Adams, 1990). Fermentation process has been used to produce biofuels (Bibra et al., 2018a; Wang et al., 2018), biochemicals (Dai et al., 2017), enzymes (Bibra et al., 2017; Rai et al., 2019), food (Dekkers et al., 2018b), beverages (Walker & Stewart, 2016), etc.

The use of fermentation for production of food and beverages is known since ages. The historians had shown signs of fermented food and beverage production from as early as 10 000 BCE (http://www.ancientpages.com/2016/09/13/ancient-civilizations-invented-alcohol-10000-years-ago/). Humans used to dry or smoke meat or other foodstuff in order to preserve it for longer periods of time. Salt preservation was also very common in the diet of sailors and soldiers during those times (https://globaledge.msu.edu/industries/food-and-beverage/background). The Romans used pickling to prevent food from microbial infestation, while the Egyptians preserved the food items by drying them in the sun (Abbas et al., 2009). The modern era breakthrough in food preservation was provided by William Cullen 1784, who developed the method of artificial refrigeration. Canning technique and salting of food began in the early 1800s to keep food fresh for a longer period (Hamad et al., 2018).

Food processing essentially remained unchanged until the nineteenth century with the invention of canning by Nicholas Appert and pasteurization by Louis Pasteur (Alba-Lois & Segal-Kischinevzky, 2010) (https://globaledge.msu.edu/industries/food-and-beverage/background). These innovations revolutionized the food preservation and processing methods enabling the food to be stored for longer time. Further, with the increased transportation during the industrial revolution, and concurrent rise in food processing technology, it became feasible to transport food from one regional market for sale to another. Today, the industry segment has evolved even further due to increased transportation technology such as airplanes. One of the discoveries in nineteenth century that changed the face of food industry was the discovery by Louis Pasteur that fermentation was a result of biochemical changes brought by live yeast cells opened new ventures. This opened new doors for the food production and formed basis to produce alcoholic beverages. Since then the understanding for fermentation had increased with its application expanding in several sectors.

The metabolic activity of fermenting microbes produces certain modifications in food ingredients viz. production of alcohols, acids, carbon dioxide, lactones, bacteriocins, removal of glycol side residues (van Hylckama Vlieg et al., 2011). These metabolites aid in the food not only aid in the preservation by limiting the growth of unwanted organisms but also produce specific flavors that are not present in the starting materials for a better mouth feel. The increased digestibility helps their digestion in the intestine. Some foods that are not edible in raw form, viz. olives are made edible by microbial action where the bitter-tasting phenolic compounds are removed. Another example is the growth of bakers' yeast (*Saccharomyces cerevisiae*), alone or with lactic acid bacteria, that achieves dough leavening during bread manufacture (Marco et al., 2017). The science of food and beverage production is very extensive. Hence, this chapter will focus on giving brief introduction that can form a basis for the food production. The chapter includes the common terminology, food fermentation types, different types of microbial cultures, starter cultures, and processes commonly used and known among the wider population that can create an interest in the readers for further studies. Further, recent changes in the food and beverage production, processing, and preservation have also been discussed.

5.2 Current Trends in Food Fermentation

The possible serendipitous discovery of fermented food now has become an artisanal practice, where the microbes improve the physical, chemical, and biological composition to enhance the sensory qualities, improve the shelf life, and make it healthier for the consumer (Borresen et al., 2012). The fermentation of food

materials has come a long way from using unknown microbial sources to known specific cultures, curing to develop specific tastes and transformation of single step/culture process to multistep/culture processes. The microbial cultures, process parameters, make the important part of the food fermentation, and we will discuss the various current trends in this section.

5.2.1 Fermentation Types

The fermentation for food and beverage production can be categorized into three types based on the type of microbial culture used.

5.2.1.1 Spontaneous Fermentation

During spontaneous fermentation, the indigenous microbes in the raw material carry out the initial biochemical transformation followed by a succession of lactic acid bacteria (LAB) and/or molds (Alexandraki et al., 2013). No external microbial source is added. After the growth of the indigenous microbes, the LAB succeed them producing lactic acid from the lactose. The production of the lactic acid lowers the pH and prevents the contamination by unwanted microbes while simultaneously increasing the shelf life. Following the growth of the LAB, molds can grow and produce certain flavor-enhancing chemicals by enzymatic hydrolysis that change the organoleptic properties of the food and beverages. However, the succession by molds is limited only to the food where appropriate oxygenic conditions are available (Josephsen & Jespersen, 2004).

5.2.1.2 Back-Slopping Fermentation

During back-slopping fermentation, the microbial inoculum is obtained from the previous batch of the fermented product (Sulieman, 2017). Using microbial inoculum from a previous fermented batch ensures a faster and more reliable fermentation compared to the spontaneous fermentation, due to use of a previously adapted culture. Hence, every time similar set of microorganisms perform the fermentation, ensuring the growth of the same bacteria every time. Making bread, homemade yogurt, and dosa are some of the examples that include the use of back-slopping fermentation.

5.2.1.3 Starter-Culture Fermentation

In starter-culture fermentation, the indigenous microbial flora is inactivated by heat treatment, and new microbial cultures are added (Alexandraki et al., 2013). Nevertheless, the addition of starter cultures ensures that only the desirable microbiota dominate the fermentation. Use of yeast for beer and wine making, lactic acid bacteria for dairy products, sausages, and sauerkraut include some of the examples that involve the use of starter culture.

5.2.2 Microbial Cultures

The selection of an appropriate microorganism is one of the major requirements that drive the physical, biochemical, and organoleptic changes during the fermentation. Most commonly yeast and *Lactobacillus* cultures have been used to produce fermented foods. The microbial cultures can have a diversified role in the fermentation ranging from being a starter culture to health-promoting culture. Hence, choosing the appropriate and healthy starting microbial culture and optimizing their performance is one of the main priorities to ensure the consistency, safety, and quality of the final product. In some instances, the microorganisms transform the starting material into products ready for direct human consumption viz. alcoholic fermentation by yeast (Ali et al., 2012), while in other instances, the microorganisms hydrolyze the unwanted materials to improve the substrate characteristics for further human use viz. curing of coffee beans (Vinícius de Melo Pereira et al., 2017). Table 5.1 gives the information on use of various microbial cultures for different fermented products.

5.2.2.1 Starter Cultures

The starter cultures are the preparations that include living or inactive microorganism that build up the desired metabolic activity in the food and beverages (Singh et al., 2012). These are the main fermenting organisms that carry out majority of the desired biochemical transformation. Several yeasts (*Saccharomyces, Kluyveromyces, Candida*, etc.), bacteria (*Lactobacillus, Bifidobacteria, Brevibacterium*, etc.), and molds (*Penicillium*, *Aspergillus*, *Mucor*, etc.) have been used as the starter culture (Sulieman, 2017; Vinícius de Melo Pereira et al., 2017). A good starter culture should have the following characteristics:

- Should be stable
- Should possess generally regarded as safer (GRAS) status
- Should produce the desired product at a fast-kinetic rate
- Should be able to perform at diverse range of operational parameters viz. temperature, pH, water activity, etc.
- Should be resistant to antibiotics
- Should be able to perform in the presence of inhibitory chemicals
- Should not produce antibiotics, bacteriocins or other products that can inhibit the other useful microbial cultures
- Should produce the desired color, aroma, consistency, body texture in the final fermented products
- Should be able to live in harmony with other required microbial cultures required for final fermented products

Depending upon the amount of the starter culture required, they can be classified into mother starter cultures (volume <1 l) or bulk cultures (volume >1 l) (Sulieman, 2017).

Table 5.1 The various fermented products produced by yeast, bacteria, and molds and associated fermentation and culture types.

Product	Fermenting species	Fermentation type	Culture type	References
Yeast				
Ale Beer	*Saccharomyces cerevisiae*	Starter-culture fermentation	Starter	Walker & Stewart (2016)
Bread	*Saccharomyces cerevisiae*	Starter-culture fermentation	Starter	Ali et al. (2012)
BioBran – Fermented rice bran	*Saccharomyces boulardii*	Starter-culture fermentation	Starter	Borresen et al. (2012)
Coffee (wet fermentation)	*Kluyveromyces marxianus,* *S. cerevisiae var. ellipsoideus, Schizosaccharomyces spp.* *Candida guilliermondii var. membranifaciens,* *Candida parapsilosis,* *Candida pelliculosa, Rhodotorula mucilaginosa* and *Torulopsis fumata, Cryptococcus laurentii, Kloeckera apis apicuata, Cryptocccus albidus Candida guilliermondii, Hanseniaspora uvarum, Hanseniaspora opuntiae*	Starter-culture fermentation	Starter	Vinícius de Melo Pereira et al. (2017)
Coffee (dry fermentation)	*Debaryomyces hansenii, Debaryomyces polymorphus,* *Pichia guilliermondii, Pichia ofunaensis,* *Pichia holstii,* *Pichia guilliermondii,* and *Arxula adeninivorans*	Starter-culture fermentation	Starter	Vinícius de Melo Pereira et al. (2017)
Lager beer	*Saccharomyces pastorianus* and *Saccharomyces carlsbergensis*	Starter-culture fermentation	Starter	Borresen et al. (2012) and Walker & Stewart (2016)
Lambic beer	*Brettanomyces bruxellensis*	Starter-culture fermentation	Starter	Walker & Stewart (2016)

Red rice yeast	*Monascus purpureus*	Starter-culture fermentation	Starter	Borresen et al. (2012)
Whisky	*Saccharomyces cerevisiae*	Starter-culture fermentation	Starter	Hui et al. (2004) and Walker & Stewart (2016)
Wine	*Saccharomyces cerevisiae*	Starter-culture fermentation	Starter	Walker & Stewart (2016)
Wine	*Candida stellata*	Starter-culture fermentation	Starter	Walker & Stewart (2016)
Bacteria				
Acidophilus milk	*Lactobacillus acidophilus*	NA*	Probiotic	Tamime (2002)
Bacteriocin Nisin-prevent delayed *Clostridium* bloating in cheese	*Lactobacillus lactis* subsp. *lactis*	NA*	Bioprotective	Sulieman (2017)
Bacteriocin	*Lactobacillus lactis* subsp. *Cremoris* and *Lactobacillus lactis* subsp. *lactis* biovar *diacetylactis*	NA*	Bioprotective	Chen et al. (2017) and Sulieman (2017)
Butter and buttermilk	*Lactococcus lactis* subsp. lactis, *Lactococcus lactis* ssp. *-actis* var. *diacetylactis*, *Lactococcus lactis* subsp. Cremoris and *Leuconostoc menesteroides* subsp. *cremoris*	Starter culture fermentation	Starter	Tamime (2002)
Cabbage (Sauerkraut)	*Leuconostoc mesenteroides, Lactobacillus plantarum, Lactobacillus brevis* and *Lactobacillus fermentum*	Starter-culture fermentation	Starter	Wakil et al. (2014)
Cheese-Gouda, Edam, sour cream, and lactic butter (Mesophilic starter)	*Lactococcus lactis* ssp. *lactis*, *Lactococcus lactis* ssp. *lactis* var. *diacetylactis*, *Lactococcus lactis* ssp. *cremoris* and *Leuconostoc mesenteroides* ssp. *cremoris*	Starter-culture fermentation	Starter	Kongo (2013) and Tamime (2002)
Cheese – High cook cheese, hard and semi-hard cheese, (Thermophilic starter)	*Streptococcus thermoplillus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus helveticus,* and *Lactobacillus delbrueckii* subsp. *lactis*	Starter-culture fermentation	Starter, Adjunct	Kongo (2013), Sulieman (2017), and Tamime (2002)

(*Continued*)

Table 5.1 (Continued)

Product	Fermenting species	Fermentation type	Culture type	References
Cheese (Mixed starter)	*Lactobacillus lactis* subsp. *lactis, Lactobacillus lactis* subsp. *cremoris* and *Streptococcus thermoplillus*	Starter-culture fermentation	Starter	Kongo (2013) and Tamime (2002)
Cheese – Gruyere, Swiss, and Emmental	*Propionibacterium acidipropionici* and *Propionibacterium freudenreichii* subsp. *Shermanii*	Starter-culture fermentation	Starter	Kongo (2013)
Cheese ripening	*Lactobacillus casei, Lactobacillus plantarum*, *Lactobacillus rhamnosus,* and *Brevibacterium linens*	Secondary fermentation	Adjunct, Probiotic	Kongo (2013) and Sulieman (2017)
Coffee	*Leuconostoc mesenteroides, Lactobacillus* subsp. and *Streptococcus faecalis*	Secondary fermentation	Adjunct	Vinícius de Melo Pereira et al. (2017)
Dry sausages	*Lactobacillus sakei, Lactobacillus curvatus, Lactobacillus plantarum, Lactobacillus pentosus, Lactobacillus casei, Propionibacterium pentosaceous,* and *Propionibacterium acidilactici*	Starter-culture fermentation	Starter	Hammes & Hertel (1998) and Hugas & Monfort (1997)
Fermented milks	*Lactobacillus delbrueckii* subsp. bulgaricus, *Streptococcus thermophilus, Lactobacillus casei, Lactobacillus acidophilus, Lactobacillus rhamnosus,* and *Lactobacillus johnsonii*	Starter-culture fermentation	Starter	Kongo (2013) and Tamime (2002)
Fruit and vegetable – increase in shelf life	*Lactobacillus acidophilus* NCDC291	NA*	Bioprotective	Garcha & Natt (2012)
Kefir	*Lactobacillus paracasei* subsp. *paracasei*, *Lactococcus lactis* spp. *lactis* and *Leuconostoc mesenteroides*	Starter-culture fermentation	Starter	Kıvanç & Yapıcı (2015)
Kimchi	*Leuconostoc mesenteroides, Lactobacillus plantarum, Weissella kimchii* sp. *nov., Lactobacillus. Kimchi, Lactobacillus sakei,* and *Weissella koreensis*	Starter-culture fermentation	Starter	Choi et al. (2002), Lee et al. (2013), and Muyanja et al. (2003)

Kombucha	*Gluconobacter xylenus*	Secondary fermentation	Adjunct	Borresen et al. (2012)
Sourdough	*Lactobacillus brevis, Lactobacillus hilgardii, Lactobacillus sanfransiscensis, Lactobacillus farciminis, Lactobacillus fermentum, Lactobacillus brevis, Lactobacillus plantarum, Lactobacillus amylovorus, Lactobacillus reuteri, Lactobacillus pontis, Lactobacillus panis, Lactobacillus alimentarius,* and *Weissella cibaria*	Starter-culture fermentation	Starter	Mheen & Kwon (1984) and Palla et al. (2017)
Wheat dough	*Pediococcus pentosaceus* strain DSM 20336 *Weissella confusa* strain PL9001 *Pediococcus acidilactici DSM 20284, Lactobacillus crustorum* strain B481 *Lactobacillus fermentum* strain NBRC 15885	Secondary fermentation	Adjunct	Xing et al. (2019)
Yakult	*Bifidobacterium bifidum, Bifidobacterium longum, Bifidobacterium infantis* *Bifidobacterium breve*	Starter-culture fermentation	Starter	Sulieman (2017)
Yogurt	*Lactobacillus delbrueckii* ssp. *bulgaricus* *S. thermophilus* *Bifidobacterium*	Starter-culture fermentation	Starter	Chen et al. (2017) and Tamime (2002)
Molds				
Cheese-blue	*Penicillium roqueforti*, *Penicillium camemberti* (for cheese)	Secondary fermentation	Adjunct	Speranza et al. (2017) and Sulieman (2017)
Cheese-swiss	*Propionibacterium shermanii* subsp. freudenreichii	Secondary fermentation	Adjunct	Speranza et al. (2017) and Sulieman (2017)
Cheese-soya milk	*Aspergillus oryzae*	Starter-culture fermentation	Starter	Sulieman (2017)

The mother starter cultures are used sometime to produce starter cultures. Furthermore, depending on the microbial species used, they can be classified into single-strain starter culture (only one microbial strain is used as starter culture) or multi-strain cultures (more than one microbial starter culture is used as starter culture) (Sulieman, 2017).

5.2.2.1.1 Yeasts The yeasts are one of the most commonly used organisms to produce fermented food and beverages. The fermentation of carbon sources to produce alcoholic beverages by yeasts is one of the oldest known technologies (Walker & Stewart, 2016). *Saccharomyces cerevisiae* commonly known as Baker's yeast is used to produce alcoholic beverages and bread. Yeasts are chemoorganotrophic plant-like microfungi and use the organic substrates as the source of carbon and energy (Ali et al., 2012). The yeast favor warm and acidic environments for beverage production with 20 and 30 °C and pH 4.5 and 6.5 being the favorable conditions for *S. cerevisiae*. However, the lager-producing yeast strains viz. *Saccharomyces pastorianus* can ferment even in cooler temperatures (for example, 8–15 °C) (Walker & Stewart, 2016). During fermentation, the yeasts acidify their growth environment through a combination of proton secretion during nutrient transport (through the action of the plasma membrane proton-pumping ATPase), direct secretion of organic acids (for example, succinate and acetate), removal of buffering agents, and carbon dioxide evolution and dissolution. *Saccharomyces cerevisiae* though is considered as the facultative anaerobe, growth is not possible under obligate anaerobic conditions due to the requirement of oxygen as a growth factor for membrane fatty acid (for example, oleic acid) and sterol (for example, ergosterol) biosynthesis. Under anaerobic conditions, *S. cerevisiae* is not able to produce oleic acid and ergol. Hence, some oxygen is supplied at the start of fermentation, or fatty acids and sterol growth factors can be supplemented to the medium (using commercially available yeast foods) for efficient fermentation.

Depending upon the requirement for different alcoholic beverage, flavor and aroma of different yeast types are used. *Saccharomyces cerevisiae* is most commonly used for the ale production; *Saccharomyces pastorianus*, a hybrid of *S. cerevisiae* and *Saccharomyces eubayanus*, is used for lager production (Walker & Stewart, 2016). The two different type of yeasts carry out "top fermentations," yeasts congregate on the surface of the fermenting wort, and "bottom fermentations," yeast settles down at the bottom of the fermenting vessel and can be recovered, respectively, for ale and lager production. During whiskey and related alcoholic beverage production, the mashing of cereal produces maltose and maltotriose that need to be hydrolyzed. After the prohibition on use of external enzymes by "The Scotch Whiskey Regulations of 2009," only the yeasts that possess native amylolytic activity are used for scotch and whiskey production (Russell

& Stewart, 2014). The yeast strain "M type," a hybrid of *S. cerevisiae* and *Saccharomyces diastaticus,* used in Scotch whisky production has amylolytic activity that aids in hydrolysis of sugars. At industrial scale, maltose (malt wort for brewing), sucrose (molasses for rum production), lactose (cheese whey-based beverages), and fructose (Agave spp. polyfructans for Tequila) are the economical substrates used for fermentation. For juice and wine production, glucose together with fructose is used. In addition to *Saccharomyces* spp., some non-saccharomyces cultures viz. *Candida stellate* are also commonly used for desired organoleptic features and aroma in the wine. Furthermore, the secondary metabolites viz. esters, diketones, phenolic compounds, etc., produced by the yeasts act as flavor congeners in the final spirit (Russell & Stewart, 2014).

Bread making is another widely practiced application of *S. cerevisiae*. It is a two-step, temperature-dependent process: (i) low-temperature (30–37 °C) sugar fermentation by yeast that produces CO_2 resulting in porous dough structure and concomitant development of dough volume, and (ii) high-temperature (100 °C) baking that ceases the yeast activity that finalizes the bread structure (Ali et al., 2012). Several physical, chemical, and biochemical transformations take place during bread making. The mixing of the bread ingredients transforms them into a viscoelastic material made of three-dimensional protein network where the starch granules are consistently detached. The dough produced after kneading of the ingredients is a multiphase and multicomponent system composed of conditions, proteins, lipids, carbohydrates, water, and air. During kneading, air bubbles are introduced in the dough that act as the early nuclei for the gas bubble. After kneading, the dough is left for leavening during which gas bubbles are built-in in the dough by yeast's biochemical activity. The alcohol produced during biochemical transformation of sugars by yeast changes the colloidal nature of the wheat proteins and the interfacial tension in the dough. A stable three-dimensional network increases the gas retention in the dough. A better gas production and retention helps in increased loaf volume, measured by the increase in the dough volume during leavening. After this, the dough is given in a desired shape and baked in an oven. During baking, the inside temperature reaches 100 °C, and the volume fraction of bread reaches a final value between 0.8 and 0.9 (Shehzad et al., 2011). After baking, the gluten cross-links, and starch granules are disrupted giving the bread its desired aroma, flavor, and organoleptic characteristics (Bajd & Serša, 2011).

During both the applications, yeasts can encounter high sugar concentration that can result in the decrease of yeast activity due to increased osmotic stress. Under increased osmotic stress, yeast cells produce glycerol, or other osmolytes such as trehalose, which protect yeast membranes from desiccation (Walker & Stewart, 2016). Using osmotolerant strains can obliviate the osmotic stress experienced by the yeasts. *Saccharomyces cerevisiae* is unable to fix nitrogen

(non-diazotropic) and thus requires constant supply for nitrogen during fermentation for anabolic processes either as an organic nitrogen (for example, amino acids) or as an inorganic nitrogen (for example, ammonium salts) source. The growth of distilling strains of *S. cerevisiae* increases almost linearly with free amino nitrogen (FAN) levels up to 100 mg/l (Walker & Stewart, 2016). The nitrogen source also severs to produce specific flavors in distilled spirits. Minerals such as Zn, Mg, Na, K, Ca, Fe, Co, Mo, Cu, Mn, Ni, Se, etc. are also required to maintain a healthy microbial growth and enzymatic activity during fermentation (Bibra et al., 2018b; Rai et al., 2019). The metal ions may be available from the substrate, but the bioavailability can be different due to precipitation, chelation, or absorption. zinc, in alcoholic fermentations. Zn is an essential cofactor of the terminal alcohologenic enzyme, ethanol (alcohol) dehydrogenase, and its deficiency may result in sluggish or stuck fermentations (Walker, 2004). Regarding magnesium, this is essential for yeast in the production of ethanol, and it is important to maintain high levels of bioavailable magnesium to ensure maximal fermentation performance (Chandrasena et al., 1997; Walker, 2004).

5.2.2.1.2 Lactic Acid Bacteria (LAB) After yeast, LAB are the most common microorganisms widely used in the food and beverage industry. LAB from several genera, including *Lactobacillus, Streptococcus*, and *Leuconostoc* are predominant in fermented foods (Rezac et al., 2018). They aid in production and preservation of several food items viz. fermented fresh vegetables such as cabbage (sauerkraut, Korean *kimchi*); cucumbers (pickles); fermented cereal yogurt (Nigerian *ogi*, Kenyan *uji*); sourdough bread and bread-like products made without wheat or rye flours (Indian *idli*, Philippine *puto*); fermented milks (yogurts and cheeses); fermented milk–wheat mixtures (Egyptian *kishk*, Greek *trahanas*); protein-rich vegetable protein meat substitutes (Indonesian *tempe*); amino acid/peptide meat-flavored sauces and pastes produced by fermentation of cereals and legumes (Japanese *miso*, Chinese soy sauce); fermented cereal–fish–shrimp mixtures (Philippine *balao* and *burong dalag*); and fermented meats (e.g., salami) (Council, 1992; Khalid, 2011).

LAB are gram-positive, usually nonmotile, non-spore-forming rods and cocci, facultative anaerobes, peroxidase-positive organisms (Khalid, 2011). They are chemotrophic and oxidize organic compounds to obtain carbon and energy source. They do not synthesize cytochromes and porphyrins (components of respiratory chains) and thus depend upon sugar fermentation for ATP generation. They have two fermentation modes where they produce either a mixture of lactic acid, carbon dioxide, acetic acid and/or ethanol (heterofermentation) or lactic acid (homofermentation) (Ali, 2010). The different end products are obtained depending upon the different metabolic pathways involved in the sugar utilization. During homofermentative/homolactic fermentation, Embden–Meyerhof

pathway is utilized, whereas during heterofermentative/heterolactic fermentation, 6-phosphogluconate/phosphoketolase/pentose phosphate pathway is involved (Abbott et al., 2009). Various growth conditions may significantly alter the end-product formation by some LAB. These changes can be attributed to an altered pyruvate metabolism and/or the use of external electron acceptors such as oxygen or organic compounds.

LAB can grow rapidly in most food substrates, and they lower the pH to 4.0–4.5 and some of the *Lactobacilli* and *Pediococci* to about pH 3.5, where competing organisms are no longer able to grow (Council, 1992). They can also produce hydrogen peroxide through oxidation of nicotinamide adenine dinucleotide by flavin nucleotide. The hydrogen peroxide has antibacterial properties and kills unwanted organisms but has relatively less effect on the *Lactobacilli* due to the presence of catalase enzyme that can reduce hydrogen peroxide (Council, 1992).

Cheese production, one of the most widely used application of LAB, is classical example of fermentation development through ages where all three forms of fermentation – spontaneous, back-slopping, and starter cultures were used. The lactic acid production and concomitant pH drop by LAB leads to different texture, aroma, and flavor (Kongo, 2013). LAB cause a rapid acidification of milk through the production of lactic acid, with the consequential decrease in pH, thus affecting several aspects of the cheese manufacturing process and ultimately cheese composition and quality (Briggiler-Marcó et al., 2007). The milk used for cheese production is heated at 73 °C for 15 minutes to remove the undesired microorganisms. The fat content in the milk may be adjusted at this point to match the standards (Kongo, 2013). Depending upon the type of fermentation used, starter microbial cultures are obtained in situ from the substrates, added from the previous batch or a fresh starter culture is added. The milk undergoes coagulation producing a jelly-like mass. Sometimes external coagulants such as enzyme rennin, lime juice, vinegar, or plant rennet are used to fasten the process of coagulation. One of the requirements for enzymes is that they should be able to withstand the acidic environment (pH 4.5) during cheese production. The coagulum produced is cut into appropriate sizes to achieve desired acid development rate and the cheese texture. The coagulant milk is stirred to break into curd that is then heated at 37–45 °C to expel the whey from curd. After heating, the whey is removed from the curd, and the curd is milled to increase its safety and shelf life. The curdled milk is then added to a vessel for desired shape. The cheese is then ripened for the desired time. It is during ripening the LAB break down the protein and develop the desired characteristics of the cheese. The longer the cheese is ripened more costly it is.

LAB are also important for imparting specific aroma and organoleptic characteristics to various dairy products. During fermentation, LAB catalyze three major biochemical conversions of milk components: (i) glycolysis – carbohydrate

conversion into lactic acid or other metabolites, (ii) proteolysis – caseins conversion into peptides and free amino acids, and (iii) lipolysis – milk fat conversion into free fatty acids (Figure 5.2) (Chen et al., 2017). The biochemical conversions during glycolysis produces lactate, ethanol, acetate, and diacetyl; proteolysis produces amino acids, thiols, keto acids, aldehydes, alcohols, carboxylic acids, hydroxy acids and thioesters; and lipolysis produces lactones, acids, alcohols, and secondary alcohols, which enhance the flavor in yogurt. In addition to these flavoring compounds, the LAB also produce some bacteriocins that are used to protect the food and beverages from contaminating bacteria. Nisin produced by *Streptococcus lactis*, is the only commercially FDA-approved bacteriocin that is used in food and beverages (Chen et al., 2017; Kongo, 2013).

5.2.2.1.3 Other Starter Organisms The fungi are the most sought starter culture after yeast and LAB. They help to preserve food by producing inhibitory metabolites for pathogens, remove toxic compounds; to improve nutritional value; and to ensure the organoleptic quality of the food (Speranza et al., 2017). Since ages they have been involved in producing gorgonzola and roquefort cheese. In addition to cheese, they have been actively used to produce wine, fermented meat,

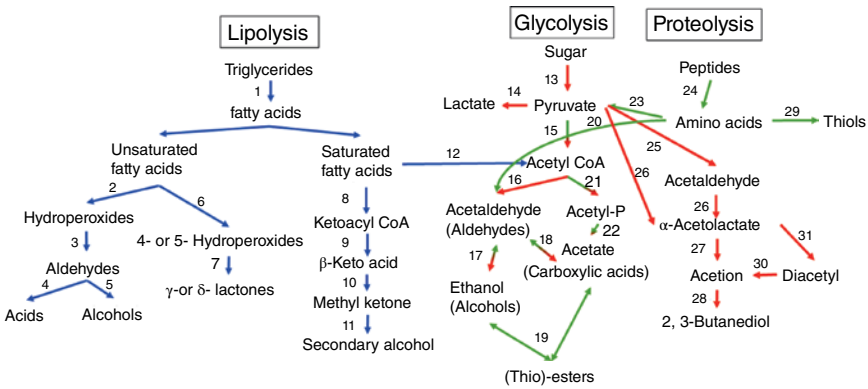

Figure 5.2 Biochemical pathways for transformation of fats (blue arrows), carbohydrates (red arrows), and proteins (green arrows) into various compounds produced by lactic acid bacteria. The share between the protein and carbohydrate hydrolysis is shown by green-red-colored arrows. 1, Lipase; 2, Oxidation; 3, Hydroperoxidelase; 4, Oxidation; 5, Reduction; 6, β-oxidation; 7, Ring closure; 8, β-oxidation; 9, Thiohydrolase; 10, β-ketoacyldecarboxylase; 11, Reductase; 12- β-oxidation; 13, Glycolysis; 14, Lactate dehydrogenase; 15, Pyruvate formate lyase/pyruvate dehydrogenase/α-ketodehydrogenase complex; 16, Acetaldehyde dehydrogenase; 17, Alcohol dehydrogenase; 18, Aldehyde dehydrogenase; 19, Esterase A; 20, Aldolase; 21, Phosphotransacetylase; 22, Acetate kinase; 23, Aminotransferase; 24, Peptidase; 25, Pyruvate decarboxylase; 26, α-acetolactate synthase; 27, Acetolactate decarboxylase; 28, Acetoin reductase; 29, Lyases; 30, Diacetyl reductase; 31, Oxidative decarboxylation. *Source:* Adapted from Chen et al. (2017).

etc. The different enzymes produced by fungi help in the hydrolysis of different components in the starting material. The fungi grow under aerobic conditions. Thus, adequate air supply is necessary when fungi is used as the starter culture.

5.2.2.2 Adjunct Cultures

Adjunct/secondary cultures are the cultures that are added at some point of the manufacture of fermented foods after the starter culture have been introduced for production (Bintsis, 2018). The adjunct cultures are mainly nonstarter LAB cultures that are commonly used in the cheese manufacture where they balance the biodiversity lost during pasteurization, improve hygiene, improve the flavor, and accelerate the ripening process (Bintsis, 2018; El Soda et al., 2000). The adjunct LAB cultures do not use lactose, amino acids, and sugars from nucleic acids as energy sources, but can use carbohydrates associated with the milk fat globule membrane (El Soda et al., 2000). The extracellular polysaccharides produced by adjunct cultures bring various organoleptic, flavor, and texture changes in yogurt, cheese, fermented cream, and milk-based desserts (Bintsis, 2018; Jolly et al., 2002).

5.2.2.3 Bio-protective Cultures

One of the challenges food and beverage industry faces is the spoilage of the finished products, due to contamination by unwanted organisms viz. *Clostridia* etc. during preparation, processing, and shelf storage. Some LAB can produce chemical compounds viz. bacteriocins that have bactericidal or bacteriostatic effect (Garcha & Natt, 2012). Due to the production of bacteriocins, the LAB has been considered bio-protective cultures. The bacteriocins aid in food preservation and spoilage reduction by targeting the cell wall synthesis and membrane disruption of unwanted microorganisms, causing their cell death (Bintsis, 2018). Lacticin 3147 from *Lactococci*, macedovicin from *Streptococcus macedonicus,* ACA-DC reuterin from *Lactobacillus reuteri*, sakacin M from *Lactobacillus sakei* 148, pediocin PA-1/AcH from *Pediococcus acidilactici*, plantaricins (A, EF and JK) from *Lactobacillus plantarum* are some of the bacteriocins that are produced by LAB (Bintsis, 2018). They have also been effective in controlling and reducing the fungal contamination in the fermented food and beverages. *Lactobacillus acidophilus* NCDC 291 was used to control the growth of four different fungi *Fusarium, Alternaria, Penicillium,* and *Aspergillus* (Garcha & Natt, 2012).

5.2.2.4 Probiotic Cultures

Probiotic cultures are live microbial cultures that help to improve intestinal health of the host organism (Bintsis, 2018). The human intestine harbors around 10e12 microbes that live in a harmonious relation with the host and protect against the pathogens, extract nutrients from food components, improve immune system, and provide various vitamins (Rezac et al., 2018). The gut microbiota is

usually stable but when encountered by antibiotics, stress or disease, the microbial balance gets disrupted (Sonnenburg & Bäckhed, 2016). The disrupted balance makes the host to go under diseased conditions. The probiotic cultures help to maintain and resurrect the microbial balance in the intestine. Most of the probiotic cultures are from LAB comprising mainly strains of *Lactobacillus* spp., *Bifidobacterium* spp. *Propionibacterium* spp., and *Leuconostoc* spp. (Rezac et al., 2018). The ingested probiotic cultures influence the carbohydrate metabolism by rapidly metabolizing the carbohydrates to lactic acid, acetic acid, or propionic acid. These compounds help in the growth of the normal gut microbiota. The ingestion of *Lactobacilli* or *Propionobacteria* produced acids and vitamins that aid in the stimulation of *Bifidobacterium* in the gut (Derrien & van Hylckama Vlieg, 2015). The studies on *L. acidophilus* and *L. fermentum* had shown the inhibitory effect on the growth, α-toxin production, and inflammatory responses of *Clostridium perfringens* (Guo et al., 2017).

5.3 Future Directions

Fermentation, though has been employed since ages for the production and preservation of food and beverages, modern era has seen continuous improvement in the process. The use of mixed cultures, nanotechnology, producing meat alternatives, fermentation-based cell agriculture, tissue engineering, and genetic engineering are some of the various changes that have revolutionized the production of fermented food and beverages sector. This section will focus on some of the recent advancements in the fermentation of food and beverages.

5.3.1 Use of Defined Mixed Cultures

The use of defined mixed cultures has seen an uprising in the production of fermented foods and beverages. Where primary microbial culture carry out the required fermentation, the secondary culture provide the necessary biochemical transformations that carry out the secondary fermentation to obtain desired flavors, physical changes, and organoleptic changes. The production of wine, "sour" beers, and kombucha fermented tea all involve both yeast and bacterial fermenters (Borresen et al., 2012). The production of kombucha, a Russian beverage, involves the use of a symbiotic colony of acid-producing bacteria and yeast to ferment an infusion made with tea leaves and herbs and sweetened with sugar. *Gluconacetobacter xylenes* convert the alcohols produced by the yeasts into acetic acid during the production of kombucha. The wine production also involves the late-stage secondary malolactic fermentation by LAB followed by aging, clarification, and packaging (Walker & Stewart, 2016). During the malolactic fermentation,

the LAB convert the dicarboxylic L-malic acid to monocarboxylic L-lactic acid, giving the desired aroma and mouth feel. In sour-mash bourbons also the inclusion of lactic acid bacteria depresses the wort pH and impart desired flavor congeners to the final distillate (Walker & Stewart, 2016).

5.3.2 Nanotechnology

In recent years, the use of nanotechnology had been on rise in the food and beverage industry. It has found applications in areas ranging from food packaging, contamination prevention, increasing shelf life, etc.

5.3.2.1 Nanosensors

The nanosensors had been instrumental in detection of chemical changes in the food and beverages. The high sensitivity and specificity of the nanosensors make them handy when it comes to field application in comparison to the other prevalent methods (Hamad et al., 2018). They had been actively used to detect the gases in milk, pesticides on vegetables and fruits, potential carcinogens in foods and beverages, etc.

5.3.2.2 Nanoparticles

Nanoparticles have been used in developing several aspects of food and beverage industry such as packaging, regulated release of coloring agents, and antimicrobial activities (Bajpai et al., 2018). The food material usually gets spoiled when it has enough air and moisture for the unwanted organisms to grow. Active packaging material made of nanoparticles with ability to absorb or release certain compounds can decrease the spoilage of the food materials. The silver (Ag) nanoparticles are most commonly used for metal-oxide-based food packaging as Ag increases the cell membrane permeability of microbes by attaching to the cell or by degrading the lipopolysaccharide (Sondi & Salopek-Sondi, 2004). An effective antimicrobial-substances-based unique methodology is required to ensure the proper application of antimicrobial packaging and quality of the packaged products. The bioactive substances are incorporated into the packaging material by capsulation, capping the material with nanomaterials, or employing other nanotechniques to ensure the incorporation of antibacterial nanoparticles in the packaging (Bajpai et al., 2018). The polymeric nanoparticles had also been used to regulate the release of the bioactive compounds such as vitamins and flavonoids in an acidic environment (Pool et al., 2012). The nanoparticle coating of ZnO has shown positive results as an anti-brown agents in Fuji apples (Bajpai et al., 2018; Li et al., 2011).

5.3.2.3 Nanocomposites

The nanocomposites are produced by the combination of nanoparticles with the polymers viz. low-density polyethylene, gelatin, isotactic polypropylene, and

polylactic acid (Hamad et al., 2018; He & Hwang, 2016). The presence of different chemical groups in the nanocomposites and their ability to regulate the migration of metals into stored foods makes them suitable for the development of high barrier properties that can protect the food materials against the infestation by unwanted organisms. Further their ability to act as the gas barrier and reduce the carbon dioxide leakages from carbonated beverage bottles is advantageous for increasing the shelf life of the product (Hamad et al., 2018). The use of nanocomposites by manufacturing industries in place of cans and glass bottles to layer their bottles is cost-effective too.

5.3.3 Meat Analogues

The drive to obtain a constant protein supply with lesser environmental impact has led to the research and development of meat alternatives by fermentation (Stephens et al., 2018). The products are manufactured by fermentation using bacteria, algae, fungi, or yeast wild type or genetically modified to produce the desired organic molecules. The biopolymers for plants is structured into fibers by using a concentrated two-phase system consisting of soy protein isolate and wheat gluten, or a blend of the two (Dekkers et al., 2018a). Soy protein has 65–70% (w/w) of soy protein and forms the protein source in the meat analogues. The wheat gluten has two proteins, namely, glutenin and gliadin that help in binding the fibers together in the meat analogues. The mixture for meat analogues is prepared using proteins, fat, salts, and other ingredients by blending, chopping, and mixing them to form a matrix of proteins. This protein matrix encapsulates the fat and non-soluble components. After the blending and mixing of ingredients, the mixture is extruded with water addition to reach a final moisture content of 60%. The temperature is raised to 150 °C that helps in the formation of new chemical bonds between the protein molecules creating a fibrous meat-like material. The material is then sent to a die for cooling (Chiang et al., 2019).

Quorn, is one of the commercial meat analogue product, produced by the filamentous fungus *Fusarium venenatum* (Dekkers et al., 2018a). A 20% (weight basis) solid product is obtained from the continuous fermentation process where the growth of *F. venenatum* strictly controlled. The fungal biomass obtained is treated to remove RNA and undergoes further processing steps – forming, steaming, chilling, and texturizing – to obtain fibrous products as chunks, sausages, and burgers (Wiebe, 2002).

5.4 Conclusions

The fermented food and beverages offer improved health, shelf life, and mouth feel, and the process of their production and preservation has been known since historic. In ancient times, salting, pickling, and sun drying were the common methods for

preserving food. The process of producing fermented food and beverage has advanced from artisanal roots to a more science-driven process. The use of known microbial cultures, canning, pasteurization, chemical preservatives, refrigeration, and other means have reduced the need to ferment foods for preservation and storage. The yeasts, lactic acid bacteria, and molds are the most common microbial cultures used to produce fermented food and beverages. The increased knowledge about microbial physiology has helped to choose among different strains for their use as starter or adjunct cultures. With increased knowledge of microbial physiology, the fermentation process has also advanced from back-slopping to use of starter cultures. Further developments in the fermentation process has led to use of mixed cultures, nanotechnology, producing meat alternatives, fermentation-based cell agriculture, tissue engineering, and genetic engineering. The use of nanotechnology has improved the targeted approach for delivery of desired components in the gut and increasing the shelf life of food materials. It has also made it possible to develop sensors that can check the food quality and contamination in no time. The latest development had been producing alternatives for the animal with an aim to develop environmentally friendly and sustainable processes.

5.5 Questions for Thought

1. What are the advantages of using fermented food and beverages?
2. What are the different types of fermentation used in the production of food and beverages?
3. What are the main organisms used to produce fermented food and beverages?
4. What are the different types of cultures used in the fermentation of food and beverages?
5. What are the desired characteristics of a starter culture?
6. What are the essential steps in cheese production?
7. Why LAB are used so often to produce fermented food and beverages?
8. Why yeast is used to produce alcoholic beverages?
9. How nanotechnology has shaped the food and beverage fermentation?
10. What are meat analogues?

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6

Bioactive Oligosaccharides

Production, Characterization, and Applications

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6.1 Introduction

Carbohydrates refer to biomolecules that consist of carbon, hydrogen, and oxygen (Rao, 2019). Based on their molecular weight, carbohydrates are classified as monosaccharides, oligosaccharides, and polysaccharides. Oligosaccharides consist of 2–10 sugar units linked together by glycosidic bonds (Sako *et al*., 1999). In general, oligosaccharides can be composed of glucose, fructose, galactose, or xylose as monomeric units. There are various sources of oligosaccharides that include plants, animals, microorganisms, marine, and also some insects. In recent years, substantial attention has been dedicated on oligosaccharides. During the last decade, applications of oligosaccharides have been revealed due to their functional properties and bioactivity. In plants, these oligomers play a role in defense against abiotic stress (El Sayed *et al*., 2014). Oligosaccharides derived from various sources possess various medical applications because of their antibacterial, antifungal activities (Weinberger *et al.,* 2010). The oligosaccharides have been used as functional constituents of the food industry exclusively in beverages and dairy products (Moure *et al.,* 2006; Mussatto & Mancilha, 2007). Together with naturally existing oligosaccharides, hydrolytic or synthetic methods are also available for the production of these carbohydrates of low molecular weight (Du *et al*., 2011). Generally, oligosaccharides are separated using membrane separation processes such as ultrafiltration (Kim *et al*., 2003) and further purified by chromatographic methods. Characterization of oligosaccharides is being done by mass spectrometry (MS) due to its precision, analytical versatility, and very high sensitivity. Matrix assisted laser desorption ionization-time of flight mass spectrometry

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(MALDI-TOF-MS) and nuclear magnetic resonance (NMR) are added familiar methods for the characterization of oligosaccharide structure (Okada *et al*., 2010). This chapter deals with the sources, types, methods for production, extraction and separation, purification, and characterization of oligosaccharides. A detailed review of applications of these bioactive oligosaccharides is also presented.

6.2 Sources, Types, Structure of Oligosaccharides

There are varied types of oligosaccharides available from diverse natural sources such as plants, animals, microorganisms, algae-derived marine oligosaccharides along with the unfamiliar oligosaccharides such as pseudo-oligosaccharides and differ in nature basically by the type of monomeric sugar entities. The sources of oligosaccharides are classified, and representative examples for each class are provided in Figure 6.1.

6.2.1 Plant Source

Vegetables and cereals are major sources of Fructan-type oligosaccharides (FTO), while legumes are displayed as plant source of α-galacto-oligosaccharides (α-GOS). FTO consist of fructose units polymerized to different magnitude. 1-Kestose, 1-nystose, 1-fructofuranosyl-nystose are oligomers with two, three, and four fructose units, respectively (Belorkar & Gupta, 2016). Honey, soybean, lentils, mustard, fruits, and vegetables such as artichoke, onion, rye, barley, asparagus, sugarcane, banana, yacon, garlic, wheat, tomato, chicory, and bamboo shoots (Mussatto & Mancilha, 2007) are the major plant sources of oligosaccharides. Xylo-oligosaccharides were extracted from barley hulls, corn cob, gram husk,

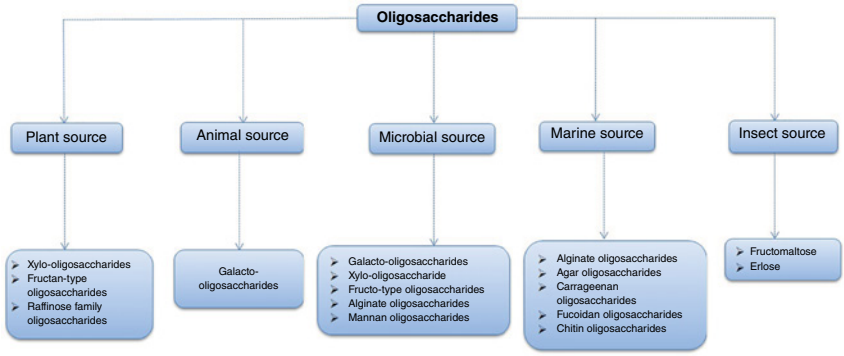

Figure 6.1 Sources of bioactive oligosaccharides with examples.

almond shells, straw, wheat bran, brewery spent grains, and bamboo (Katapodis & Christakopoulos, 2008). Oligosaccharides were also manufactured from cell-wall polysaccharides of plants such as arabinan, polygalacturonan, arabinoxylan, and rhamnogalacturonan (Van Laere *et al.,* 2000). Lupin species were extracted for raffinose family oligosaccharides (Wichienchot *et al*., 2010). Traditional Chinese medicinal plants were reported to contain an ample number of oligosaccharides and their esters (Chen *et al.,* 2015).

6.2.2 Animal Source

Galacto-oligosaccharides are naturally present in mammalian milk. In oligosaccharides of animal milk, galactose or *N*-acetylglucosamine linkages are dominant, while galactose and *N*-acetylgalactosamine can be also found. In this type of oligosaccharides, the most important structural element is the presence of sialic acid (Boehm & Stahl, 2007).

6.2.3 Insect Source

Honeydew, a syrup excreted by scale insects feeding on plant phloem sugars, contains several oligosaccharides. Fructomaltose is widely associated with scale insects, aphids, bee honey (Gray & Fraenkel, 1953), and occur in *Coccus hesperidum*, along with melezitose and a gluco-sucrose in the honeydew (Wolf & Ewart, 1955). Gluco-sucrose, a family of oligosaccharide, was named as "erlose" (Stephen, 1959). Erlose and other oligosaccharides are identified in *Diadegma* sp. (Hogervorst *et al.,* 2007). Honey oligosaccharides are formed by action of enzymes in the intestines of bees from nectar-containing sucrose, glucose, and fructose or honeydew (Kandler & Hopf, 1980).

6.2.4 Marine Source

Marine oligosaccharides present a unique type of oligosaccharides due to their complex chemical and physical properties (Vera *et al.,* 2011). Analysis of the composition of marine oligosaccharides is still challenging owing to the complex structures and heterogeneity (Lang *et al.,* 2014). Marine algae and seaweeds are the major sources of the marine oligosaccharides. These oligosaccharides are derived from different algal polysaccharides through the respective polysaccharide-depolymerizing enzymes or by chemical hydrolysis. Algal polysaccharides such as alginate, agar, carrageenan, fucoidan, and ulvan function as promising candidates for generating structurally diverse oligosaccharides (Jutur *et al.,* 2016). The other major class of marine oligosaccharides is chitin oligosaccharides. Chitin extracted

from crab and shrimp shells can be hydrolyzed into chitin oligosaccharides. This oligosaccharide is known as oligo-*N*-acetylglucosamine due to the presence of several *N*-acetylglucosamine with different degrees of polymerization (Yin *et al.,* 2016).

6.2.5 Microbial Source

Xylo-oligosaccharides or feruloyl oligosaccharides are well known to be produced by *Aspergillus*, *Trichoderma*, *Penicillium*, *Bacillus,* and *Streptomyces*. Mannan oligosaccharides are primarily extracted from cell-wall fragments of yeast (Dimitroglou *et al.,* 2010). GOS can be produced by several lactic acid bacteria through lactose hydrolysis (Toba *et al.,* 1981). *Bacillus subtilis* natto was used to produce fructo-oligosaccharides along with levan (Bersaneti *et al*., 2018). Microorganisms such as *Penicillium frequentens*, *Aspergillus phoenicis*, *Aspergillus niger*, *Fusarium oxysporum*, *Scopulariopsis brevicaulis*, *Aspergillus japonicus*, *Penicillium rugulosum*, *Aureobasidium pullulans,* and *Arthrobacter sp*. were employed for the production of fructo-oligosaccharides using sucrose (Prapulla *et al.,* 2000). *Pseudomonas mendocina* NK-01 is used to produce alginate oligosaccharides (AlgOs) and medium-chain-length polyhydroxyalkanoate using glucose as a carbon source (Guo *et al.,* 2011). *Bacillus circulans* T-3040 was engaged for the production of novel cyclic isomalto-oligosaccharide (Funane *et al.,* 2007). Glucooligosaccharides (GlOS) were produced using *Leuconostoc mesenteroides* NRRL B-1299 (Iliev *et al.,* 2008).

6.2.6 Synthetic Oligosaccharides

Biocatalysis is one of the methods for producing oligosaccharides, and their analogues are used in research particularly for drug and vaccine discovery. The glycosyltransferases and glycosidases are used to synthesize oligosaccharides in laboratories (Palcic, 1999). Enzymatic synthesis of cyclodextrins using cyclodextrin glycosyltransferase with starch as a substrate is an example of cyclic oligosaccharides (Ibrahim, 2018). These oligosaccharides produced by synthetic way present an extremely safe option for the production of Good Manufacturing Process-grade carbohydrate drugs (Driguez *et al.,* 2014), which are pure and structurally well defined.

6.2.7 Pseudo-oligosaccharides

Actinomycetes produce secondary metabolites whose chemical structures closely resemble sugars called pseudo sugars (glycomimetics). These metabolites generally contain one or more six-membered ring structures analogous to pyrano-sugars,

or five-membered ring structures analogous to furano-sugars. Aminoglycoside antibiotics (e.g., kanamycin, neomycin, and gentamicin), the pseudo-oligosaccharides (e.g., acarbose, validamycins, amylostatins, and trestatins), and the aminocyclopentitol pactamycin fall under this category. These oligosaccharides possess significant biological activities, mostly used as drugs to treat plant diseases and human diseases (Alanzi *et al.,* 2018). Diels–Alder transformation is the most known method to produce this type of oligosaccharides (Schürer & Blechert, 1999). Structures of some common oligosaccharides and pseudo-oligosaccharides are illustrated from Figures 6.2a–i (Ohta *et al.,* 1999; Eggleston & Cote, 2003; Yokose *et al.,* 2009; Wang *et al.,* 2015; Belorkar & Gupta, 2016; Muanprasat & Chatsudthipong, 2017; Ibrahim, 2018; Singh *et al.,* 2018).

6.3 Production Methods of Oligosaccharides

Oligosaccharides can be produced or synthesized by physical, chemical, or enzymatic methods (Courtois, 2009). Generally, hydrolysis of polysaccharides by either enzymatic or chemical treatment or synthesis through disaccharide substrates were employed (Mussatto and Mancilha, 2007). Naturally occurring oligomers can be directly extracted from the source using different extraction methods.

6.3.1 Chemical Methods

The acid hydrolysis method is a reasonably inexpensive, simple, and controllable process (Warrand & Janssen, 2007). Sulfuric, hydrochloric, and trifluoroacetic acids are the most commonly used acids for polysaccharide depolymerization. In general, the process is operated at higher temperatures, above 60 °C for two to six hours on average (Du *et al.,* 2011). But, acid hydrolysis poses some drawbacks such as the destruction of monosaccharides with sequential accumulation of toxic compounds such as furfural and 5-hydroxy methylfurfural and also produces a low yield of oligosaccharides (Avila-Fernandez *et al.,* 2011). Microwave-induced acid hydrolysis of glucans releases GlOS (Prapulla *et al.,* 2000). Raffinose oligosaccharides (RFOs) are mined directly from plant sources through water, aqueous methanol, or ethanol extractions (Mussatto & Mancilha, 2007).

6.3.2 Physical Methods

Xylo-oligosaccharides are recovered from brewery's spent grain by autohydrolysis with water at 190 °C (Prapulla *et al.,* 2000). Hydrothermal processing of wheat bran was employed for production of feruloylated-arabinoxylo-oligosaccharides

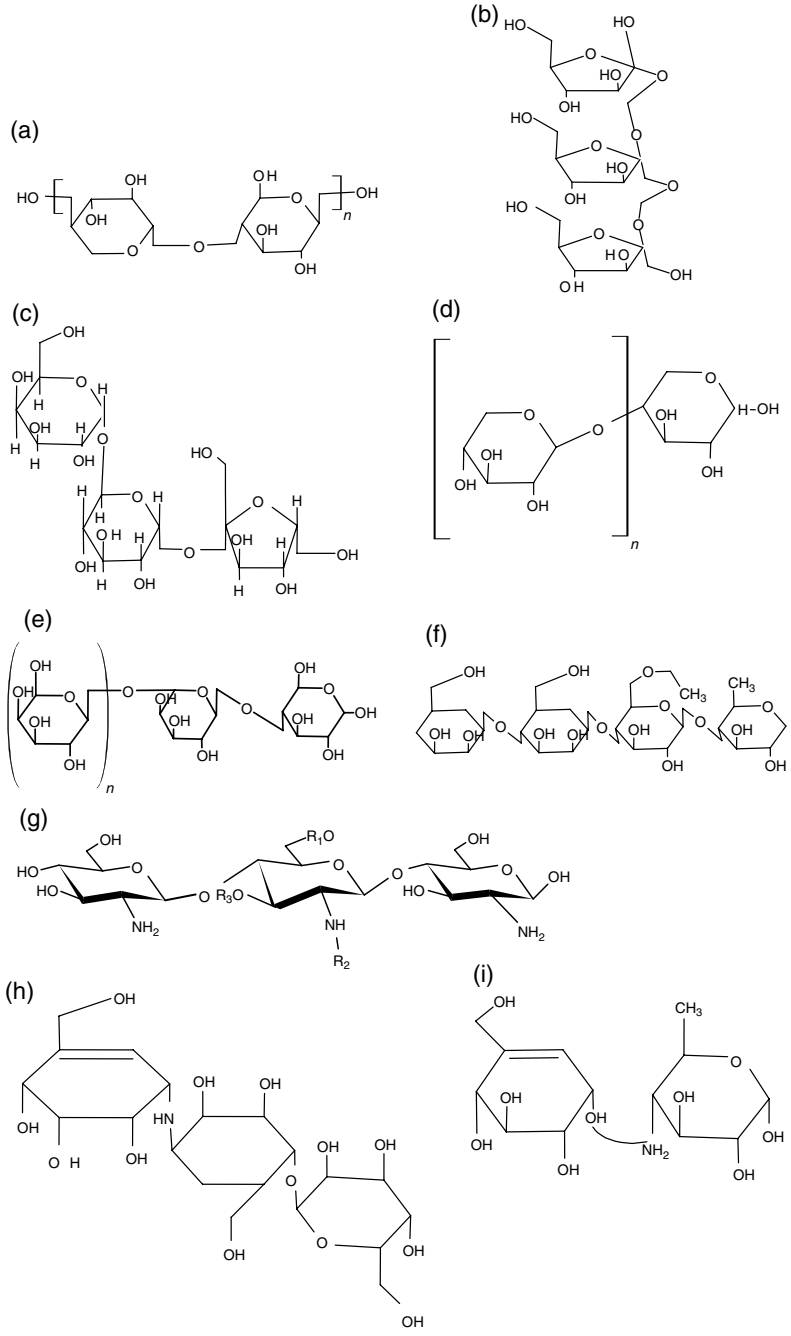

Figure 6.2 (a–i) Structures of different oligosaccharides (a) *Malto-oligosaccharides (MaOS);* (b) *Fructo-oligosaccharides (FOS);* (c) *Raffinose oligosaccharides (RFOs);* (d) *Xylo-oligosaccharides;* (e) *Galacto-oligosaccharides (GOS);* (f) *Manno-oligosaccharides (MOS);* (g) *Chito-oligosaccharides (CTOS);* (h) *Validamycin;* (i) *Trestatins.*

(Rose & Inglett, 2010). Production of various oligomers such as alginate and chitin oligomers were also attempted using Gamma-ray's irradiation with cobalt-60 (Choi *et al.,* 2002; Naeem *et al*., 2015).

6.3.3 Enzymatic Hydrolysis

Conventionally, chemical methods have been employed for the production of oligosaccharides. Disadvantages of chemical methods such as labor-intensive, continuous monitoring and use of hazardous chemicals, expensive and low yields lead to the use of enzymatic methods for the synthesis of oligosaccharides (Prapulla *et al.,* 2000). Enzymatic depolymerization of polysaccharides is the method of preference for large-scale oligosaccharide production (Barreteau *et al.,* 2006). Oligosaccharides with controlled molecular weights can be synthesized by enzymatic hydrolysis. MaOS and isomalto-oligosaccharides were produced by the action of α-amylases, α- and β-amylases, and α-glycosidases on starch (Mussatto & Mancilha, 2007). Fructo-oligosaccharides (FOS) are derived by the hydrolysis of inulin and fructans using inulinase (Cazetta *et al.,* 2005; Avila-Fernandez *et al.,* 2011). Production of FOS can be carried by transfructosylation using fructosyltransferases. Transgalactosylation using β-galactosidases is used for commercial production of GOS from lactose. Transgalactosylation using α-galactosidase or enzymatic conversion of RFOs by levansucrase results in the production of α-GOS (Martins *et al.,* 2019). Carrageenases are enzymes used to degrade carrageenan. These enzymes are classified into λ-carrageenase, i-carrageenase, and k-carrageenase based on their substrate specificities (Ghanbarzadeh *et al.,* 2018). β-agarase is used to produce agar oligosaccharides (Xu *et al.,* 2018). AlgOs are released during the depolymerization of alginate using alginate lyases through β-elimination reaction (Falkeborg *et al.,* 2014). Chitin and chitosan can be hydrolyzed into its respective oligosaccharides using chitosanases, chitinases, and other nonspecific enzymes (Mourya *et al.,* 2011). Screening of novel enzymes from extremophilic microorganisms that can depolymerize the polysaccharides with desired catalytic activity under unusual physiological conditions such as high temperature, high salinity, and low water activity (Meyer *et al.,* 2015) would be important for the production of oligosaccharides using enzymatic methods as it is a specific and safe option.

6.3.4 Microbial Production of Oligosaccharides

Apart from physical, chemical, and enzymatic methods, oligosaccharides can be directly produced using fermentative production by microorganisms. FOS was reported to be produced using *A. phoenicis, A. japonicas, A. niger, F. oxysporum, S. brevicaulis, Penicillium frequentans, P. rugulosum, A. pullulans, Arthrobacter sp*.

An extensive array of microbes such as *A. niger, A. oryzae, Trichoderma harzianum, Lactobacillus and Streptococcus thermophiles, Caldocellum saccharolyticum, Bifidobacterium bifidum, Saccharopolyspora rectivirgula, Saccharomyces lactis, Kluyveromyces lactis* produces β-galactosidases, which can be used for the microbial production of oligosaccharides by transgalactosylation through hydrolysis of lactose. *Aureobasidium pullulans* was used for the production of isomaltooligosaccharides (Prapulla *et al.,* 2000).

6.4 Extraction, Separation, and Purification of Oligosaccharides

Ultrafiltration using a low-pressure range is a normally used method for the separation of oligosaccharides from polysaccharides (Kim *et al.,* 2003). Cellulase-assisted extraction increased the yield of oligosaccharides from rice bran. This type of extraction enhanced the level of oligosaccharides in a shorter time (Patindol *et al.,* 2007). Mostly oligosaccharides are soluble in water, so extraction of sugars using water is more sufficient to produce oligosaccharides in large quantities without loss of any proteins and biochemical action (Ku *et al.,* 1976). High-purity oligosaccharides are produced using enzyme-aided alkaline extraction. Xylan pulp was initially treated with NaOH, i.e. alkaline extraction in the first step followed by treatment with xylanase for the production of xylo-oligosaccharides. Up to 95% of pure xylo-oligosaccharides was obtained using this process (Hakala *et al.,* 2013). The ultrasonic or microwave-assisted extraction (UMAE) has attracted more researchers for the extraction of oligosaccharides because of the high yield of desired products. In the case of prebiotic oligosaccharides from lotus seeds, a high yield of oligosaccharides was obtained using the UMAE method (Lu *et al.,* 2017). Adsorption on activated charcoal followed by elution with 30% ethanol was employed for purification of CTOS (Prapulla *et al.,* 2000). Table 6.1 summarizes the different extraction methods used for oligosaccharide production.

The purification of oligosaccharides is an essential process in biological and structural studies. Several membrane separation and chromatography techniques are used to purify oligosaccharides. The techniques involve ultrafiltration, nanofiltration, hydrophobic interaction liquid chromatography (HILIC), high-speed counter-current chromatography (HSCCC) coupled with pre-column derivatization, capillary zone electrophoresis, and affinity chromatography. HILIC is widely used to purify plant-source oligosaccharides (Fu *et al.,* 2010). HSCCC coupled with pre-column derivatization was proven for purification of fructo-oligosaccharides (FOS). Using petroleum ether–*n*-butanol–methanol–water as a solvent system, FOSs were separated by HSCCC (Duan *et al.,* 2018). Nanofiltration is one of the purification methods used for purifying oligomers from mixtures comprising monosaccharides (Goulas *et al.,* 2002). For purification, ultrafiltration

Table 6.1 Extraction methods for oligosaccharides.

Extraction method	Oligosaccharide	References
Ultrafiltration	Oligosaccharides from defatted soybean meal	Kim *et al.* (2003)
Aqueous extraction	Raffinose and stachyose present in soybeans	Ku *et al.* (1976)
Cellulase-assisted extraction	Raffinose, cellobiose, cellotriose, and longer-DP Cello-oligosaccharides	Patindol *et al.* (2007)
Enzyme-aided alkaline extraction	Xylo-oligosaccharides	Hakala *et al.* (2013)
Ultrasonic-microwave assisted extraction	Prebiotic oligosaccharides from lotus seeds	Lu *et al.* (2017)
Subcritical H_2O–CO_2 pretreatment	Xylo-oligosaccharides	Ahmad *et al.* (2018)

Table 6.2 Methods for the purification of oligosaccharides.

Type of separation	Sources	Oligosaccharides	References
Nanofiltration (NF)	Sugar solution with galactose	Galacto-oligosaccharides	Goulas *et al.* (2002)
Ultrafiltration	Almond shells	Xylo-oligosaccharides	Nabarlatz *et al.* (2007)
Hydrophobic interaction liquid chromatography	Dried roots of *Rehmannia glutinosa*	Oligosaccharides of Raffinose family	Fu *et al.* (2010)
High-speed counter-current chromatography coupled with pre-column derivatization	Ethanol, ethyl acetate, methanol, isopropanol, and petroleum ether	Fructo-oligosaccharides	Duan *et al.* (2018)
Capillary zone electrophoresis	Mature seeds of peas	Oligosaccharides of the raffinose family	Arentoft *et al.* (1993)

with thin-film polymeric membranes is an appropriate process due to good fouling resistance (Nabarlatz *et al.,* 2007). High-performance capillary electrophoresis (HPCE) is a simple method used for the isolation of oligosaccharides from a group of mixtures in the purest form. The advantages of this method include low-cost per analysis, use of nontoxic chemicals, and ease of operation (Arentoft *et al.,* 1993). Table 6.2 presents the various methods used for the purification of oligosaccharides.

6.5 Characterization of Oligosaccharides

The mass spectrum of simple oligosaccharides and *N*-glycans derived from glycoproteins exhibits a high abundance of crossing fragmentation ions, which provides very precise information on the linkages of monosaccharide residues (Mechref *et al.,* 2003). Another simple technique is HPCE that is used to determine the linkages in the hyaluronan oligosaccharides (Maccari *et al.,* 2004). Fast-atom bombardment-mass spectrometry (FAB-MS) helps to confirm the identity of UV-absorbing oligosaccharide products (Wang *et al.,* 1984). Electrospray ionization quadrupole ion trap MS provides the information on sequence, composition, branching, and interglycosidic linkages (Morelle *et al.,* 2005). Fourier transform infrared spectrometry provides a spectrum of oligosaccharides present in water in the form of glycosidic bonds with respect to changes in conformation of their constituent monosaccharides (Kačuráková & Mathlouthi, 1996). The structures of oligosaccharide were identified using NMR for the quantitative and qualitative analysis of FOS.

6.6 Functional Properties of Oligosaccharides

Chemically, oligosaccharides comprise of monosaccharide units, linked by oxygen bridges between the hemiacetal hydroxyl of the anomeric carbon of one unit and an alcoholic hydroxyl of another unit (Prapulla *et al.,* 2000). Oligosaccharides can be an excellent replacement for sucrose as they are about 0.3–0.6 times as sweeter than sucrose with the lower caloric intensity, mouthfeel, and texture characteristics warranting their application in food preparations. Oligosaccharides are used as humectants because of the high moisture-retaining property. Oligosaccharides are classified as digestible or non-digestible based on their physiological properties. The stability of oligosaccharides is subject to the sugar residues, ring form, anomeric configuration, and linkages. Further, oligosaccharides are highly diverse in nature compared to oligopeptides and oligonucleotides due to the varied nature of available sources (Patel and Goyal, 2011). Further, these molecules are highly soluble in water, heat stable, hydrolyze only at high acid conditions, and non-cariogenic. The health benefits of the bioactive oligosaccharides are boosting of beneficial bacteria such as *Bifidobacterium* sp. and *Lactobacillus* sp. in the colon by acting as "prebiotics," promotion of lactose metabolism, enhancement of minerals absorption, maintenance of good ratio of HDL/LDL, decrease of serum lipids and blood cholesterol, improvement of blood pressure, removal of toxins from the body, and lowering of glycemic index (Ibrahim, 2018).

6.7 Applications of Oligosaccharides

Oligosaccharides derived from different sources are used as dietary fiber, sweetener, weight controlling mediator, and humectant in food products. Bioactive oligosaccharides have been functional with several health-promoting activities such as enhancement of gastrointestinal normal flora, suppression of pathogens, prevention of dental caries, augmentation of immunity, facilitation of mineral absorption, antioxidants, antibiotics, regulation of blood glucose level in diabetics, and serum lipids in hyperlipidemics. Oligomers have been reported to be employed as a biomaterial for drug delivery. Apart from the pharmacological and medical applications, oligosaccharides have been established as cosmetics additives, animal and fishery feed, agriculture, etc. Figure 6.3 depicts the application areas of different bioactive oligosaccharides.

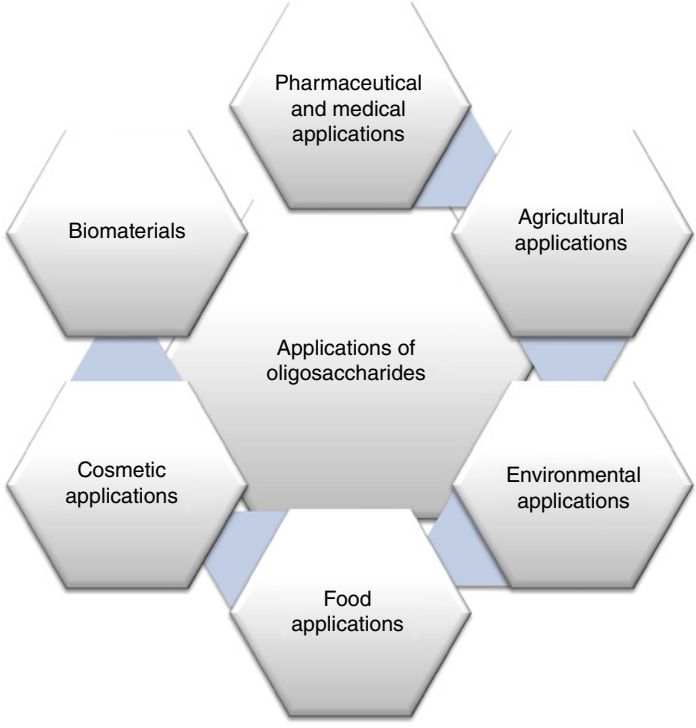

Figure 6.3 Application areas of bioactive oligosaccharides.

6.7.1 Functional Foods, Nutraceuticals, and Prebiotics

Pitaya oligosaccharides possess prebiotic properties with a resistance to acid in the human stomach, salivary α-amylase, and the competence to stimulate the growth of *Lactobacilli* and *Bifidobacteria*. Pitaya oligosaccharides are the major source of prebiotics and are also used as a major constituent in functional food and nutraceuticals (Wichienchot *et al.,* 2010). Most of the fruits such as pear, watermelon, and nectarine and vegetables such as garlic and white onion contain oligosaccharides that can be used as prebiotics. Nectarine with the highest content fructo-oligosaccharides can be used as functional foods and nutraceuticals (Jovanovic-Malinovska *et al.,* 2014). Pectic oligosaccharide-rich extract from bergamot peel by enzymatic method owns a good prebiotic effect (Mandalari *et al.,* 2007). FOS and GOS were added to the formula of milk of infants to bring a similar composition of breast milk (Cencic & Chingwaru, 2010). κ-Carrageenan oligosaccharides, agarose oligosaccharides (AO) and AlgO have the capability of increasing the short-chain fatty acids content by altering the composition of the microbiota (Han *et al.,* 2019). AlgOs do not cause side effects, and they act as prebiotics to promote human health (Wang *et al.,* 2006). GOS, the human milk oligosaccharides, play a predominant role as prebiotics for lactose intolerance (Chen & Gänzle, 2017). The synergistic effect of isomalto-oligosaccharides and cranberry extract have a capability of increasing diet-induced obesity by changing the modulation of gastrointestinal health (Singh *et al.,* 2018). The palatinose oligosaccharide was coated on to the chewing gum. These oligosaccharides provide a hard-shell coating and also provide a sweetness to the gum pellet (Yatka & Richey, 1994). GlOS mixtures can be used as "bodybuilding" and calorie-free sweetening agents (Schwengers, 1987). Fructo-oligosaccharides acts as new replacement of sweeteners in food products (Bali *et al.,* 2015).

6.7.2 Pharmaceutical and Medical Applications

6.7.2.1 Effects on Intestinal Microflora

Non-digestible oligosaccharides cannot be hydrolyzed by digestive enzymes of human but can be utilized by intestinal bacteria such as *Bifidobacteria* to bring about beneficial activity in the human intestinal system (Mitsuoka *et al.,* 1987). The FOS, GlOS, and GOS have various beneficial effects on human health. Among these, GlOS were found to improve the glycolytic activity and improve the dietary digestibility in human (Djouzi & Andlueux, 1997). Lactitol-oligosaccharides improve organic acids, bifidobacteria, and decrease putrefactive products in the intestine (Yanahira *et al.,* 1995). MOS improve the maturation of microflora in the gut by promoting the growth of lactobacilli in the ileal mucosa

(Chee *et al.,* 2010). Cello-oligosaccharide (COS) influences the intestinal flora of weaned pigs by increasing *Lactobacillus* and decreasing *Clostridium* in the intestine (Jiao *et al.,* 2014). Trans-oligosaccharides can be fermented fully in the human colon. They do not change the beneficial microflora in the intestine but plays a role in maintaining the intestinal microflora (Alles *et al.,* 1999).

6.7.2.2 Effects on Urogenital Infections

Human milk oligosaccharides show an effective role in building defense against bacterial and viral infections in the gastrointestinal and urogenital tract of infants. These oligosaccharides play a major role in preventing gastrointestinal and urogenital tract infections in breastfed infants (Obermeier *et al.,* 1999). FOS and GlOS prevent vaginal infection. This is because of the consumption of GOS and FOS by *Lactobacillus* present in the vagina (Rousseau *et al.,* 2005).

6.7.2.3 Type II Diabetes and Obesity

Neoagaro-oligosaccharides (NAOs) having the capability of relieving Type II diabetes by improving glucose tolerances, decreasing pancreatic β-cell loss, ameliorating liver damage, uplifting the antioxidant abilities, and decreasing pancreatic β-cell loss. The NAOs activate the mitogen-activated protein kinase pathway in T2DM. NAOs can be used in the treatment of Type II diabetes (Lin *et al.,* 2019). Wheat-derived arabinoxylan oligosaccharides decrease the effect of high fat-induced metabolic endotoxemia, macrophage infiltration in the adipose tissue, and interleukin 6 in the plasma. So it acts as a promising agent for controlling obesity and decrease metabolic disorders (Neyrinck *et al.,* 2012). Xylo-oligosaccharide, isomalto-oligosaccharide, fructo-oligosaccharide, GOS derived from plant sources can play a major role in glycemic and insulin metabolism (Sajadimajd *et al.,* 2019). Unsaturated alginate oligosaccharides (UAOS) act as an anti-obesity agent by increasing AMP-activated protein kinase α and acetyl-CoA carboxylase (ACC) phosphorylation in adipocytes (Li *et al.,* 2019). Oligosaccharides from brown seaweed *Sargassum confusum* modulates the production of insulin by regulating insulin receptor substrate 1/phosphatidylinositol 3-kinase and c-Jun N-terminal kinase pathways and thereby act as a functional constituent in the regulation of gut microbiota in obese and diabetic patients (Yang *et al.,* 2019). GOS can help in the medication of metabolic endotoxaemia or low-grade inflammation in obese people (Fernandes *et al.,* 2017). Chitosan oligosaccharide capsules can reduce the lipid accumulation in the body by activating JAK2-STAT3 signaling pathway to prevent leptin resistance and suppress adipogenesis (Pan *et al.,* 2018). Antihyperglycemic activity of konjac mannan oligosaccharides attenuated HFD-induced glucose metabolism dysfunction by regulating resistance to insulin and leptin (Zhu *et al.,* 2019).

6.7.2.4 Immunomodulatory and Antitumor Activities

FOS and GOS exhibit anti-cancer activity by influencing lipid metabolism, mineral absorption, anti-inflammatory, and atopic disease (Macfarlane *et al.,* 2008). Ginseng marc-derived glucan-type oligosaccharide mainly with glucose residues activates macrophages and decreases the apoptotic cell death (Seo *et al.,* 2015). Alginate oligomers possess cytokine-inducing activities in a structure-dependent manner, and the structure-activity relationship of alginate oligomers may deliver understanding into the toll like receptors (TLR)-mediated recognition and signal transduction leading to the cytokine production (Iwamoto *et al.,* 2005).

6.7.2.5 Effect on Cardiovascular Risk

Soybean oligosaccharides (SO) prevent against myocardium ischemia reperfusion injury by altered activation of the JAK2/STAT3 pathway. SO promotes heart function in humans (Zhang *et al.,* 2015).

6.7.2.6 Lowering of Cholesterol

Pectin-derived oligosaccharides (POS) play a vital role in cholesterol-lowering effects through the regulation of intestinal flora and their metabolites. POS on cholesterol metabolism was modulated by bacterial species including *Bifidobacterium, Lactobacillus* and *Bacteroides*. They reduced serum LDL-C, lowered cholesterol, and also inhibited hepatic cholesterol synthesis (Hu *et al.,* 2019). AlgOs show an important role in reducing LDL-plasma cholesterol levels and hence can be employed as an efficient drug for lowering cholesterol (Yang *et al.,* 2015). Chitosan oligosaccharides (CSOS) were found to improve the plasma lipids and avoid atherosclerotic risks by increasing the macrophage-derived 3 H-cholesterol in the liver and bile with the reverse cholesterol transport mechanism (Zong *et al.,* 2012). Fructo-oligosaccharides consumption has a profound outcome on lipid metabolism, and serum cholesterol levels and its supplementation in the diet were found to be a viable approach for decreasing cholesterol (Costa *et al.,* 2015).

6.7.2.7 Role in Osteoporosis

Achyranthes bidentata Blume is used mostly as a medicinal herb prescribed for osteoporosis treatment in China. The crude saccharide of this herb was found to possess an excellent osteoprotective effect (Wang *et al.,* 2017). Isoflavones present in the non-digestible oligosaccharides obtained from chicory roots, and soya extract can prevent osteoporosis (Borai *et al.,* 2009). Fructo-oligosaccharides, raffinose, galacto-oligosaccharides, and xylo-oligosaccharides can help in the prevention of osteoporosis (Ohta *et al.,* 1999).

6.7.2.8 Antihypertensive Effects

Sodium alginate oligosaccharide can be used to prevent kidney injury in the early stage of the disorder (Ueno *et al.,* 2012). AlgOs of very-low molecular weight exhibit striking antihypertensive effects in salt-induced hypertension in Dahl S rats via the subcutaneous route and may afford a new approach for antihypertensive treatment (Moriya *et al.,* 2013).

6.7.2.9 Hepatic Protection

Sialyl Lewis (x) oligosaccharide plays a defensive role in hepatic ischemia and reperfusion injury by decreasing the adhesion of polymorphonuclear cells (Misawa *et al.,* 1996). Fermentable dietary FOS can control hepatic steatosis by inducing omega-3 fatty acids (ω-3 PUFA) exhaustion. So, hepatic triglyceride can be reduced by activating the α-stimulation receptor of fatty acid oxidation (Pachikian *et al.,* 2013). Agriophyllum oligosaccharides can protect liver function by activating insulin in the INS-R/IRS2/PI3K/AKT/Glut4/PPAR-γ signal pathway and facilitating the hepatocyte proliferation (Bao *et al.,* 2020). RFOs produced by *Rehmannia glutinosa* exhibited hepatoprotective effects (Zhang *et al.,* 2013).

6.7.2.10 Antioxidant and Neuroprotective Agent

Alginate-derived oligosaccharide (ADO) has a higher radical scavenging effect than oligosaccharides derived from chitosan and fucoidan (Wang *et al.,* 2007). AlgOs have potential applications in the food and pharmaceutical sectors as antioxidants and the activity is related to its structure and composition (Liu *et al.,* 2019). Alginate oligosaccharide was reported to possess a protective effect against endoplasmic reticulum, and mitochondrial-dependent apoptosis enhances Bcl-2 expression and reduces Bax expression and caspase-3 activation (Khodagholi *et al.,* 2011). AlgOs has neuroprotective effect against A*β*-induced neural damage in adult male albino Wistar rats in vivo (Liu *et al.,* 2019). CSOS showed antioxidant activity in mice through in vivo induction of sepsis by lipopolysaccharide. It has been observed that the CSOS exhibited less redox imbalance through alteration of the calcium-sensing receptor and NF-κB signaling pathway in the gastrointestinal tract (Huang *et al.,* 2016). The neuroprotective action of CSOS was confirmed in rats with sciatic crush injury resulting in peripheral nerve regeneration (Jiang *et al.,* 2009). Fucosyllactoses promote immune and cognition aspects through an increase in the gut microbiome. Deacetylated oligosaccharides derived from chitosan have neuroprotective effect on human SH-S5Y5 neurons (Santos-Moriano *et al.,* 2018). CTOS have neuroprotective activity, such as inhibition of β-amyloid and acetylcholinesterase activities, anti-neuroinflammation, and anti-apoptosis and possess a better cognitive response in Aβ1-42-induced model of Alzheimer's disease by obstructing oxidative stress and neuroinflammatory effects

(Zhou *et al.,* 2008; Nidheesh *et al.,* 2016). CTOS showed antioxidant activity against Cu(II)-induced neurotoxicity in primary cortical neurons by enhancing intracellular reactive oxygen species (Xu *et al.,* 2010). Attachment and entry of Caco-2 cells by *Campylobacter jejuni* have been inhibited by administrating pectin-derived acidic oligosaccharides (Ganan *et al.,* 2010). Administration of glucuronomannan oligosaccharides promotes downregulation of the apoptotic signaling pathway by increasing the tyrosine hydroxylase expression of dopaminergic neurons in Parkinson's disease mice (Liu *et al.,* 2020).

6.7.2.11 Antimicrobial Activity

Pectic oligosaccharides from haw fruit exhibited pH and dose-dependent antibacterial activity against *Escherichia coli* (Zang *et al.,* 2013). CTOS produced by acid hydrolysis from giant freshwater prawn shells inhibited the growth of *Vibrio parahaemolyticus* (Chimtong *et al.,* 2016). Several reports highlighted the application of CTOS as antibacterial agents (Seong *et al.,* 1999; Jeon & Kim, 2001; Han *et al.,* 2007; Dutta *et al.,* 2012). Cows' milk oligosaccharides decrease the adhesion of enterotoxic *E. coli* strains of the calf (Martín *et al.,* 2002). Oligosaccharides isolated from Galdi sheep milk was reported to exhibit antimicrobial activity against *Staphylococcus aureus, E. coli, Salmonella typhimurium*, *Candida albicans,* and *A. niger* (Shahi & Deepak, 2018). Dandelion-derived oligosaccharides possessed high antibacterial activity against *E. coli, B. subtilis,* and *S. aureus* (Qian *et al.,* 2014). κ-Carrageenan oligosaccharides synthesized through enzymatic hydrolysis demonstrated inhibitory activity against *E. coli*, *S. aureus*, *S. cerevisiae*, *Penicillium citrinum,* and *Mucor* sp. (Wang *et al.,* 2011).

6.7.2.12 Antibiotics

Oligosaccharides display an extensive spectrum of biological activity such as disruption of cell-wall biosynthesis and inhibition of bacterial translation. Four different classes of bioactive oligosaccharides such as orthosomycins, moenomycins, saccharomicins, and acarviostatins were reported. On the basis of number of orthoester linkages and sugar residues, orthosomycins can be classified as Class I and Class II orthosomycins. Class I orthosomycins are characterized as hepta- and octa-saccharides with two orthoester linkages and a dichloroisoeverninic acid ester. Examples of this class are avilamycins, everninomicins, flambamycin, and curamycin. Class II orthosomycins constitute pseudotrisaccharides with one orthoester linkage and an aminocyclitol. Examples of this class are hygromycin B and the destomycins. Everninomicins, avilamycins, and hygromycin B are produced by *Micromonospora carbonacea, Streptomyces viridochromogenes* Tü57, and *Streptomyces hygroscopicus,* respectively. Orthosomycins were found to possess potent activity against as methicillin-resistant *Staphylococci*, vancomycin-resistant *Enterococci*, and penicillin-resistant *Streptococci* and quite effective in the treatment of infective endocarditis.

Avilamycin was applied as a growth promoter in animal feed. Moenomycins encompass a unique class of phosphoglycolipids synthesized by *Streptomyces* species. Saccharomicins are heptadecaglycoside antibiotics produced by the actinomycete *Saccharothrix espanaensis* LL-C19004 and had encouraging activity against all tested multiple-resistant *Staphylococci* strains. Acarviostatins are pseudo-oligosaccharides reported to be produced by cultures of *Streptomyces coelicoflavus* ZG0656 (McCranie & Bachmann, 2014). Oligosaccharide containing *R-N*-acetylglucosamine at the non-reducing end was found to inhibit protein biosynthesis in *Helicobacter pylori* (Manabe *et al.,* 2007).

6.7.2.13 Oligosaccharides as Vaccine Components

Fragments of complex oligosaccharides derived from polysaccharide provinces on the surface of pathogenic bacteria can be used for displaying antigenicity. Besides, these synthetic oligosaccharides play a role as haptens in the protein conjugates leading to the elicitation of both oligo and polysaccharide-specific IgG antibodies in animal models and humans. Synthetic oligosaccharide-based vaccines have been developed for infections against *Haemophilus influenzae* Type B, *Shigella dysenteriae* Type 1, *Streptococcus pneumoniae* Type 3, *Vibrio cholerae* O1 and *Pseudomonas aeruginosa*. Advances are made for the development of synthetic oligosaccharide-based vaccines for *Bacillus anthracis*, *Borrelia burgdorferi* and *Mycobacterium tuberculosis* (Pozsgay, 2008). Chitosan oligosaccharide conjugated with porcine circovirus type 2 (PCV2) subunit vaccine enhances the cellular and humoral immune responses by promoting the spleen lymphocyte proliferation against PCV2 viral infection (Zhang *et al.,* 2017). 2-Fucosyllactose enhances the performance of influenza vaccine by increasing the immunological response of mesenteric lymph nodes in mice (Xiao *et al.,* 2019).

6.7.3 Environmental Fortification

Algae-derived oligosaccharides fuel the plant growth by increasing the carbon and nitrogen assimilation and also increase the production of metabolites such as alkaloids, terpenoids, and terpenes for defense against pathogens (González *et al.,* 2013). The xylo-oligosaccharides play a noteworthy role in improving the salinity tolerance of Chinese cabbage by enhancing the activities of antioxidants such as peroxidase, superoxide dismutase, ascorbate, catalase, and carotene (Chen *et al.,* 2015). The major problem of the seafood industry is the disposal of chitinaceous waste. The chitinolytic microorganisms from the marine environment have the capability of producing chitin oligosaccharides from dumping waste (Kumar *et al.,* 2018). The extraction of oligosaccharides can also be carried out from agro-industrial wastes (Chimtong *et al.,* 2016; Cano *et al.,* 2020). Cyclodextrins are used in deodorant to counteract smoke and odors arising from clothes, cooking, and pets (Patel and Goyal, 2011).

6.7.4 Cosmetics

Direct exposure to UV radiation affects and damages skin. UV radiation mainly affects melanin pigment in the skin leading to aging of skin, wrinkle formation, and spot formation. Seaweed oligosaccharides especially agaro-oligosaccharides (AOS) and carrageenan-oligosaccharides prevent skin aging, wrinkle formation, and spot formation, and also serve as skin moisturizers (Cheong *et al.,* 2018). Non-sulfated fucose-based oligosaccharides obtained from *Klebsiella pneumoniae* have significant activities on the skin and also acts as an antiaging agent (Yvin *et al.,* 1999). Cotton honeydew extract is composed of various oligosaccharides along with glucose, inositol, saccharose, fructose, trehalulose, melezitose, and trehalose. This extract has the capacity of stimulating keratin synthesis and hence employed in haircare products and cosmetics (Oberto *et al.,* 2005).

6.7.5 Elicitors and Agriculture

Elicitors are compounds that trigger the plant defense and can be used to enhance secondary metabolite synthesis. The productivity of penicillin G and their intermediates during biosynthesis can be enhanced by the addition of alginate and mannan oligosaccharides (Tamerler *et al.,* 2001). Yokose *et al*. (2009) reported that AlgOs promote the growth of *Nannochloropsis oculata*. Oligomers or oligosaccharides have attracted increasing interest in agricultural applications as elicitors. Oligosaccharides derived from the plant and fungal cell-wall polysaccharides such as β-glucan, chitin, and chitosan were accomplished elicitors for prompting plant-defense responses (Hahn *et al.,* 1993). Oligo-alginates serves as elicitors and bio-stimulators by acting as signal molecules toward enhancement of nitrogen assimilation and basal metabolism to improve the growth of different plants. Khan *et al*. (2011) reported that AlgOs derived from sodium alginate through irradiation by Co-60 gamma rays considerably enhance growth, yield, and alkaloid production of opium poppy (*Papaver somniferum* L.). Iwasaki and Matsubara (2000) reported the stimulation of root growth in lettuce using oligo-alginates. Hu *et al*. (2004) examined the promotive effects of maize seed germination treated with AlgOs. Ali *et al*. (2014) reported the improvement of essential oil composition in *Eucalyptus citriodora*. Zhang *et al*. (2014) used AlgOs produced using alginate lyase from *Sphingomonas* sp. to promote root formation and growth in rice (*Oryza sativa* L.). AlgOs enhanced the development of root by inducing the expression of the auxin-related gene and also accelerated the auxins biosynthesis and transport, reduced indole-3-acetic acid oxidase activity and induced calcium signaling generation in rice roots (Zhang *et al.,* 2014). Oligo-alginates prepared by acid hydrolysis were reported to promote the growth and physiological activity of *Eucomis autumnalis* under salinity stress (Salachna *et al.,* 2018). Liu *et al*. (2013)

reported that AlgOs enhance the drought stress resistance of wheat during the growth period by regulating the ABA-dependent signal pathway. Oligosaccharides produced from the fungal and plant cell-wall polysaccharide play an important role in signaling a defensive response and act as an elicitor against pathogen attack (John *et al.,* 1997). *N*-acetyl CTOS are acting as potent elicitor signals in several plant systems. This includes the induction of lignification in wheat (Barber *et al.,* 1989), ion flux, and protein phosphorylation in cultured tomato cells (Felix *et al.,* 1993), chitinase activity in melon (Roby *et al.,* 1987), and gene expression of glucanase in cultured barley cells (Kaku *et al.,* 1997). Xyloglucan oligosaccharides promote cell division and cell expansion in the suspension-cultured tobacco cells (Kaida *et al.,* 2010). Cyclodextrins can form complexes and encapsulate herbicides, insecticides, fungicides, repellents, pheromones, and growth regulators leading to their controlled release in the agricultural fields (Morillo, 2006).

6.7.6 Novel Biomaterials

Stearic acid (SA) grafted chitosan oligosaccharide CSOS was employed as a gene delivery system (Hu *et al.,* 2006). Oligosaccharides derived from hyaluronic acid (HA) was used as a drug carrier (Pouyani & Prestwich, 1994). Surface modification of graphite using oligosaccharide surfactant polymers was reported to be effective in subduing protein adsorption from human plasma protein (Holland *et al.,* 1998). Biodegradable oligosaccharide-based graft copolymer as nanowires can be used as functional biomaterials in various application fields (Qiu *et al.,* 2012). A biocompatible and biodegradable drug delivery system was fabricated by surface modification of oxidized cellulose nanocrystal with chitosan oligosaccharide for transdermal delivery applications (Akhlaghi *et al.,* 2013)

6.8 Market Potential of Oligosaccharides

In view of the prospective applications, different oligosaccharides have received huge market potential as a viable biocommodity. Oligosaccharides were initially recognized as food additives and functional foods due to the health benefits leading to an increase in its market league. Bioactive oligosaccharides have a sustainable market due to its phenomenal popularity as prebiotics especially in infant food formula to improve the healthy gut microbiome. The total global market for these functional oligosaccharides is projected to magnify globally with over $90 billion/year. It is expected that the demand for functional oligosaccharides will nurture significantly over years not only from the food industry but also from other sectors like agriculture, pharmaceuticals, medicine, etc., owing to their biological activities. Currently, fructo-oligosaccharides, galacto-oligosaccharides, mannan

oligosaccharides, xylo-oligosaccharides, and human milk oligosaccharides occupy huge demand from food industries, nutraceuticals, and pharmaceuticals. Further, the marketing potential will depend on the production costs of these metabolites. Hence, the search for novel sources of oligosaccharides seems to be a remarkable hunt. Synthesis of novel oligosaccharides with enhanced target-specific functional properties will cater to new research and business opportunities.

6.9 Future Prospects

Oligosaccharides are unique, structurally diverse bioactive metabolites. Owing to their impending applications, the potential source of these bioactive oligosaccharides has to be explored from the plethora of natural and industrial wastes. Technology for tailor-made oligosaccharides with desired functional properties and specific health benefits has to be developed. Further, understanding the molecular mechanisms in various biological applications and effects of long-term consumption of these oligosaccharides are indispensable. Advancement in enzyme technology will serve as a cost-effective production route leading to higher yield rather than conventional extraction methods and also promote the development of designer bioactive oligosaccharides in the future. Further technological advancements for conversion and extraction of oligomers from waste of agro-industries will function as a sustainable approach. Currently, most oligosaccharides are attracted and confined to food-related applications. Looking forward to utilizing these bioactive oligosaccharides in extended applications such as drug delivery, antibiotics, cosmetics, animal feed, and agrochemicals. The development of new generation vaccines using synthetic oligosaccharides will serve as another milestone. Carbohydrate sequencing and the synthesis of defined oligosaccharides are two upcoming strategic technologies that can contribute toward the expansion of oligosaccharide-based diagnostics and therapeutics.

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7

Biopolymers

A Retrospective Analysis in the Facet of Biomedical Engineering

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7.1 Introduction

In congruence with the slowly disintegrating environment, which was almost invisible to the existence at an early stage, has now evolved with more predominant issues. Understanding this necessity has navigated us to choose bio-based materials to be incorporated in various fundamental applications such as in biomedicine, food industry, waste water management, packaging, textiles, electronics, and so on. Humans who are intrinsically driven by curiosity have stepped up remarkably in exploring its utility in many more ways. With the gathering knowledge, biopolymers are no more looked as a one-dimensional entity, but instead it is a multidimensional approach involving polymer chemistry, structural analysis, computational simulations, material design, polymer solution properties, solid-state polymer physics, etc. Though it might sound like a one millennium tough task of procuring a functional polymer, it all requires a well-designed formulation with basic techniques. Biopolymers inherit attributes such as being biodegradable, renewable, sustainable, nontoxic, non-immunogenic, and many more.

Biopolymers can be classified based on its source from which they are derived such as polysaccharides (cellulose, starch, chitin, chitosan, alginate, etc.), polypeptides (silk fibroin, elastin, collagen, gelatin, etc.), and polynucleotides (DNA, RNA). They can be plant-based, animal-based, or microorganism-based biopolymer. Microscopic molecules that makes up the polymer sets as the critical factor for the functioning of its translated three-dimensional product. Hence, it is very crucial in understanding its monomeric substituents. The common factor that binds these polymers in one pot is that these are nature-procured materials,

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but microscopically these display different entities that gives them unique attributes. One tough realization here is that these being bio-based is not sufficient to render it for direct application. They may execute varying degrees of physical, chemical, and mechanical features, which can also limit themselves from its prompt implementation. Therefore, the strategy employed here is to modulate each polymer with respect to its intended application.

This chapter gives an overview of the multifarious biopolymers and special focus onto its application in the biopharmaceutical sector under the subtopics tissue engineering, bio sensing, bio imaging, drug delivery, biological implants, wound healing, cancer therapeutics, and other industrial applications. Further, some fundamental fabrication techniques will be discussed such as 3D/4D bioprinting and electrospinning.

7.2 Natures' Advanced Materials: A Glance at Its Structure and Properties

7.2.1 Polypeptides

Short peptide motifs can be contrived into biopolymers where some can be categorized into thermoresponsive biopolymers such as those based on leucine zippers, human collagen, human elastin, and silkworm silk to give peptide-based biopolymers (Figure 7.1). Peptides can be either obtain in their pure form and synthesized chemically or through recombinant technology with genetic engineering tools.

7.2.1.1 Collagen

Type I collagen represents 25–35% of the total protein content of our body and forms the main component of the extracellular matrix found in skin, bone, tendon, cornea, and ligament. It reveals a monomeric, triple helix configuration, composed of two identical alpha1 and 1 alpha2 chain. It comprises of the unique RGD (arginine, glycine, aspartic acid) domains, which leverage cell adhesion by recognizing cell integrin receptors (Emsley et al., 2000). Collagen is the most widely used bioink for fabricating tissue scaffolds due to its low antigenicity, excellent biocompatibility, low immunogenicity, and able to get degrade completely by the collagenases and hydrolytic mechanisms (Marques et al., 2019). It is an anionic polyelectrolyte and shows high versatility but has low viscosity, and to elevate its mechanical strength, it is often cross-linked by utilizing glutaraldehyde or even blended with other biopolymers.

7.2.1.2 Elastin

These are genetically encoded biopolymers with the repeating sequence, Val-Pro-Gly-X-Gly (X≠ Pro) recapitulates the natural elastin found in blood

vessels, lung tissues, etc. They possess unique property of showing inverse phase transition behavior with solubility below transition temperature (Tt) and insoluble aggregates above it (Reiersen et al., 1998). They demonstrate excellent biocompatibility, non-immunogenic, mechanical, and viscoelastic properties similar to natural elastin (Zhang et al., 2015). The properties of this smart biopolymer can be

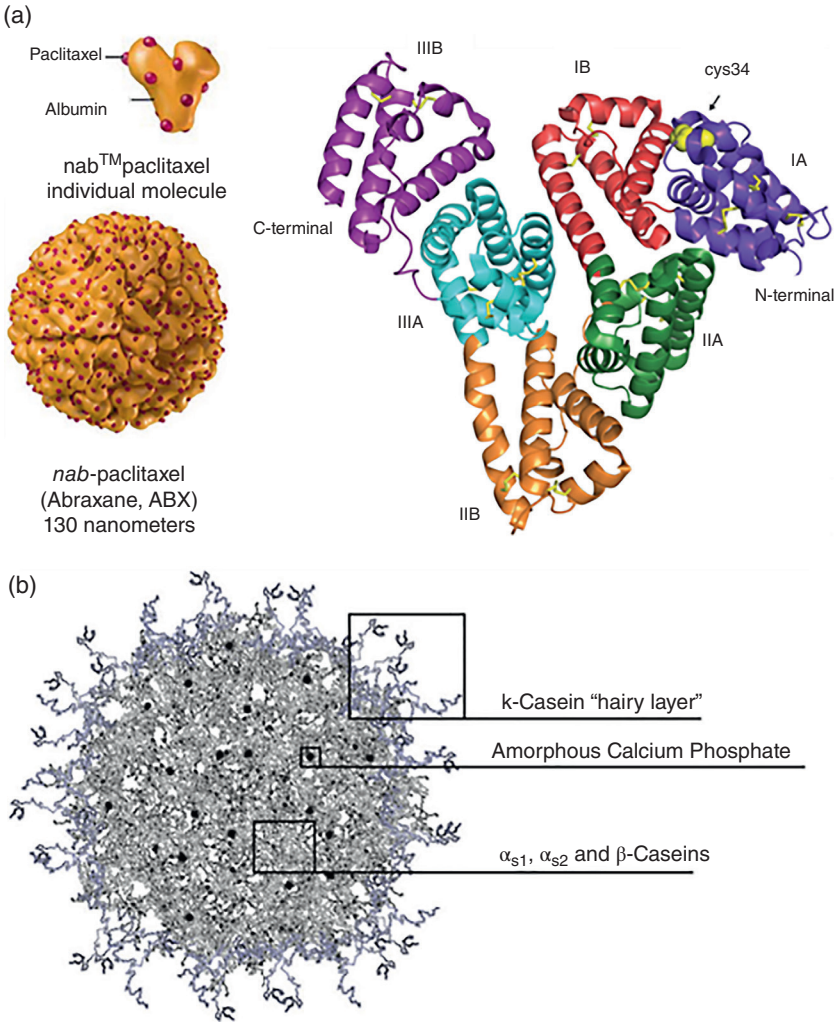

Figure 7.1 Polypeptide-based biopolymers: (a) albumin-based NP-Abraxane™ (Butterfield et al., 2017), crystal structure of albumin (Larsen et al., 2016); (b) casein micellar structure (Głąb & Boratyński, 2017); (c) silk fibroin (Qi et al., 2017) and its SEM image (Aramwit, 2016); (d) gelatin chemical structure (Deshmukh et al., 2017).

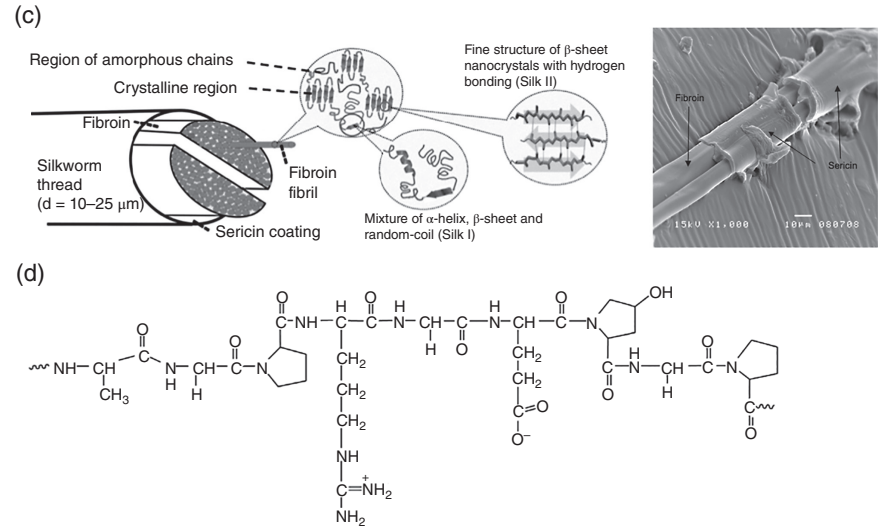

Figure 7.1 (Continued)

modulated by precisely controlling their MW, length, and peptide sequence. They are utilized as thermal responsive drug carriers and injectable hydrogels, biosensors, and tissue engineering.

7.2.1.3 Silk Fibroin

Silk is one of the FDA-approved natural biopolymer, which can be designed and synthesized by the recombinant DNA technology (Betre et al., 2002). The hydrophobic domain in the heavy chain with the hexapeptide repetitive sequence GAGAGX dictates the crystallinity by forming antiparallel beta sheets and provides the mechanical strength to the silk fiber. The elasticity is obtained from the less-repetitive hydrophilic regions in the heavy chain. It is biocompatible, biodegradable, non-immunogenic, and is extensively used in sutures, drug delivery carriers with pH and thermoresponsive-controlled drug release, and in tissue engineering (Mackay & Chilkoti, 2008).

7.2.1.4 Gelatin

Hydrolysis of collagen leads to the formation of irreversible gelatin biopolymer with the primary sequence containing GXY (where X and Y are normally proline and hydroxyproline) and molecular weight ranging from 20 to 250 KDa. Gelatin retains the RGD sequence from the collagen, which potentiates good biological performance with cell recognition motif (Santoro et al., 2014). It is water soluble, biodegradable, biocompatible, nontoxic, non-immunogenic, inexpensive, and

abundant biopolymer. However, it has weak mechanical strength (Xing et al., 2014), high viscosity, and succumbs to enzymatic degradation. These drawbacks can be overcome by blending it with other polysaccharides or biomaterials and sets it as a promising scaffold for tissue engineering with similarities to ECM and in the formation of hydrogels (Afewerki et al., 2019).

7.2.1.5 Albumin

Human serum albumin constitutes 50% of the blood plasma protein and is responsible for 75–80% of the osmotic pressure of plasma. This anionic and globular protein measures 66.5 KDa in its MW and comprises a single polypeptide chain of 585 amino acids. It has inherent specificity toward tumor with its recognition-binding sequence toward gp60 and osteonectin (SPARC) and therefore fabricated as nanoparticles for tumor targeting and controlled drug release (Jiang et al., 2017). Furthermore, being a natural protein inherent to our system, it is categorized under nontoxic biopolymer with an average half-life of 19 days, which leverages good pharmacokinetic profiles. Projecting its enhanced uptake in solid tumors, Abraxane^{TM} is synthesized, which is a commercially available drug delivery nanoparticle with an average diameter of 130 nm and binds paclitaxel for metastatic breast cancer treatment (Miele et al., 2009).

7.2.1.6 Casein

Casein is a proline-rich phosphoprotein, which constitutes about 80% of the cow's milk content. The structure reveals four major electrophoretic components, which are alpha1, alpha2, beta, and kappa-caseins in the proportion 4 : 1 : 4 : 1. They are amphiphilic in nature and can self-assemble into energetically stable micellar configuration with k-casein forming the outer hydrophilic layer. It is sparingly soluble in water and therefore utilized as soluble sodium caseinate form. They are relatively cheap, nontoxic, and heat stable due to relatively less secondary and tertiary confirmation. They are majorly used as drug delivery carriers in cancer nanotherapeutics (Elzoghby et al., 2011), good film-forming ability with the presence of large number of polar groups and can be transformed into hydrogels (Song et al., 2010) for use in tissue engineering (Nazari et al., 2020). Its possible immunogenicity and allergic reaction to casein-sensitive patients can limit its biomedical use.

7.2.2 Polysaccharides

7.2.2.1 Cellulose

First discovered by Turbak et al. in 1838, cellulose is the most abundant biopolymer in our biosphere (Figure 7.2). Exhibiting similarities to the starch, its chemical construct displays a linear polymer with beta-1-4 bonds linking the D-anhydroglucopyranose units. It is semicrystalline in nature with favorable

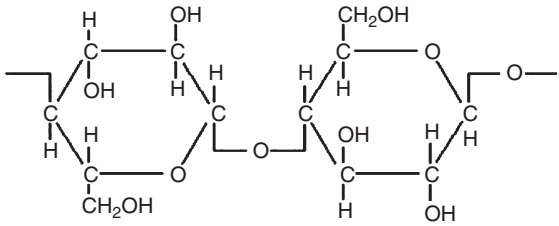

Figure 7.2 Cellulose (Richards et al., 2012).

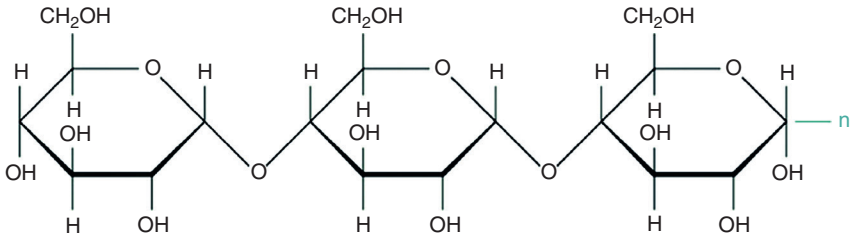

Figure 7.3 Starch (Othman et al., 2018).

mechanical properties, optical transparency, negligible inflammatory response, and nontoxic material. It is biocompatible but moderately biodegradable due to the lack of cellular enzymes in our body, and in addition, it is insoluble due to the extensive hydrogen bonding between the chains. However, the most widely employed derivatives of cellulose such as methylcellulose (MC), carboxymethylcellulose (CMC), and hydroxypropylmethylcellulose (HPMC) impart solubility with additional properties. In comparison, bacterial cellulose is more attractive with its fibrillar structure and elastic modulus comparable to that of articular cartilage (Wu et al., 2019b). These derivatives have the ability to swell in contact with water, and hence, they are used in wound dressings, transdermal patch, suitable candidates for tissue engineering, and controlled drug delivery (Oprea & Voicu, 2020).

7.2.2.2 Starch

A polysaccharide-based biopolymer, starch is made of amylopectin and amylose, and the percentage composition of it may differ depending on its source such as rice, wheat, corn, potato, and so on, where it is stored as a high-energy storage in these plants (Figure 7.3). These are easily accessible, inexpensive, and can be modified in conjunction with other synthetic polymers. Though its application has been found in food industry, textiles (Le Corre & Angellier-Coussy, 2014), wound healing, and tissue engineering (Hemamalini & Giri Dev, 2018), its major obstacle of high hydrophilic nature hinders its versatility in biomedical usage.

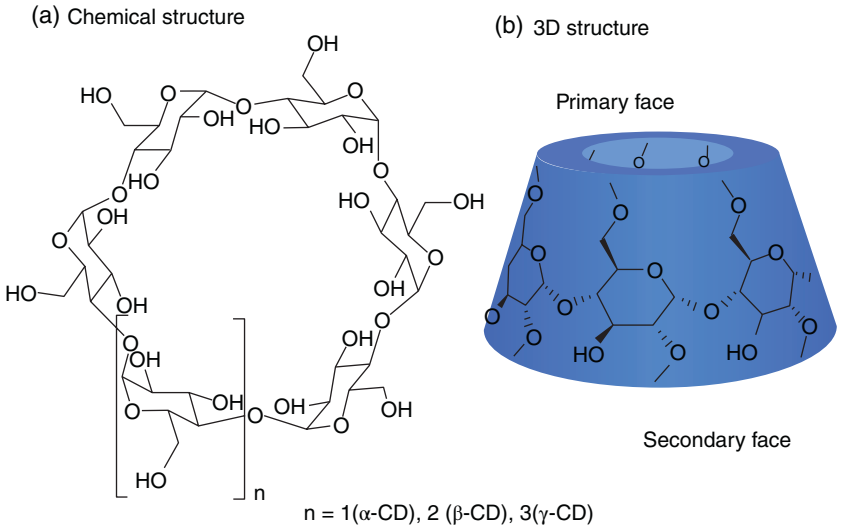

Figure 7.4 Cyclodextrin (Baykal et al., 2018).

7.2.2.3 Cyclodextrin

As the name suggests, these are cyclic oligosaccharides composed of α-(1-4) linked D-glucopyranose units in $^4C_1$ chair conformation and is obtained from the enzymatic degradation of starch (Figure 7.4). CDs are crystalline, nonhygroscopic, nontoxic, biodegradable, biocompatible, inexpensive, readily functionalized, and water-soluble biopolymer. Differing in physicochemical properties such as solubility and ring size, there are three natural CD available, α, β, and γ with the glucose content of 6, 7, and 8, respectively. It has a hydrophilic exterior surface and an interior hydrophobic cavity, which leverages inclusion of various organic, inorganic, and biological molecules for host–guest interaction for drug delivery (Zhang & Ma, 2013) and as stabilizing agents (Suárez-Cerda et al., 2017).

7.2.2.4 Hyaluronic Acid

It is an anionic, non-sulfated, linear carbohydrate polymer, which is the chief component of the extracellular matrix. Being produced in the plasma membrane, it is made of 1000–25000 repeating units of D-glucuronic acid and d-n-acetylglucosamine monomers, which is linked by alternating 1-4 and 1-3 glycosidic bonds (Figure 7.5). In addition to being biodegradable, its high recognition to the CD44 receptors, which is overexpressed on cancer cells, polymeric structures incorporating hyaluronic acid render its wide application as an efficient drug delivery active targeting system (Huang & Huang, 2018). It is nontoxic and in accordance with it, stimulating the differentiation of epithelial cells is used in tissue engineering and wound dressings.

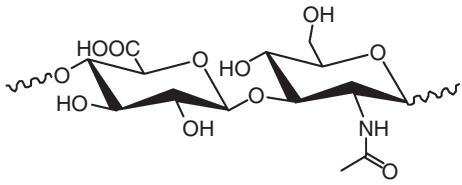

Figure 7.5 Hyaluronic acid (Sionkowska et al., 2020).

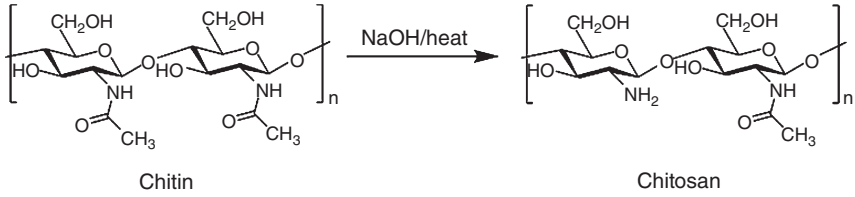

Figure 7.6 Chitin and chitosan (Ruiz & Corrales, 2017).

7.2.2.5 Chitosan

Chitin, the key component found in the exoskeleton of crustaceans' shells can be chemically deacetylated to obtain linear cationic polysaccharide termed as chitosan. It is a semicrystalline, biodegradable, biocompatible, low-immunogenic, nontoxic, cheap, and abundant biopolymer with excellent antimicrobial and antifungal properties(Ing et al., 2012). Chitosan architecture reveals surface active, amine and hydroxyl groups, which are bonded to the *N*-acetyl glucosamine and β-1,4 glycosidic bonds linking the glucosamine units (Figure 7.6). The reactive groups instigate interaction with several chemical and biological moieties through covalent bonding, electrostatic coupling with charged molecules, and hydrogen bonding. One limiting factor here is its insolubility at physiological pH, but its surface modification has been extensively investigated to facilitate its solubility in cellular environments (Knidri et al., 2018). It displays affinity toward negatively charged cell membranes owing due to its cationic surface. Its captivating attributes has inclined it for various applications such as wound healing, drug delivery nanocarrier, and nanofibers, in biomaterials, tissue scaffolds, and formation of hydrogels (Jayakumar et al., 2010).

7.2.2.6 K-carrageenan

It is a marine-originated natural, linear, and sulfated polysaccharide, which is extracted from red seaweeds. This high molecular weight galactan are hydrophilic in nature that constitutes 30–70% of the cell walls' dry weight. Its chemical components reveal repeating chains of sulfated and non-sulfonate

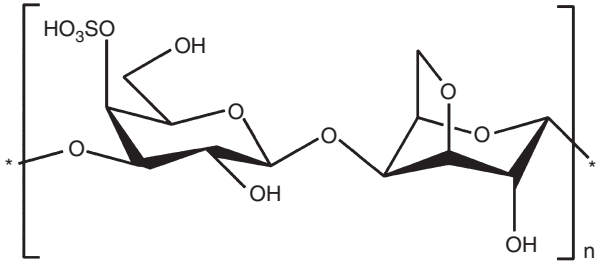

Figure 7.7 k-Carrageenan (Solov'eva et al., 2013).

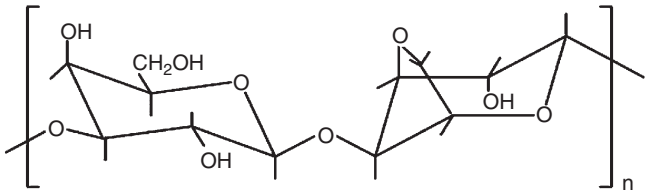

Figure 7.8 Agarose (Garcia et al., 2000).

3,6-anhydrogalactose units that are joined alternatively by alpha-1-3 and beta-1-4 glycosidic linkages (Figure 7.7). Its ease for chemical modification with the presence of sulfate and hydroxyl functional groups makes it a robust biopolymer. In addition to being biocompatible, its thermoreversible gelation and tunable viscoelastic properties facilitate its use as an ideal drug delivery system (Li et al., 2014b). Its application can be seen broadly in food industry, clarification of the beverages, and also in pharmaceutical industry (Zia et al., 2017). It has shown antiviral (Falcó et al., 2019) and antitumor properties (Calvo et al., 2019).

7.2.2.7 Agarose

It is a copolymer extracted from seaweeds and constructed with an alternating repeat of three-linked beta-D-galactopyranose and four-linked 3,6 anhydro-alpha, L-galactopyranose residues (Figure 7.8). The polymer chain displays temperature-dependent change in its conformation that presents itself as random coil at higher temperature (>65–95 °C) and forms double-helical aggregates at about 35 °C; they demonstrate good mechanical strength with its interconnected pores, slow biodegradation, and superior tissue adhesiveness (López-Marcial et al., 2018).

7.2.2.8 Alginate

Alginate is a linear anionic polysaccharide-based block copolymer that is made of 1,4-linked hexuronic acid residues, namely, β-D-mannuronate (M) and α-L-glucuronate (G) residues (Figure 7.9). Enriched with hydroxyl and carboxyl

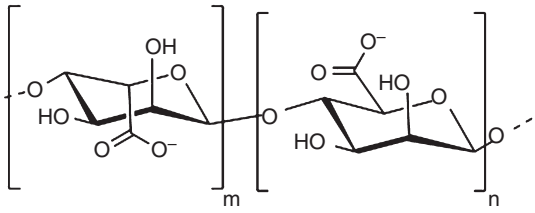

Figure 7.9 Alginate (Piras & Smith, 2020).

groups on the alginate backbone, it potentiates extensive chemical modification to corroborate with multifarious hydrophilic and hydrophobic drug moieties. However, due to lack of cell-adhesion sequences such as RGD, alginate has been widely used as composite blends with other polymers and used in a variety of biomedical applications such as bioink in tissue engineering (Abbasi et al., 2020) and carriers in drug delivery (Sun & Tan, 2013).

7.2.3 Polynucleotides-based Biopolymers

Anionic nucleic acids form the basic structural repeats of the polynucleotide-based biopolymers. A base, sugar, and a phosphate molecule that makes up one nucleotide underpins the supramolecular structure, which repeats itself to form a rod-like helical strand. DNA- or RNA-based biopolymers can self-assemble or undergo complex formation with cationic small molecules. This is driven by the electrostatic interactions that takes place between the negative phosphate chain and the positive interacting moieties. As nucleotides carry high biological relevance, great amount of interest has been projected toward its derivatives and its applications in drug delivery, optical sensing (Liu et al., 2018a), tissue regeneration, diagnostics, etc.

As we see it, from our daily physical activities to the invisible thought process, our existence is undeniably governed by the meticulously arranged, intricate coding in the DNA sequence. Integration of DNA with nanotechnology has sprouted back in 1982, when Seeman shared her innovative idea of constructing three-dimensional networks with DNA. DNA can be modified and programmed to effectuate specific molecular recognition or elicit a therapeutic function, self-assembled to form nanosystem or hydrogel and exquisitely conjoined with potential nanoparticles, elevating it to execute a sophisticated, cohesive function. Intrinsic to our system, biocompatible polynucleotides can be contrived to form aptamers with various SELEX techniques and decorated on the nanoparticles. Aptamers-conjugated nanoparticles (NPs) leverage enhanced cellular uptake with its high specificity toward various cancer cell surface biomarkers (Ravichandran & Rengan, 2020). Self-assembled spherical nucleic acids (SNA)

leverages drug solubility along with high drug-loading capacity (Zheng et al., 2013). They can be modulated to attain different nanoarchitecture, among which DNA tetrahedron (Meng et al., 2019) has received major focus due to its ability to get rapidly internalized into the cell via caveolin-dependent pathway. In addition, it reveals tolerance to enzymatic degradation and thermal stability. DNA-polymer hybrid nanosystems and DNA hydrogels have been fabricated to manifest bio-responsive sustained drug release in cancer therapy. DNA, a hydrophilic polyelectrolyte biopolymer, gains enormous interest in hydrogel fabrication, which instigates from its ability to absorb large water (Li et al., 2020). Such supramolecular DNA hydrogels (Shao et al., 2017) demonstrate features such as biocompatible, biodegradable, ability to self-heal, diffusion to nutrients, and along with imparting multi-stimuli responsiveness to the contrived hydrogel.

7.3 Smart Biopolymers

Smart biopolymers form the imperative technological gears to comprehensively get incorporated into the third-generation biomaterials to make it intelligent and versatile. These smart features are either inherited or incorporated with selective chemical modifications. Incorporating elements into the natural renewable polymers and thereby, synthetically modifying it to render them stimuli responsive has gained massive interest and has potential applications. Setting out an example, human body has varying pH levels and biopolymers can leverage pH-dependent drug release in the tumor zone or gastric region for precision drug delivery. Another advantage is that they can be noninvasively triggered to exhibit its function with response to stimuli such as light, temperature, electric, magnetic field, redox, mechanical stress, and even ultrasound. Such response to even small external change is evidently visualized at the macroscopic level in the form of optical, permeable, chemical, precipitation, turbidity, phase separation, or the formation of hydrogels.

7.3.1 Chemical-Responsive Biopolymers

7.3.1.1 pH-Sensitive Smart Biopolymers

Polymers falling into this category have ionizable functional groups. Polyanionic polymers with acidic functional groups such as carboxylic or sulfonic groups accept or release protons at low and high pH, respectively. Alginate is an anionic copolymer, which experiences shrinkage at low pH (Mukhopadhyay et al., 2015). Therefore, we observe a swelling behavior of the polymers as the pH increases. Similarly, polycationic polymers with basic functional groups such as amino salts in their composition protonates at low pH and ionizes at neutral or high

pH. Chitosan polysaccharide is a perfect example of pH-sensitive biopolymer, which swells upon protonation of free amino groups in an acidic environment. Chemical modification of cellulose with the introduction of carboxymethyl in its chain renders the resulting CMC polymer pH sensitive (Gao et al., 2014). Another strategy is to chemically modify the backbone with pH sensitive linkage such as disulfide bonds (Wu et al., 2019a). Other extensively utilized pH-responsive natural biopolymers are gelatin and K-carrageenan (Sarıyer et al., 2020).

7.3.1.2 Glucose-Responsive Biopolymers

Biopolymeric nanosystems functionalized to act upon glucose molecules can benefit the clinical society in many ways. Dextran has unique attributes that qualifies it as a drug delivery carrier such as rendering it degradable by the dextranase enzyme. Jamwal et al. fabricated GOx immobilized dextrin nanoparticles for the pH-triggered delivery of insulin. Glucose oxidase catalyzes glucose to gluconic acid and hydrogen peroxide. The trick here lies in the protonation of the carrier due to reduced pH, which changes its morphology and results in the release of insulin into the system (Jamwal et al., 2019). In a similar concept, Kim et al. fabricated pH-sensitive chitosan microgels to immobilize GOx for efficient delivery of drug. Such potential systems are designed to deliver insulin in a glucose-dependent manner for diabetic patients (Kim & Kim, 2017). In another biomedical application, iron-cross-linked alginate hydrogel was developed for biosensing glucose. Increased glucose uptake is an important hallmark of cancer, which can be witnessed together with the overexpression of glucose transporters on the cancer cell membrane. Li et al. prepared glucose-conjugated chitosan nanoparticles to effectuate interaction with glucose transporters (GLUTs) and thereby instigate the delivery of encapsulated Dox molecules (Li et al., 2014a).

7.3.2 Physically Responsive Biopolymers

7.3.2.1 Temperature-Sensitive Smart Biopolymers

Thermoresponsive polymers are classified under either lower critical solution temperature (LCST) or upper critical solution temperature (UCST), below and above which these polymers exist as a single-phase solution and are completely miscible (Darge et al., 2019). Being a pivotal parameter, LCST is defined as a critical temperature at which the phase transformation of the polymeric solution takes place. Composition of the polymer is the key to achieve a change in their phase and solution upon exceeding their critical solution temperature. Benefitting from this, Cok et al. recently developed temperature and pH dual-sensitive chitosan hydrogels with the substitution of N-isopropyl in the polymeric chain (Cok et al., 2020). Thermoresponsive hydrogels constructed with such polymers are the subject of extensive research in biomedical applications with its unique

attributes of being minimally invasive and displaying in situ gelation process, which gets transformed into gel in physiological conditions from solution at RT. Upon modification of cellulose with methyl (MC) and hydroxypropylmethyl (HPMC), temperature-responsive gelation of the polymer takes place at 40–50 °C and 75–90 °C, respectively. Agarose, for instance, has intrinsically unique behavior toward temperature, which is a huge attractive polymer imparting the ability to form gels even at very low concentrations.

7.3.2.2 Light-Responsive Smart Polymers

These are the photo-responsive materials or composites introduced with light-responsive chromophores, which when triggered with light will undergo induced phase transition. Light-responsive polymers can be triggered by three different light sources: UV, visible light, and near-infrared light (NIR). However, in biomedical applications, NIR light-responsive materials have gained huge popularity due to its distinctive features such as deep tissue penetration, nontoxic to tissues, and high efficiency to triggered drug release. On the contrast, UV light-responsive polymers are limited by inefficient drug release, potential damage to genetic material in the body (Cadet & Douki, 2018), and low depth of penetration (Ash et al., 2017). Light gains more advantage compared to other stimuli, whose parameters can be controlled such as intensity of light, wavelength, irradiation time, and surface area depending upon the treatment requirement. It is advantageous in leveraging light-induced spatiotemporal release of drugs in a controlled fashion. One fundamental feature is manifested with its photothermal effect where the photons are efficiently converted into heat upon light irradiation. Photo-responsive materials are extensively investigated as it is imperative in the facet of photothermal therapy (PTT) (Pierini et al., 2018) and photodynamic therapy (PDT) (Zhang et al., 2017) for tumor ablation. The mechanism underlying the fabrication of composite is simple but unique. The photos irradiated onto the plasmonic metal nanoparticles are absorbed and converted to heat owing to its surface plasmon resonance (SPR) phenomena. The generated heat is then transferred, which allows the conjugated and thermal-responsive polymer to disassemble or disintegrate to release the encapsulated drug molecules. Thus, the combination of these two synergistic, light-responsive and thermoresponsive, components complements each other so well to manifest cancer cure. There have been overwhelming reports contemplating its potential futuristic goals in clinical translations.

7.3.2.3 Electric-Responsive Smart Polymers

Electro-sensitive polymers display physical and chemical changes when triggered with applied voltage, translating the applied current input to mechanical energy. One can observe the output response as either with the shrinkage or the swelling

of the polyelectrolyte polymers under the influence of electric current. The most commonly employed conducting polymer is polypyrrole (PPY) and poly(3,4-ethylenedioxythiophene (PEDOT), but they lack biodegradability and solubility. Thus, to make it more suitable for clinical applications, they are blended with more flexible and biodegradable natural biopolymers such as alginate, chitosan (Ulutürk & Alemdar, 2018), gelatin, collagen, agarose (Hur et al., 2014), silk (Magaz et al., 2020), and so on to produce electroactive copolymers. Therefore, biohybrid composites are contrived with the amalgamation of natural biopolymers and electroconductive moieties and widely utilized as coatings, implants, or scaffolds in tissue engineering or as drug delivery vehicle in clinical therapeutics such as cancer. In the application of a drug carrier, the polymers containing ionizable groups can exhibit change in pH with a small inclusion of current. This change leads to the disruption of hydrogen bonds and polymer degradation, subsequently resulting in the release of entrapped drug molecules. Field parameters such as strength, duration, and the frequency can aid in the pulsatile or sustained release of drugs. Copolymers can be fabricated with blending, grafting, curing, or cross-linking methods. One limitation adhered to the utilization of hydrogel-based conducting system is that it requires high voltage supply for response (2–25 V). One fundamental consideration is the selection of driving current force that does not affect the nerve functioning, but at the same time effectuates the drug release.

7.3.2.4 Magnetic-Responsive Smart Polymers

With the application of external alternating magnetic force, the administered hybrid nanosystems can be noninvasively controlled for a triggered spatiotemporal release of chemo-drugs. Ferrogel is the term that denotes magnetic-responsive hydrogels and basically could be fabricated by blending, co-precipitation, and grafting techniques. Strong attraction can be achieved between anionic polypeptide biopolymer possessing amine, carboxyl, and anionic functional groups with the cationic magnetic nanoparticles (maghemite, magnetite, or cobalt ferrite). Helminger et al. adapted in situ coprecipitation technique to fabricate inorganic–organic hybrid composite by loading iron into the gelatin hydrogels and obtained strong protein–ion interaction, which stabilized the construction. It was visualized with the change in color from bright orange to black, indicating the formation of magnetite NPs inside the hydrogel (Helminger et al., 2014). Superconducting quantum interference device (SQUID) experimental analysis revealed superparamagnetic properties, which connote its potential application in actuators, drug delivery, hypothermia therapy, waste water treatment, etc. In order to incorporate with hyaluronate, it is imperative to surface modify MNPs, wherein biocompatible moieties such as chondroitin sulfate is preferred

(Tóth et al., 2015). As an attractive therapeutic strategy, guided with the external magnetic force, channeling of the subjected composites to the tumor site is achievable in order to enhance the bioavailability and improve its pharmacokinetics. Post-tumor homing, these composites with selective composition attains the ability to translate the magnetic force into the units of heat to effectuate localized photothermal ablation of tumor. These are smart and versatile biopolymers, which demonstrate a significant role in the therapeutic, diagnostics, coatings (textiles and fibers), and microelectronic applications. Moreover, embedding of SPIONs into the matrix of magnetic-sensitive polymers can manifest its application as an MRI contrast agent in cancer diagnosis.

7.3.2.5 Redox-Responsive Biopolymer

Glutathione (GSH)-responsive biopolymer for the degradation or disassembly of nanoparticles for the intracellular release of drug payload is most widely employed redox stimuli-based drug delivery in cancer nanotherapeutics. It is elegantly achieved by conjugating the polymer with disulfide linkage to render it cleavage by GSH, which is seen in way different ratio. In comparison, tumor intracellular space has 100–1000 folds higher GSH levels than found in blood circulation, leveraging it to release its cargo specifically inside the cancer cell and preventing it from premature drug release. Recently, Gao et al. fabricated GSH responsive, hyaluronic-acid-based hydrogel for wound detection by conjugating it with amino ethyl disulfide (AED) cross-linker. GSH level of detection in the wound would be implicated by the change in hydrogel volume (Gao et al., 2019). In another study, Zhao et al. utilized this method to deliver Dox molecules inside the cancer cells by encapsulating it in the mesoporous silica NPs, which in turn is coated by the HA linked with the disulfide. HA, which has recognition with the cell-surface receptor CD44, will aid in the cellular uptake, and subsequent cleavage by the intracellular GSH will release the dox molecules (Zhao et al., 2015). Likewise, several other biopolymers have been employed such as carboxymethyl chitosan, alginate nanoparticles (Zhang et al., 2017), alpha cyclodextrin, and so on to facilitate reduction-responsive drug delivery.

7.3.3 Biochemical Stimuli-Responsive Biopolymers

7.3.3.1 Enzyme-Responsive Biopolymer

Enzymes are integral to our physiological system, endowed with exceptional biorecognition and catalytic properties. Integration of such powerful concept with the potential nanoparticles is compelling in mediating enzyme-responsive spatiotemporal drug delivery to the tumor site. In particularly, diseases like cancer, experimental evidences corroborate an unequivocal dysregulated enzyme profile

(Benjamin et al., 2012), which underpins the conceptualization of incorporating enzyme-responsive linkers in the polymeric chains for cancer therapeutics. Several classical enzymes are enumerated under proteases, phospholipases, and oxidoreductases, which are extensively exploited as potential targets in several pathological diseases. Matrix metalloproteinase (MMP) has predominant role in tumor progression and metastasis (Shay et al., 2015), which is overexpressed in the microenvironment and conjugation of MMP2-sensitive peptides to nanosystems can potentially elicit enzyme-triggered drug release. Vaghasiya et al. employed collagen, which plays dual role of primarily functioning as a gatekeeper to prevent premature drug release by capping the pores in the mesoporous silica NPs (MSNs) and secondly actuates cleavage by the MMP2 present in the tumor zone to effectuate "on-demand" release of cisplatin drug molecules (Vaghasiya et al., 2020). The milk protein, casein, is intriguing in the application where the stability must not be compromised with the hostile pH conditions such as in the acidic stomach. Casein has gained prominence for its stability in the wide range of pH (2.0–8.0). Huang et al. assembled casein molecules onto the magnetic NPs, which gets gradually broken down by the gastric protease to subsequently release the encapsulated Dox molecules (Huang et al., 2015).

7.4 Fundamental Applications of Biopolymers in Biomedical Engineering

7.4.1 Biopolymers in Cancer Theranostics

Perplexing complexity could be a perfect definition given to the word "cancer" that masters to refrain itself from any therapy with its ever-evolving opportunistic mutations. The unrelenting quest in unravelling the underlying molecular biology had a radical shift toward our perception and have furnished some phenomenal nanotechnological platforms (Chen et al., 2017) that hold the potential to curb and culminate cancer. It is out there to the knowledge, as how the standard chemo and radiotherapy can severely compromise the quality of life (Shi et al., 2017). However, the genesis of nanotechnology has not just put forth a new dimension to the cancer treatment but also executed the first-line therapies in a more refined manner. Cutting the chase-how crucial is a drug carrier in cancer nanotheranostics? A nanosystem is integral to this modernized therapy, which begets the interface between therapy and diagnostics and concisely, mitigates the drug payload with minimizing the off-target side effects (Parveen et al., 2012). Utilizing these formidable biopolymers have procured us with the contrivance of multifarious potential drug delivery system (DDS), which leverages the channeling of the chemo-agents safely to the tumor zone for imaging guided therapy.

7.4.1.1 Drug Delivery

Precise delivery of chemotherapeutic agents encapsulated or entrapped in a potential nanocarrier has begun the new genesis of modern therapeutics. The first and foremost criteria, is beyond doubt, the biocompatibility of the nanosystem. Chitosan is widely used as a coating material to render the nanoparticle biocompatibility. Cetyl trimethylammonium bromide (CTAB) forms an essential component in the synthesis of GNRs for driving PTT, but due to its toxicity, chitosan can be used to replace CTAB (Almada et al., 2017). In another study, chitosan coating onto GNR has not only prevented agglomeration of the gold nanoparticles but also shown to dramatically red shift, which induces shift in the SPR band. Chitosan being an anionic material gives an overall positive charge to the nanoparticle and enhances cellular internalization (Manivasagan et al., 2018).

Speaking about the targeting, some polymers such as collagen and gelatin are intrinsically amenable for active targeting to the tumor vicinity. These have the embedded RGD tripeptide sequence in its chain, which has the affinity toward the integrin receptors on the cancer cell membrane. An injectable and self-healing hydrogel with collagen can be electrostatically coupled with AuNPs to potentiate photothermal therapy. In addition, the in vivo study carried out using a photosensitizer, TMPyP, incorporated into the matrix exhibited 72 hours of retention in the tumor site, whereas there was complete clearance observed in the only solution controls. The formulation can thus inevitably aid in coupling PPT and PDT for multimodality treatment (Xing et al., 2016).

Similarly, the linear anionic polymer, hyaluronic acid, is widely integrated with potential nanosystems owing to its inherent nature for tumor targeting projecting it as a natural ligand for the CD44 receptors, which is overexpressed in many cancer types. Hypoxic regions in tumor can be a serious hindrance for various treatment modalities specially, PDT, radiotherapy, and chemotherapy, and result in therapeutic failure. Realizing this, Phua et al. encapsulated catalase enzyme by covalently conjugating it in hyaluronic-acid-based nanoparticle. Here, the nanosystem accumulated in the tumor, post i.v. injection, converted endogenous H_2O_2 to O_2 and therefore relieved it from hypoxia for facilitating photodynamic therapy. HA has not just aided in the tumor targeting but also played role in stabilizing the enzyme amidst the body proteases (Phua et al., 2019). However, considering the low mechanical integrity of HA, several strategies have been formulated for its improvement. As a paradigmatic approach, Aviv et al. integrated it with FmocFF peptide and formulated a composite hydrogel, which demonstrated sustained release of encapsulated curcumin (Aviv et al., 2018). Active targeting of hyaluronic-acid-based nanoparticles can overcome drug-resistance-induced treatment failure (Li et al., 2019).

Biopolymers are very versatile and can be tailored to effectuate controlled or sustained drug release into the system. For such methodology, stimuli responsive smart

biopolymers are employed, which disintegrates in response to a change in pH, temperature, redox, light, or ionic strength. Gou et al. employed silk-fibroin to develop multiresponsive nanoparticles for the mitigation of ulcerative colitis by stimulatory release of the anti-inflammatory drug curcumin. Here, the author took advantage of the inherent characteristics of silk fibroin to get cleaved by GSH and respond to ROS in the host system. In addition, the FDA-approved silk fibroin also exhibits pH-triggered drug release, all together which demonstrated a controlled release and macrophage targeting efficiency (Gou et al., 2019).

Extensive research in this facet has unfolded several criteria, determining the pivotal contributing factors such as particle size, dispersity, surface charge, functionalization, and so on which is marked fundamental in achieving a sophisticated and potential nanocarrier. Kuna et al. intricately studied the influence of molecular size on the biodistribution and pharmacokinetics of elastin-like polypeptide as a drug carrier (Kuna et al., 2018). In addition to the experimental evidences, computational studies can elicit in-depth understanding of the vital interactions necessary for the formation of a stable drug nanocarrier with high drug-loading capacity. Emphasizing this approach, Rahbar et al. displayed with B3LYP and M06-2X density functional studies that with more hydrogen bonds formation between chitosan nanoparticles and the FU drug, the conjugation is more energetically suitable and render stable configuration, which makes chitosan NP a suitable drug carrier (Rahbar et al., 2020).

Albumin has the ability to penetrate vascular walls via gp60 receptor-mediated transcytosis in the endothelial cells. This effectuates the nano-sized albumin formulations to accumulate in tumor via EPR effect. Albumin is greatly used as a stabilizing agent by providing an anionic layer over the nanoparticle surface. It can be used as a reducing agent to synthesize gold nanoparticles as an alternative to citrate-covered AuNPs. Albumin is also shown to self-assemble to form nanoparticles that encapsulated ICG dye and a PTT agent. Very recently, An et al. self-assembled BSA with ICG dye to construct a novel phototheranostic agent that elicits enhanced imaging-guided PTT (An et al., 2020).

7.4.1.2 Cancer Diagnosis and Molecular Imaging

A facile approach to discern the efficacy of the treatment is to simultaneously or sequentially monitor the molecular responses in a real time, which is also imperative in mediating a successful personalized therapy. Nanoparticles made of natural polysaccharides and polypeptides cloak the toxicity and stability issues of the paired synthetic polymer or the incorporated metallic counterparts to maneuver a hybrid nanosystem. Amphiphilic nanocarrier can encapsulate or conjugate both hydrophobic and hydrophilic drugs and can tag the imaging biomolecule, which could be either quantum dots, iron NPs as an MRI contrast agent, AuNPs as a CT imaging agent, near-infrared fluorescent dyes, for instance, IR820, which serves the purpose of being both therapeutic and diagnostic, and

many more. Moreover, molecular imaging briefs us with the pharmacokinetics and pharmacodynamics of the drug being delivered to the tumor, noninvasively. Here, we will illuminate on how natural biopolymers in many dimensions have played crucial role in the consummation of these interventions.

Yang et al. meticulously designed SMID (SF@MnO_2/ICG/DOX) nanoparticles, which leveraged dual-model imaging-guided tri-modal combinatorial PDT/PTT/chemotherapy (Figure 7.10). FDA-approved silk fibroin (SF) was mineralized with MnO2 NPs, and clinically approved ICG for dual-role (photosensitizer and PTT adjuvant) and Dox were loaded through interaction with its amino acids and active carboxyl groups, respectively. Encapsulated MnO_2 NPs relieved tumor hypoxia for PDT by catalyzing endogenous H_2O_2 and also by getting decomposed into Mn^{2+} and O_2 at tumor acidic pH. Furthermore, the Mn^{2+} produced during the process elicited as a contrast agent for T_1-weighted MRI. In vivo biodistribution study of the contrived SMID NPs with ICG-mediated fluorescence imaging traced the enhanced accumulation of the NPs at 24 hours post i.v. injection. Therefore, in accordance with the enhanced bioavailability, a remarkable combinatorial effect was effectuated upon laser irradiation with a significant 89.6% tumor regression with no dramatic weight loss (Yang et al., 2019). Study by Liang et al. exemplifies the multimodal imaging by synthesizing HA-based nanoparticle to achieve hyaluronidase enzyme-activated enhanced fluorescence of encapsulated Cy5.5 NIR dye (Figure 7.11). Here, carboxylic group on HA backbone was activated and made to react with NH_2, which could further endow conjugation with -COOH group in IR825 dye molecule. The obtained IR825-HA was able to spontaneously self-assemble into nanoparticles endorsed with the CT contrast agent, PFOB. This is attributed to the strong hydrophobic interaction between IR825 and perfluorooctylbromide (PFOB). This conscientious designing potentiated photoacoustic imaging, fluorescence, and CT imaging-guided photothermal treatment to ablate cancer in an efficient manner (Liang et al., 2017).

7.4.2 Biopolymeric-based Biosensor

In accordance with the global emphasis projected toward the detection of biomolecules for the diagnosis, it has instigated the development of novel biosensors in concoction with potential biopolymers. Biosensing represents the miniaturized nanoscale process of recognizing these critical responses and the generation of the electrical or optical signal at the biosensor interface, which is transduced and analyzed. A highly sensitive and strong interaction of the biosensor with its biological analyte is rendered by the cohesive and synergistic interplay of the inorganic and organic hybrid nanometric scale materials. Conducting polymers and DNA biopolymers have gathered attention for biosensing applications with its low optical loss and high electrical conductivity. Biopolymers with electron donating capability such as chitosan, which has amino

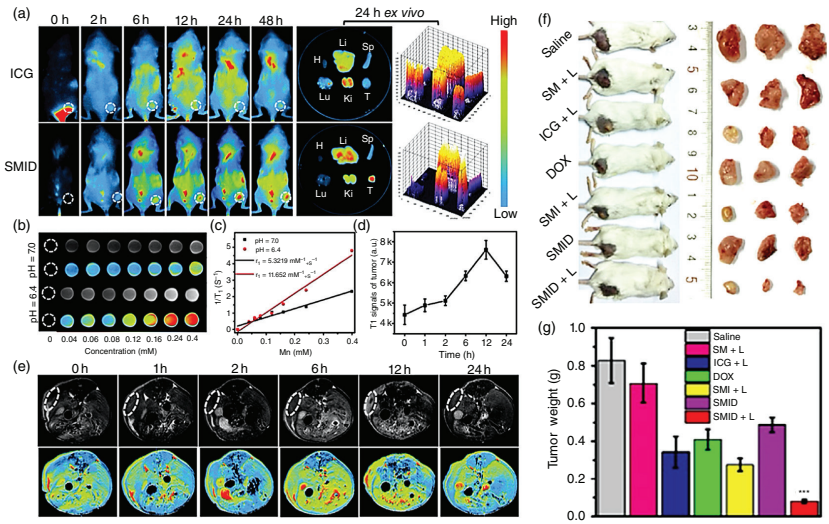

Figure 7.10 (a) Biodistribution of SMID NPs in vivo: i.v. injection of ICG or SMID nanoparticles at different time points, and ex vivo fluorescence imaging and corresponding optical intensity of tumor and major organs dissected at 24-hour postinjection; (b) MR images of SMID NPs dispersions incubated for 12 hour under different pH values; (c) T_1 relaxation rates as a function of SMID NP concentration; (d) quantified MR signals in tumor region after i.v. injection of NPs corresponding to images in e); (e) MR images of tumor bearing mice before and after i.v. injection of SMID NPs; (f) photographs of treated mice and the corresponding tumors excised at day 14 postinjection; (g) average weight of dissected tumors at day 14 (Yang et al., 2019).

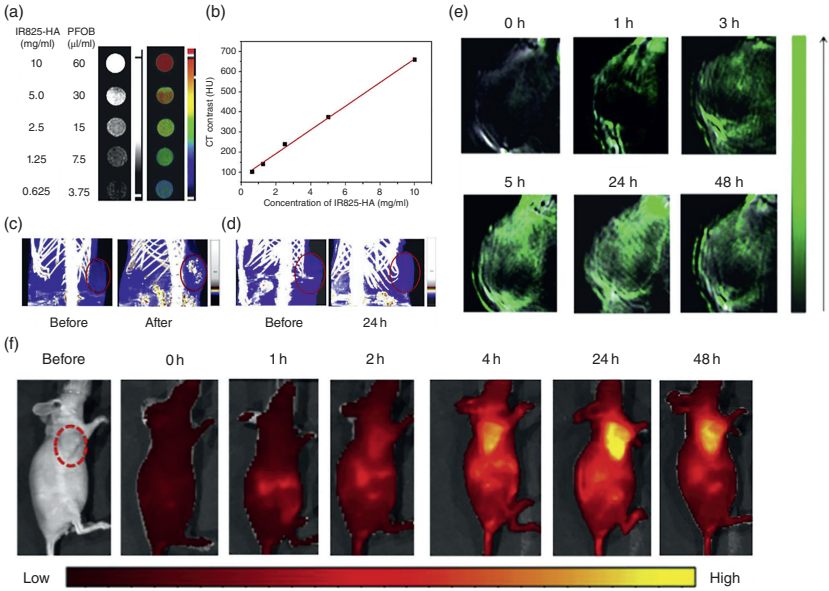

Figure 7.11 (a) In vitro phantom CT contrast images of PFOB@IR825-HA-Cy5.5 NPs at different concentrations, represented by IR825-HA. (b) HU values as a function of its concentration. (c) CT imaging of mice before and after i.t. injection. (d) CT imaging of mice before and 24 hour i.v. injection. (e) In vivo photoacoustic imaging of tumor at different time points after i.v. injection of NPs. (f) In vivo NIR fluorescence imaging and biodistribution of NPs in tumor bearing mice (Liang et al., 2017).

and hydroxyl groups as the electron donor, are particularly used for sensing purpose and for embedding or immobilizing antibodies, metallic NPs, enzymes, DNA, etc. Pathological diseases like cancer can be a lot bewildering, and with the unprecedented response to a given treatment, it is very crucial to unravel the mystifying signals in order to formulate a refined, yet powerful targeting platform. Suresh et al. immobilized anti-PSA antibodies onto the composite film of chitosan and AuNPs to develop electrochemical immunosensor for prostate cancer detection. Several biopolymer hydrogels are contrived for embedding the biorecognition moiety to protect it from harsh environments such as pH and temperature or to render it biocompatible to evade immune surveillance (Suresh et al., 2018).

DNA biosensors include DNA tetrahedron, nanohydrogel, and origami scaffolds, which participates in the molecule recognition process. Signal output from an optical biosensor is based on either fluorescence, absorbance, luminescence, or SPR. Recently, Lin et al. utilized a thrombin-recognizing aptamer and functionalized it on magnetic alginate-based hydrogel for the selective detection of thrombin from the complex mixture of proteins. The signal from the captured thrombin was then amplified by a chemiluminescence-based biosensor. Several DNA nanostructure-based electrochemical biosensor can help in the quantification of proteins or RNA that aligns itself with the cancer progression. DNA-based biopolymers show enhanced mechanical stability and biocompatibility (Lin et al., 2020). Liu et al. generated a platform with hybrid DNA hydrogel for an electrochemical-based biosensor for the detection of lung cancer-based biomarker, miR-21(Liu et al., 2018b). Meng et al. utilized fluorescence resonance energy transfer (FRET)-based strategies for the fabrication of DNA-tetrahedron-based ratiometric biosensor for monitoring telomerase activity at the single cellular level (Meng et al., 2019). With the overwhelming diabetic patients every year, researchers are urged to develop a simple and cost-effective, noninvasive glucose biosensor. As a paradigm, Tracey et al. designed a noninvasive, self-indicating, thin film-based glucose biosensor based on rapid color change upon made in contact with sweat or saliva. This was fabricated by using cellulose nanocrystals as the matrix for immobilizing magnetic nanoparticles and the glucose oxidase enzyme. The biosensor was able to detect as low as 5 mM of glucose molecules (Tracey et al., 2020). Several promising bio-based biopolymeric-based environmental-friendly, rapid detection, affordable biosensors can be formulated in various biomedical applications.

7.4.3 Wound Healing

Millions of cases with skin trauma and wounds by burns or accidents are reported every single year globally, which demands for innovative and cost-effective approaches for the affected individuals. Where small wounds repair themselves in a well-regulatory manner, other nonhealing wounds and diabetic wounds are resilient and need externally manifested support for the healing process.

Potential clinical interventions are underway, which provides hydrogel or scaffold constructs, which can be tailored with sustained release of specific growth factor to guide the cells, which potentiates chemical cues and signaling or immunomodulatory molecule such as IL-4 to recruit macrophage to substantiate the healing process. Recently, immense focus is generated toward the fabrication of vascularized skin constructs and its structural framework. Natural biopolymers such as silk fibroin (SF) displays inherent wound healing properties mediated by the NF-KB signaling, and other varieties of silk is known to promote cell interactions. Hydrated scaffolds are an important parameter for mimicking ECM for which collagen-based constructs are employed. Collagen dressings promote re-epithelialization of the dermal wounds and also is known to be impermeable to viruses and bacteria.

Another attractive biopolymer with wound healing property is biocompatible and biodegradable starch, which is also available plentiful in nature. Though it is very challenging to electrospun starch into nanofibers scaffold, this is achieved by incorporating semicrystalline, nontoxic polyvinyl alcohol (PVOH) biopolymer as plasticizer to reduce the repulsive force in starch and make it amenable for electrospinning. The resultant starch nanofiber scaffold demonstrated biocompatibility and promoted cellular proliferation of L929 fibroblast cells (Teixeira et al., 2020). Another potential biopolymer is chitosan, which has been shown to accelerate the wound healing process along with the induction of epithelialization and tissue formation in preclinical studies. Reports have also shown that chitosan possesses inherent antimicrobial properties. Charged sulfate functional groups on the surface of carrageenan tend to get deprotonated at the physiological pH, which results in electrostatic repulsion in the hydrogel and access to water molecules. This stimuli-responsive behavior necessitates the swelling of the hydrogel and necessitates improved drug release kinetics. Sharaf et al. prepared a hydrogel-based drug delivery by mixing carrageenan with cyclodextrin. Cyclodextrin with its inner hydrophobic cavity aids in the inclusion of hydrophobic drugs, where in this case, the author loaded it with honey bee propolis extract to effectuate wound dressing (Sharaf & El-Naggar, 2019). Alginate hydrogel-based wound dressings alleviates the bacterial burden, which is a plausible hindrance in the wound healing (Cao et al., 2020) and provides moist environment as a relief to the skin burns and accelerates the healing process. Several innovations in wound care have been put forth with potential biopolymers such as hyaluronic acid (Makvandi et al., 2019) and cellulose (Huang et al., 2018) (Figure 7.12).

7.4.4 Tissue Engineering and Regenerative Medicine

First described in 1984 by Eugene Bell, "tissue engineering" term has stemmed as a potential therapeutic intervention as an alternative strategy to the standard autograft and allograft for the alarming need in organ transplantation. With the

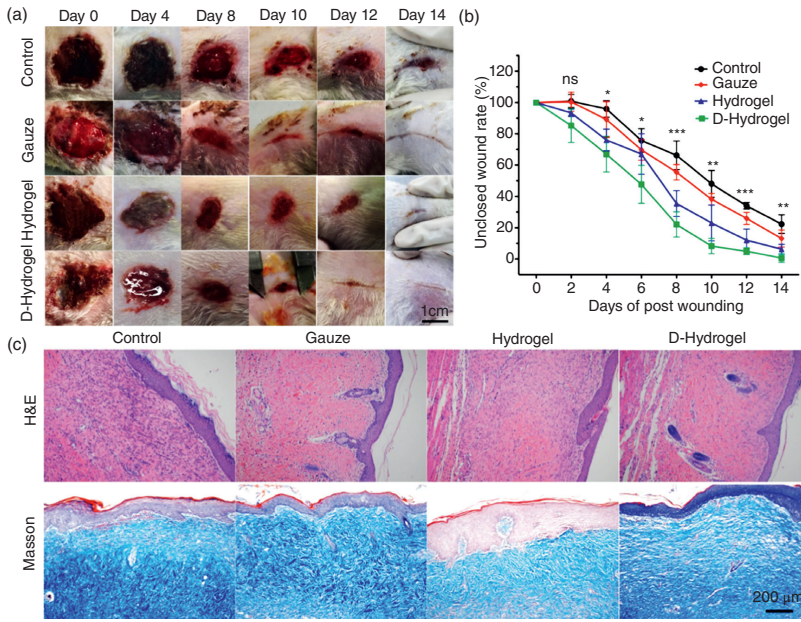

Figure 7.12 Wound healing process: (a) images of a representative wound site from each group taken on post-injury days; (b) unclosed wound area rate of an initial wound untreated or treated with petrolatum gauze, hydrogel, and hydrogel with glycine; (c) H&E staining and Masson's trichome staining of wounds at day 14 (Huang et al., 2018).

escalating incidences of tissue damage due to trauma, disease, or ageing, tissue engineering in concoction with various potential natural biopolymers has predominantly and substantially contributed in the fabrication of cell-laden tissue constructs to recapitulate the normal function by the effective replacement and restoration of damaged cells, tissues, or organs. Notably, this emerging multidisciplinary field pairs the principles of engineering and life science to conceive an artificial, yet a biodegradable scaffold that parallels the architecture of ECM. Formulating the intricate three-dimensional cell-laden scaffolds or matrices centralizes this biomaterial research that aims to encapsulate the pivotal features such as porosity, biodegradability, biocompatibility, evade immune response, mechanical stability, allow nutrient diffusion, permit cell interaction, adhesion, and proliferation. Polymeric porous scaffold, nanofibrous scaffold, and hydrogel-based scaffold have been contrived with various techniques and employed to regenerate impaired bone, cartilage, skeletal muscle, skin, vascular, neural tissue, etc.

7.4.4.1 Biopolymers as Bioink for 3D Scaffolds

Bioink and cells lays the foundation for scaffold with the optional inclusion of various bioactive molecules. Various naturally derived biopolymers are printed or patterned as a single stand-alone agent, but more often are used as blends with one or more components that synergistically work brilliantly together to execute the integrity of the host ECM. This is also exercised so that a limitation that pertains with one material can be overlooked by the addition of other to bring perfection. A closer look at ECM reveals collagen as the abundant protein in several tissues, which makes it as an ideal bioink for the fabrication of soft and hard tissue constructs. Collagen is bestowed with RGD peptides in its structure, which leverages cell adhesion and MMP degradable sequences. Decellularized extracellular matrix (dECM) is employed as a bioink, which can be obtained from discarded organs. However, its mechanical strength is compromised, and therefore collagen is cross-linked with dECM and used as a biocomposite for scaffold generation. Collagens' low-viscous nature tags it with limitation for an ideal printable bioink, but improvisation has been done with tunable concentration. Collagen-based bioinks are now made commercially available such as LifeInk 200.

7.4.4.2 Corneal Regeneration

Vision associates us to the world to comprehend the nature for its colossal and astounding resources that we are provided with. However, people can get succumbed to vision impairment due to corneal ulceration, trauma, aging, hereditary, or due to any causative infection. Soft tissue-engineered corneas has stepped

up as an ailment for the 10 million people affected every year with corneal-related blindness and where keratoplasty is inefficient to curb the daunting demand due to severe donor shortage. For this purpose, natural biopolymers such as collagen, gelatin (Tonsomboon & Oyen, 2013), elastin, and so on have been utilized for the contrivance of soft tissue scaffolds. On the greener side where collagen is palpably the preferred choice, its mechanical stability needs consideration prior clinical applications. To exert mechanical toughness into collagen, Long et al. reinforced it with silk fibers that certifies extraordinary strength to beget a composite scaffold for corneal tissue. The CS10 composite displayed increased water content, which was dependent on the amount of silk reinforced, and 10% silk content yielded optimal mechanical properties (Long et al., 2015). Very recently, bacterial cellulose and cellulose nanocrystal-collagen-based scaffolds have been fabricated and studied for corneal tissue engineering (Qin et al., 2020). Zhang et al. probed into evaluating biocompatibility of bacterial cellulose and implanted it in the cornea of rabbit. The in vitro study revealed 100% cell survival rate, and its complementing in vivo assessment showed no inflammation and maintained transparency during the course of 3 months, which scored it as a promising bioink for corneal scaffold (Zhang et al., 2020).

7.4.4.3 Neural Tissue Engineering

Neural tissues form another intrinsically low self-healing properties for which currently autograft is employed as a standard treatment for the restoration of damaged peripheral nerves. Emergence of neural tissue engineering and implicated strategies has intrigued researchers to make it as a potential long-term cure in this arena. Nerve scaffolds fabricated to closely resemble the native ECM, and providing structural and biochemical signals to direct nerve regeneration has been investigated. In addition, it should entail neurocompatibility and elastic properties to propel mechanical attributes. Another pivotal criterion is the electroconductivity of the scaffold to aid the coordination of the body activity.

Adipose-derived stem cells (ADSCs) possess therapeutic potential with the capacity to differentiate into neural lineage cells and secretion of neurotropic factors. With the advent of neurogenic and neurotube coming portraying success in the clinics limits itself to not more than 3 cm of the length of nerve fiber. Remodeling of PNS nerves though seems possible, on the contrary, regeneration of CNS is challenging owing to its complex and inhospitable environment. Moreover, the blood–brain barrier restricts the passage of conventional treatments with oral or intravenous administration of antibodies and bioactive growth factors. Bioprinting with the combination of conducting biopolymers and carbon-based nanomaterials have been utilized as bioinks for the bioprinting process.

7.4.4.4 Bone Tissue Engineering

Various unprecedented causes can lead to bone defects in which research in bone tissue engineering has put forth potential remodeling and regeneration in a most efficient manner. Where autologous bone grafting is the most commonly employed and considered as gold standard treatment method, it fails to meet with the increasing demand and also associates with several drawbacks. Bone is highly vascularized and serves in homeostasis of minerals with the storage of calcium and phosphate in it and is released into the blood as and when required. Emphasizing the rapidly developing, modernized regenerative technology, ideal scaffolds have been designed and engineered to stimulate osteoconductivity, osteoinductivity, osteogenesis, and osteointegration (Wu et al., 2019c). Biodegradable polymers utilized include both natural and synthetic categories with adipose-derived stroll cells (ADSCs) and mesenchymal stem cells (MSCs) as the main cell source for the differentiation into different lineages. Duymaz et al. developed 3D scaffold with PCL, gelatin, and low-molecular-weight Halomonas levan, which displayed increased biocompatibility to human osteoblast cells (Duymaz et al., 2019). Researchers delve into achieving an ideal bioink, which sets as the deciding factor for the printing of a successful scaffold. Recently, Chimene et al. formulated nanoengineered ionic covalent entanglement (NICE) bioink with enhanced mechanical properties and high printability with 7.5% GelMA, 1% K-CA, and 2% nSi composition (Figure 7.13). Nanosilicates (nSi) induced osteogenic properties supporting the differentiation of hMSCs into osteoblasts without any incorporation of osteoinductive materials such as BMP2. Presence of charge over the nSi promotes non-covalent electrostatic bonds with GelMA and K-CA polymer. NICE scaffolds supported the cell-induced remodeling, observed after 60 days with the mineralization of the scaffolds as examined by the SEM-energy dispersive spectroscopy (EDS) technique (Chimene et al., 2018).

7.4.4.5 Cartilage Tissue Regeneration

Accident or pathological condition such as osteoarthritis can lead to cartilage lose. Cartilage forms lining in the joints, which is made up of type II collagen and aggrecans and further interacts with synovial fluids that lubricates and cushions the bodyweight during movement. Vascularization makes the tissue accessible to the nutrients or circulating progenitor cells, which is crucial for the restoration or repair of the damaged tissues. Lack of vascularization in certain tissues such as cartilage makes it difficult undergo self-healing process upon its degradation caused by either trauma, disease, or aging. Regeneration of cartilage to rectify the defects has been done by usage of chondrocytes and MSCs and with the inclusion of hydrogels in the biomaterial for improving the lubrication. MSCs have the potential to differentiate into variety of cell types including chondrocytes, which

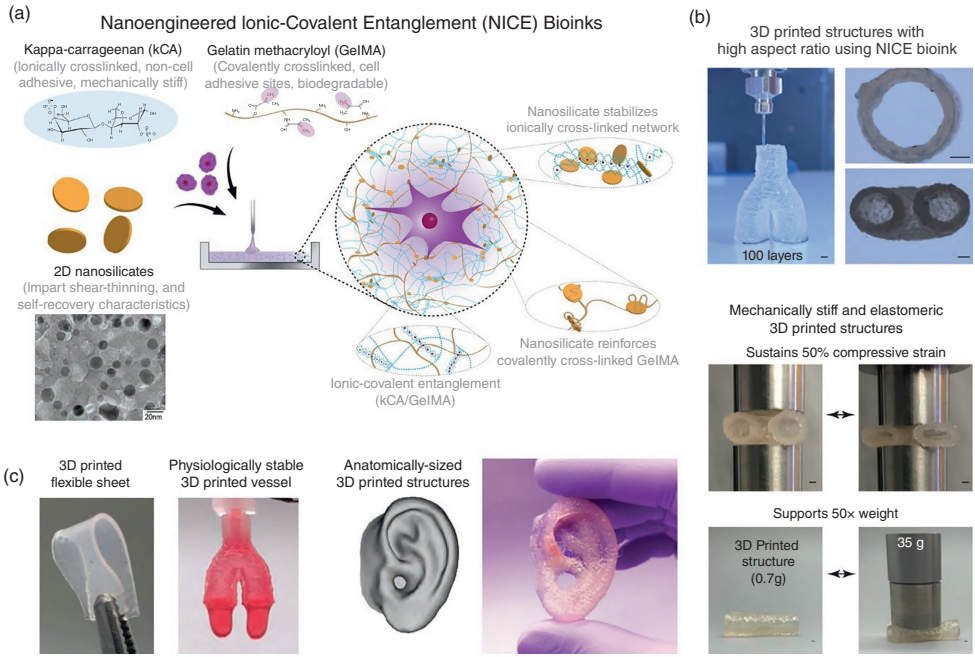

Figure 7.13 (a) NICE bioinks use nanosilicates to reinforce an ionic-covalent entanglement hydrogel made from GelMA and kCa, creating a dually reinforced hydrogel network; (b) NICE bioinks print freestanding hydrogel structures with a high aspect ratios and high print fidelity (scale bar = 1 mm); (c) 3D printed structures from NICE bioink are mechanically (film) and physiological (bifurcated vessel) stable, and have high structural fidelity (3D printed ear) (Chimene et al., 2018).

is endorsed with the application of the soft materials. In another strategy, combination of alginate, gellan, type II collagen have been prepared as a biocompatible construct with enhanced chondrocyte proliferation. Zhu et al. fabricated cell laden tissue construct with PEG diacrylate and gelatin methacrylate in 5%/10% composition, which exhibited highest cell viability and proliferation rate. To avoid rapid elimination of the TGFb1 growth factor, it is encapsulated in the PLGA-based drug nanocarrier, which displayed sustained release of up to 21 days and elicited chondrogenic differentiation (Zhu et al., 2018).

7.4.5 Biopolymers for Biological Implants

With the advent in biomaterials research, the third-generation implants have evolved that incorporates attributes such as biocompatible, bioactive, biodegradable, and stimulation of cellular responses. Implants have become an escalating necessity to repair the damaged tissues in human body such as bone, cartilage, tendon, or ligaments, and therefore considering its importance, strategies have been implemented to make it more biocompatible with highlighted features such as osseointegration. To enhance the biocompatibility, surface coating of implants with natural biopolymers is carried out. Natural biopolymers such as collagen and silk fibroin films have aided as a nucleating surface in enhancing the bone-implant contact. Nawae et al. synthesized a layer-by-layer self-assembled films from SF, Col, and PDDA and evaluated its performance. Results displayed enhanced cell proliferation, ALP activity, and protein adsorption on the coated dental implants (Nawae et al., 2018). Mg alloys for bone implants are succumbed to corrosion and therefore attempts have been taken to enhance the hydrophobicity of the materials and slow down the inherently fast degradation by coating of a hybrid bilayer made of collagen and chitosan (Córdoba et al., 2018). As a single agent for coating implants, they face drawbacks due to being mechanically weak, and hence they are often used as composites with the integration of another biopolymer. Corneal trauma or disease such as lesion, ulceration, and so on can lead to vision impairment, and polymeric scaffolds or hydrogels have been engineered for artificial corneal constructs. Recently, Goodarzi et al. engineered corneal tissue by cross-linking collagen type I and gelatin to form a hydrogel, which demonstrated lasting cell viability and proliferation (Goodarzi et al., 2019).

Protection of the orthopedic implants from the host immune system and the bacterial infection is a fundamental criterion for a successful surgical process. Wenhao et al. performed an extensive study on the effect of structural conformation of the silk fibroin coating for sustained AgNPs release for an effective long-term antibacterial activity and enhanced osteogenesis. The study revealed that coatings with SF alpha helices formed at pH 9.5 manifested smaller AgNPs formation (20 nm) than beta sheets (28.3 and 41%) and exhibited sustained release at pH 7.

Investigation of the SF-coated Ti6Al4V in vitro and in vivo revealed that alpha-structured coating showed better biocompatibility, led new bone formation, and also bone ingrowth was observed. In addition, post-implantation, the SF-based coating did not cause any adverse immune response (Wenhao et al., 2020).

7.4.6 Biopolymers in Other Applications

In many ways, these incredible biopolymers have been integrated with bio-nanotechnological applications such as in optics, electronics, textiles, food industry, filtration, water clarification, and so on for its flexibility, biocompatibility, tunable properties to harness stimuli responsive biomaterials, sustainability, etc. Surface modulation of silk to incorporate super hydrophobic property is carried out to fabricate self-cleansing silk fabrics in textiles (Cheng et al., 2019). Also, due to being comparatively less expensive, silk-based membranes are utilized as filtration units in air filtration (Gao et al., 2018) and in waste water treatment (Ling et al., 2017). Electronic devices that requires flexibility in its system employs silk-based materials and has sprouted new paradigm for several bio-integrated electronics and bio-photonics for medical applications. K-carrageenan and cellulose blends have been prepared for its potential use as electrolytes in dye-sensitized solar cell (DSSC). This is ascribed to the presence of hydroxyl-rich molecular surface structure, which leverages cross-linking with other polymer electrolytes (Rudhziah et al., 2015). Selection of such trending materials that are found abundant in nature produces cost-effective materials. Chitosan is an extensively investigated natural biopolymer in the facet of optics and electronics (Bonardd et al., 2020). The intrinsic feature of swelling nature upon moisture absorbance is adapted in the fabrication of humidity sensors. Where MIM based on Fabry-Perot resonator highly depends on the insulating layer, chitosan hydrogel in combination with photovoltaic cell is utilized in measuring relative humidity (RH). Jang et al in his study employed chitosan as a sandwich between two Ag layers to fabricate a tunable color filter as RH sensor (Jang et al., 2020).

7.5 Processing Techniques for the Contrivance of Biopolymers

7.5.1 3D Bioprinting

Gathering massive attention, three-dimensional bioprinting initially introduced by Hull and coworkers in 1986 is considered as the advanced and innovative technology in this century. Driven by additive manufacturing techniques, 3D bioprinting utilizes a combination of living cells, biomaterials, and active

biomolecules. This has exclusively impacted wide range of applications in regenerative medicine, tissue engineering, cancer research, drug delivery (Ahmad et al., 2019), implantation, etc. and in the overall biomedical engineering. Biomaterials fabricated with this technique demonstrate high precision and resolution in porous tissue constructs with controlled deposition of polymers, cells, and growth factors. Efficiency of the end bioproduct relies on the bioprinters and the properties of the bioink such as its physical, biological, and mechanical characteristics (Hölzl et al., 2016). Bioink is an integral component to this technique and comprises of the polymer of choice with cells and bioactive molecules. The general working strategy of any bioprinter is through layer-by-layer deposition of bioink with various techniques such as Inkjet or droplet method, stereolithographic, laser-based and extrusion-based bioprinting.

Inkjet bioprinting with high resolution of 50 μm is based on thermal or piezoelectric mechanisms, which uses heat and acoustic waves, respectively, to eject bioink droplets for design patterning. Advantages include high print speeds, low cost, accurate position of multiple cell type, and wide availability (Cui et al., 2012). However, it faces low droplet directionality and unreliable cell encapsulation due to low viscosity, and thereby low concentration of the ink.

Stereolithography utilizes UV or visible light for the maturation/curing of photo-responsive bioinks and fabricates bioprints in short time with high cell viability. In laser based bioprinting, viable cells are dispensed from donor slide to the receiver slide without the assistance of the nozzle, and thereby avoiding the shear stress exerted with nozzle bioprinting (Melchels et al., 2010). It offers fast, accurate bioprinting of polymers with high resolution in the range of 5–300 μm. Photo initiators are employed for the initiation of light-triggered polymerization such as camphorquinone, eosin Y, fluorescein, riboflavin, and so on, which absorb in the visible wavelength and absorbing under UV. It offers advantages such as absence of shear stress and no limitation on bioink viscosity. However, keeping in mind the destructive effects of UV on cells and cytotoxicity levels of various photo initiators, alternate strategic implements are required such as using photoinitiation free bioinks or visible light-absorbing PI (Wang et al., 2015).

In laser-assisted bioprinting (LAB), the projected laser induces bubble nucleation, which vaporizes the hydrogel and propels the droplets toward the substrate onto the donor slide. It offers high resolution between 10 and 50 μm with very high cell viabilities of >95% and able to print multiple cell types with high accuracy (Keriquel et al., 2017). However, currently it suffers scalability and low stability, in addition to being an expensive process. In combination with other biofabrication techniques, it could demonstrate great potential in bioprinting.

Extrusion bioprinting is a pressure-driven technique and utilizes mechanical or pneumatic-driven systems to deposit viable cells in the form of a filaments. It is a hybrid of fluid dispersing system and automated robotic system for extrusion

and bioprinting, respectively (Liu et al., 2017). It shows low resolution compared to other bioprinting techniques and therefore could not fabricate soft tissues, which requires small pore sizes. It uses high viscous bioinks, which could result in shear-stress-induced apoptosis of printed cells (Panwar & Tan, 2016). Also, this could impact the mechanical properties and fidelity of the print due to the distortion of the interconnecting bonds in the hydrogel. This can be overcome by adopting self-healing hydrogels, which restores its shape with non-covalent reversible bonds.

7.5.2 4D Bioprinting

The limitation observed with the inanimate and static properties of 3D bioprinting is overcome by the recently emerged 4D bioprinting technology introduced by Skylar Tibbits in 2014, which integrates time as its fourth dimension. It attempts to mimic the natural tissue regeneration process with the application of time, which allows the printed constructs to change its shape during the post-printing processes for maturation. This change in shape and functionalities during the course of time is defined by Gao et al. as the two fundamental strategies in the 4D technology. Responsive smart biopolymers are used as its bioink, which has the inherent ability to get transformed when receiving an external stimulus. Water is the most commonly used stimuli eliciting dynamic conformations in a traditional 3D bioprinting architecture as a function of time. 4D printed hydrogel constructs were observed to evolve and attain conformational changes when immersed in water (Gao et al., 2016). This fascinating technology is gaining huge popularity with its ability to innervate complex and functional structures as a next-generation bioprinting and for personalized tissue regeneration platform. Its potential strategic interventions have been explored exclusively in neuron tissue engineering, bone tissue engineering, biomedical device, etc.

Several thermos-responsive injectable hydrogels have played major role in the engineering of bone tissues. For instance, Wu et al. utilized chitosan/silk fibroin/glycerophosphate composites incorporated with copper-containing bioactive glass nanoparticles for cell-free bone repair. The composition undergoes rapid sol–gel transformation at physiological temperature. Subsequently, the Si, Ca, and Cu ions released from the gel in in vitro study showed synergistic regulation of the expression of genes responsible for angiogenesis and osteogenesis. In vivo assessment of calvaria bone defects in mice demonstrated successful repair after eight weeks of treatment (Wu et al., 2019c). In another example, Kirillova et al. adopted advanced 4D technique to biofabricate hollow self-folding hydrogel-based tubes using shape-morphing methacrylate alginate and hyaluronic acid biopolymers with mouse bone marrow cells printed in them. This proof-of-concept study

displayed cell survivability up to seven days, and the green light cross-linking did not affect its viability (Kirillova et al., 2017). However, this 4D technology is still at its dawn and needs thorough understanding of its mechanisms to overcome the major obstacles associated with it.

7.5.3 Electrospinning

First introduced in the 1930s, electrospinning technique has since gathered huge attention in the biomedical applications and tissue engineering about 50 decades later. Electrospun ultrafine micro to nanopolymeric fibers are fabricated with the application of high electric field. The main mechanism driving this technique is the electrophysical relation between the polymer solution and the electrostatic force. As the surface charge on the polymer droplet at the tip of the needle increases, it changes its conformation to a conical shape. With increasing voltage, polymer jet initiates, which is vaporized to remove the solvent from the solution. A nanofiber patterning is observed as a result of the splitting of the polymer droplet with constant electrostatic force.

In comparison, 3D electrospun scaffolds yield significantly better pore size formation and cell penetration than the conventional 2D electrospun nanofibers. A sacrificial component is commonly added during the electrospinning of the structural element to increase the pore size of the scaffolds. Another technique to increase the pore size is the incorporation of salt crystals such as sodium chloride crystal, which is termed as salt-leaching process. Other potential techniques utilized to fabricate electrospun nanofiber scaffolds are wet electrospinning, cryogenic electrospinning displaying improved cell infiltration, dispersion-shaping, and gas foaming with significantly improved fiber compression. Electrospun fibers can be contrived for both the natural and the synthetic polymeric materials such as chitosan, collagen, gelatin, PCL, PLGA as a single ingredient, or combination of both to produce composite fibrous scaffolds. Parameters that play important role in the electrospinning efficiency of the nanofibers are the inherent properties of the polymer solution such as its concentration, viscosity, molecular weight, surface tension, and depends on the processing conditions (uniform electric field, polymer feed rate, distance between the needle and the collector) and the environmental factors such as humidity and the temperature. Electrospun nanofibers scaffolds have their application widely used in the bone (Ezati et al., 2018), cartilage, vascular, cardiac, nerve, and skin tissue engineering. Surface functionalization of the electrospun nanofibers are performed to improve its surface characteristics. Several strategies are available such as plasma treatment, wet-chemical method, surface-graft polymerization, and co-electrospinning of surface-active agents and polymers.

7.6 Conclusion

The analysis on biopolymers has evidently portrayed its eminence as the building blocks for the foundation of biomedical applications. Biopolymers have unequivocally demonstrated, in many ways, that its exclusion from nanomedicine would be unimaginable. In order to fit perfectly in multifarious applications, it is bestowed with features such as its rich functional groups that can be modulated. Not to mention, the smart, responsive biopolymers that have manifested plethora of remarkable opportunities in therapeutics to give it a new dimension. However, it is also very true that it cannot be a standalone, and that incorporation of its inorganic counterparts could be mandatory. Despite that, bio-based polymers have still effectively managed to minimize the synthetics' degree of inclusion, which is plausibly the ultimate goal to bring in harmony in the environment. In addition to the progress in the facet of biomedical science, we also briefly explored its multidisciplinary applications such as optics, electronics, textile, and so on. Having said that, though the future may look seemingly far when we can visualize the translation of these nanomedicines from bench to bedside, the rays of optimism gets enhanced with time. These nature advanced materials are very promising with affordability and sustainability, and the challenges associated can be tackled with the conjoint efforts from multidisciplinary scientists.

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Metabolic Engineering Strategies to Enhance Microbial Production of Biopolymers

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8.1 Introduction

Biopolymers are polymers produced in nature and are known for great values to humans since ages. These biopolymers are used in food, cosmetics, pharmaceuticals, biomedical, electronics, clothing, and biomaterial applications. However, the natural availability of most of them is limited, extraction cumbersome and costly, and a great chance of batch-to-batch variability during extraction from living sources. Owing to these problems, microbial production of biopolymers has emerged as a method of choice. Microbes can be grown and maintained on suitable media in an artificial growth environment in large-scale fermenter for bulk production of biopolymers. The production is dependent upon various parameters like types of raw material for growth, microbial producer strain, process parameters (pH, temperature, mixing, O_2 transfer), and downstream processing. The production capacity of microbial producer strain is the most important criterion for efficient production. A high yielding producer strain makes the process and economies of large-scale production highly competitive in the market, which is an essential criterion for biopolymers to be treated as commodity. Therefore, considerable efforts have been dedicated to identifying high-producing natural producer strain or engineer microbes using various genetic elements to enhance production.

The traditional approach of strain engineering involves random mutation using a mutagenic agent (UV rays, chemicals) and screening the high producer strain from the mutated microbial species. This approach has been highly successful but

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has its own limitation such as randomness of mutation, laborious, and time-consuming. Changing the flux or the concentrations of a particular metabolite of a metabolic pathway are other approaches which are mostly based on the inhibition or overexpression of the presumed rate-limiting step. Currently, the genetic engineering approach coupled with system metabolic engineering approach is used for efficient modification of pathways in producer strain to increase the production of targeted biopolymer. Now, a question arises what metabolic engineering is? Metabolic engineering is the willful modification of cellular networks including metabolic, gene regulatory, and signaling networks to achieve desirable goals such as enhanced production of metabolites including pharmaceuticals, biofuels, and biochemicals and other biotechnology products (Lee et al. 2011). When computational biology and synthetic biology is combined with metabolic engineering, then it is collectively known as system metabolic engineering. The whole objective is to modify producer strain behavior in terms of utilization of available resources both intracellular and extracellular to minimize wasteful expenditure of cellular energy and generate targeted product in high quantity. Various engineering strategies such as rational metabolic engineering, evolutionary engineering, inverse metabolic engineering, engineering of secretory pathways, and process optimization are implemented to increase productivity (Wuest, Hou, and Lee 2011). Using these strategies, various biopolymers such as polysaccharides (xanthan, pullulans, hyaluronic acids), polyesters (poly hydroxy alkanoates/poly hydroxy butyrate [PHBs]), and polyamides (poly-L-lysine, cyanophycin) have been produced either in metabolically engineered natural-producing strains or recombinant non-natural-producing strains such as *Escherichia coli* and *Sacccharomyces cerevisiae* (Schilling et al. 2020; Yamanaka, Hamano, and Oikawa 2020).

This chapter presents a detailed description of steps from upstream (selection and microbial producer strain development), midstream (production of biopolymer), and downstream (separation and purification). We first review the potential of different microbes for production of biopolymers, followed by biopolymer production pathways, tools, and strategies to modify production pathways, role of dynamic metabolic flux and its importance, production, and purification of biopolymer from metabolic engineered microbes.

8.2 Microbes as Cell Factories for the Production of Speciality Biochemicals

Ancient Asian, Egyptian, Greek, Romans, Chinese, Babylon and North American all knew and used various microorganisms as factories for the production of exotic beverages and bread for human consumptions. Different commonly available raw materials such as grains (barley, rice), fruits (grapes), cactus, and honey were

fermented using microorganisms to produce beer, wine, octii, and mead (Alba-Lois and Segal-Kischinevzky 2010). In modern times, microorganisms are being increasingly used as cell factories for the production of a wide variety of biochemicals and speciality products such as antibiotics, enzymes, vitamins, organic acids, and therapeutic proteins. The emergence of molecular biotechnology and genetic engineering has enabled to design, manipulate, and create microorganism with increased productivity of biochemicals and biomacromolecules (Murphy 2012). Here, microbes are referred to as cell factories because like factories, we are concerned with large-scale biological production. It is an approach to bioengineer and optimize metabolic pathways present in microbes for large-scale production. This approach essentially consists of three elements: (i) design, (ii) build, and (iii) optimize (Chen and Wang 2017). This approach can be used to manipulate metabolic pathway of bacteria, fungi, yeast, and microalgae using genetic engineering approach for large-scale production of fuels, chemicals, food ingredients, pharmaceuticals, and biopolymers. Given the scope of this chapter, the main focus will be how metabolic pathway in microorganism will be altered to increase productivity, strategies involved in large-scale production of biopolymers using metabolically engineered microorganisms and purifications.

8.2.1 Bacteria as Cell Factories for the Production of Biopolymers

Bacteria, both wild type as well as genetically modified, are widely used for large-scale production of biopolymers due to ease of handling, faster growth, highly elucidated biosynthetic pathway, easy genetic manipulation, ability to metabolize a large number of sugars, easy process control, and purification. Synthesis of biopolymers is initiated when the bacteria are placed in stress or starvation condition (lacking important nutrient such as nitrogen or phosphorous or oxygen) with excess carbon content in fermentation medium. Under the starved condition, bacteria accumulate various biopolymers intracellularly to store energy and carbon which helps in maintenance of the osmotic state of cells and prevents leakage of valuable compounds out of cell (Verlinden et al. 2007). Bacteria can be used for large-scale production of biopolymers like polysaccharides, polyamides, polyesters, and polyphosphates.

8.2.1.1 Polysaccharides

Polysaccharides are long-chain polymeric carbohydrates composed of monosaccharide units bound together by glycosidic linkages. The syntheses of polysaccharides are template independent, i.e. mRNA (messenger RNA) is not required for synthesis. Rather, polysaccharide structure and synthesis is dependent upon structure and complement of polysaccharide enzymes present in bacteria. Polysaccharides are widely used in pharmaceutical industries (as a drug carrier, capsules, and coating), food industries (gelling, thickening, and stabilization

agent), petroleum industries (microbial-enhanced oil recovery), and biomaterials and tissue engineering (scaffold, explants, wound dressing). The commercial importance of polysaccharides necessitates adopting the strategies to increase production at minimum cost. Metabolic engineering of the existing pathway is one such strategy toward achieving high productivity of polysaccharides from microbes. Recently, Wang et al. (2020) produced exopolysaccharides (EPS) glucomannan and mannan using lignocellulolytic and thermophilic bacterium *Geobacillus* sp. *WSUCF1* (Wang, Salem, and Sani 2020). *Halomonas* sp. AAD6 was able to produce levan-type exopolysacchides from pretreated molasses waste. Similarly, *Xanthomonas campestris*, *Leuconostoc mesenteroides*, and *Acetobacter xylinum* are used for production of xanthan, dextran, and cellulose, respectively. Hyaluronic acid or hyaluronan is other polysaccharides produced from *Streptococcus zooepidemicus*, *Bacillus subtilis, and Lactococcus lactis*. Further, hasA *gene* from *Streptococcus pyogens* were cloned in heterologous bacteria such as *E. coli* and *Enterococcus faecalis* for increasing the production of hyaluronic acid (Liu et al. 2011; Sze et al. 2016). Alginates are a family of linear, non-repeating block copolymers consisting of variable ratios of β-D-mannuronic acid (M) and its C5 epimer α-L-guluronic acid (G) linked by β-(1,4)-glycosidic bonds. It is produced on an industrial scale by bacteria *Pseudomonas* and *Azobacter* genera. Chitosan is a linear polysaccharide of natural origin composed essentially of *β*-(1,4)-linked glucosamine units (2-amino-2-deoxy-*β*-D-glucopyranose) together with some proportion of *N*-acetylglucosamine units (2-acetamino-2-deoxy-*β*-D-gl ucopyranose). At the industrial scale, the main sources of chitin are the discarded remnants from crustacean (shrimp, prawn, crab, and lobster) processing plants which are subjected to deacetylation for chitosan production. Fermentative production of chitosan is therefore carried out by recombinant bacteria like *Escherichia coli and Bacillus subtilis* (Anderson, Islam, and Prather et al. 2018). Elakkiya et al. (2020) used *Pseudomonas aeruginosa* TEN01 for the production of bacterial biosurfactant rahamanolipid for microbial-enhanced oil recovery (Elakkiya et al. 2020).

8.2.1.2 Polyesters

Polyesters are a category of polymers that contain the ester functional group in their main chain. Synthetically, it is made by reacting alcohol with acid but biologically it is produced by bacteria in response to environmental stress condition. Polyhydroxy alkanoates (PHA), polyhydroxy butyrate (PHBs), and polymalic acids are three polyesters which are currently produced by bacteria. PHAs are polyesters of 3-, 4-, 5-, and 6-hydroxyalkanoic acids and can be subdivided into short-chain length PHAs (scl-PHA) containing up to c5 monomers, medium-chain length (mcl-PHA) with c6–c14, and more than c14 long-chain length (lcl-PHA) PHas.6 PHB is the most common type of scl-PHA and this homopolymer of 3-hydroxybutyric acid has been studied most extensively; as well, copolymers of

PHA can be formed containing 3-hydroxybutyrate (3HB), 3-hydroxyvalerate (HV), 3-hydroxyhexanoate (HHx), or 4-hydroxybutyrate (4HB) monomers. Most of the microbes synthesize either scl-PHAs containing primarily 3HB units or mclPHAs containing 3-hydroxyoctanoate or 3-hydroxydecanoate as the major monomer (Gholami et al. 2016). PHAs are synthesized intracellularly as storage granules by bacteria under stressed condition, particularly nitrogen or phosphorous limited condition with excess carbon content of fermentation medium. *Cupriavidus necator* (formerly known as *Ralstonia eutropha)*, *Wautersia eutropha*, or *Alcaligenes eutrophus, Haloferax mediterranei, Alcaligenes latus* are commonly used bacteria for industrial production (Amaro et al. 2019). However, some bacteria such as *Alcaligenes vinelandii*, *A. latus, Pseudomonas putida, P. aeruginosa* 47T2 are able to accumulate PHAs under non-limiting conditions. Recently, Rodriguez et al. (2020) produced poly hydroxybutyrate in a bubble column setting using *Methylocystis hirsute* using Gram-negative, aerobic, and methanotroph bacterium species. Gram-negative bacteria *R. eutropha* have already been commercially utilized for the production of poly [(3-hydroxybutyrate)-co-(3-hydroxyv alerate)] which is marketed as trade name Biopol. A high-yielding recombinant strain of *E. coli* strain with *Cupriavidus necator* PHA biosynthesis genes has been constructed for higher productivity of PHAs (Li, Zhang, and Qi, 2007).

8.2.1.3 Polyamides

Polyamides are composed of a large number of polymer compounds linked by amide bonds. The polyamides are divided into the homo polyamides (consisting of one type of monomers) and the copolyamides which are composed of different kinds of constituents (Bankar et al. 2018). Bacteria are able to synthesize various polyamides such as polypeptides, ε-poly-L-lysine, and poly-γ-glutamate (PGA) and multi-L-arginyl-poly (L-aspartic acid) which is also referred to as cyanophycin by enzymatic processes independently from ribosomal protein biosynthesis. These polyamides occur mainly in *Bacillus* spp., *Streptomyces albulus,* or in cyanobacteria (Oppermann-Sanio and Steinbüchel 2002). ε-Poly-L-lysine is a homo-poly-amino acid characterized by the peptide bond between the carboxyl and epsilon-amino groups of L-lysine. ε-Poly-L-lysine shows a wide range of antimicrobial activity and is stable at high temperatures and under both acidic and alkaline conditions. ε-Poly-L-lysine is a secondary metabolite produced by *S. albulus* NBRC14147 during the stationary phase of fermentation (Yamanaka et al. 2010). Recently, Chen et al. (2020) produced short-chain length ε-Poly-L-lysine (PL) from glycerol with the help of *S.* albulus M-Z18. In an attempt to create high-producing bacterial strain, Xu et al. (2019) constructed recombinant strain by cloning ε-Poly-L-lysine synthase gene from *Kitasatospora aureofaciens* into *S. albulus* strain. The recombinant strain efficiently produced ε-Poly-L-lysine with a molecular weight of 1.3–2.3 kDa and yielded of 23.6 g/L following fed-batch fermentation in a 5 L bioreactor. Overexpression of gene and high efficiency of nitrogen translocation

and utilization have been reported to enhance PL production. Xu et al. (2019) constructed a recombinant strain which overexpresses the ammonium transporter gene *amtB* in *S. albulus* PD-1. The overexpression of atmB ensures adequate supply of nitrogen during ε-Poly-L-lysine synthesis (Xu et al. 2019).

Cyanophycin, another polyamide biopolymer, comprising a poly-L-aspartic backbone with arginine (Arg) side chains attached *via* their amino groups to the carboxyl group of each aspartate (Asp) are produced by heterotrophic wild-type bacteria like *Acinetobacter* sp., *Bordetella bronchiseptica, Clostridium botulinum,* and *Desulfitobacterium hafniense*. Recombinant strains are also employed with heterologous expression of diverse cyanobacterial cyanophycin synthetases (CphAs) genes in *E. coli*, *P. putida*, *R. eutropha, Rhizopus oryzae*, and *S. cerevisiae* for higher yield in fermentation process (Du, Li, and Zhou 2019).

Poly-γ-glutamic acid (γ-PGA) is an anionic homopolyamide which consists of D- and L-glutamic acid units connected through amide linkages between α-amino and γ-carboxylic acid groups. γ-PGA can be synthesized by *Bacillus* species, *Fusobacterium nucleatum*, and some archaea and eukaryotes but *Bacillus* species are used most widely to study biological γ-PGA production. Bacteria are either L-glutamate-dependent (*B. subtilis* CGMCC 0833 and *Bacillus licheniformis* P-104) or non-L-glutamate-dependent (e.g. *B. subtilis* C1 and *Bacillus amyloliquefaciens* LL3) producers of γ-PGA. Despite the lower γ-PGA productivity, glutamate-independent producers are preferred due to the low cost of production and simple fermentation process (Luo et al. 2016). Recently, Chettri et al. (2016) isolated γ-PGA-producing *B. subtilis, B. licheniformis,* and *Bacillus sonorensis* from fermented food product *kinema* (Chettri, Bhutia, and Tamang 2016).

8.2.2 Fungus as Cell Factories for the Production of Biopolymers

Filamentous fungi (including microorganisms such as *Aspergillus niger* and *Rhizopus oryzae*) represent an enormously important platform for industrial fermentation due to their high yield coefficients and the ability to secrete products extracellularly. Fungal metabolism is exploited to manufacture ethanol, citric acid, steroids, antibiotics, and other substances with applications in the food, fuel, chemical, and pharmaceutical industries. Yeasts are eukaryotic, single-celled microorganisms classified as members of the fungus kingdom. It is used by human civilizations for thousands of years for production of wine and breads. It is one of the main industrial microorganisms used in the production of biochemicals and expression of heterologous protein expression. Compared to bacterial system, yeast has more natural adaptability to the harsh industrial-scale conditions and an ability to correctly produce and secrete biologically active eukaryotic proteins in correctly folded state. *Saccharomyces cerevisiae*, *Komagataella* sp. (Formerly *Pichia pastoris*), *Kluyveromyces lactis*, and *Yarrowia lipolytica* are some

example of yeasts used for the production of biopolymers and heterologous protein expression on an industrial scale (Kavšček et al. 2015; Viera Gomes et al. 2018).

8.2.2.1 Polysaccharides

Yeast are mostly used for production of yeast cell wall polysaccharides like α- and β-glucans and α-mannan because of their industrial and pharmaceutical properties such as antitumor, antibacterial, and antioxidant properties. However, recombinant strains are used to produce biopolymers in higher quantity. Recently, Farinha et al. (2019) extracted copolymer chitin–glucan complex (CGC) and mannose-containing polymers (mannans) for their antitumor, antibacterial, and antioxidant properties from methylotrophic yeast *Komagataella pastoris* cell wall (Farinha, Araújo, and Freitas 2019). Simlarly, Galinari et al. (2017) isolated cell wall polysaccharides α-D-mannan from yeast *Kluyveromyces marxianus,* which has antioxidant and antiproliferative polymers (Galinari et al. 2017). Levan is an extremely important biopolymer known for its prebiotic properties. It is a Fructans and is a fructose polymer consisting of multiple fructose units joined by β(2 → 6) glycosidic bonds. Owing to its commercial values, a recombinant invertase (Δ*suc2*) null mutant of *S. cerevisiae* was constructed for efficient production (Franken et al. 2013). For Hyaluronic acid production, a recombinant strain of yeast *Pi. pastoris* was constructed by inserting five genes: *HasC*, *glmU* (pyrophosphorilase), *pgI* (phosphoglucoisomerase), *HasA,* and *HasB* under the control of constitutive promoter (de Oliveira et al. 2016). Chitosan, a β1-4–linked glucosamine polymer, is formed by deacetylation of chitin and can be easily produced by fermentation of fungal cell. Various wild-type strains of Zygomycetous fungi are *Absidia coerulea, Benjaminiella poitrasii, Cunninghamella elegans, Gongrenella butleri, Mucor rouxii, Mucor racemosus,* and *Rhizopus oryzae*. Further, isolation of chitosan is reported from few edible basidiomycetous fungi like *Agaricus bisporus, Lentinula edodes, and Pleurotus sajor-caju*. Other fungi that are explored for chitosan production are *A. niger, Penicillium chrysogenum, and S. cerevisiae*. These fungi are grown in bioreactors and harvested to extract chitin/chitosan from their cell wall. *Aureobasidium pullulans,* yeast like fungus, secretes a polysaccharide pullulan, which is commercially a useful hydrocolloid (Lacroix and Le Tien 2005).

8.2.2.2 Polyester

Polyhydroxyalkanoates (PHA), polyhydroxy butyrate (PHB), and polymalic acids are polyesters produced by microorganisms. Production of PHA and PHB is known to be a bacterial phenomenon and not a fungal phenomenon unless and until a recombinant fungal strain is employed for PHA and PHB production. One early report published in 2002 by Carlson and coworkers demonstrated the production of PHB using a recombinant strain of *S. cerevisiae*. This recombinant strain contained three genes poly-β-hydroxybutyrate pathway from *R. eutropha*

(β-ketothiolase [phbA], reductase [phbB], and synthase [phbC] (Carlson, Fell, and Srienc 2002). Polymalic acid (PMA), another microbial polyester can be conveniently produced by yeast-like fungus *Aureobasidium pullulans* (Wang et al. 2016). A recombinant strain of *A. niger* was constructed by abolishing citric acid accumulation and increasing glycolytic flux for enhancing fermentative production of polymalic acid (Xu et al. 2020).

8.2.2.3 Polyamides

Till now, it was considered that polyamides can be produced only by *Bacillus* spp, *S. albulus,* or in cyanobacteria only. Recently, Purev et al. 2020 reported the production of ε-poly-L-lysine by endophytic fungus *Epichloë festucae*. The production was attributed to the presence of *epls gene* for the production of ε-poly-L-lysine (Purev et al. 2020). Wild-type strain of fungus is unable to produce cyanophycin in fermentative medium. Meussen et al. (2012) constructed a recombinant strain of fungus *Rhizopus oryzae* by cloning cyanophycin synthetase encoding gene cphA for heterologous expression of cyanophycin synthetase (Meussen et al. 2012). *Saccharomyces cerevisiae* has been employed as a cell factory for cyanophycin production. Heterologous expression of $cphA_{6308}$ in transgenic *S. cerevisiae* led to the production of soluble and insoluble cyanophycin (Steinle et al. 2008). Besides *S. cerevisiae,* two different strains of transgenic *Pi. pastoris* (strains GS115 and KM71H) expressing the $cphA_{6308}$ gene were employed for cyanophycin production (Du, Li, and Zhou 2019).

8.2.3 Microalgae as Cell Factories for the Production of Biopolymers

Microalgae are unicellular photosynthetic microorganisms, living in saline or freshwater environments, that convert sunlight, water, and carbon dioxide to algal biomass (Ruane, Sonnino, and Agostini 2010). Microalgae are a good source of algal secondary metabolites and have drawn great attention as a promising source for sustainable production of fatty acids, carotenoids, vitamins, and other compounds of interest. These metabolites can be used as antioxidant, antiviral, antibacterial, antifungal, anti-inflammatory, antitumor, and antimalarial effectors. Both genetically modified as well as unmodified microalgae can be used for bioactive production, it is still imperative to genetically modify wild strain for feasible industrial production (Fu et al. 2016; Khan, Shin, and Kim 2018). *Anabaena cylindrica* and *Anabaena variabilis, Chlorella reinhardtii, Porphyridium cruentum*, *Tetraselmis suecica*, *Desmodesmus* sp., *Synechococcus elongates*, *Euglena sanguine, Nannochloropsis*, *Dunaliella*, *Chaetoceros*, *Botryococcus*, *Scenedesmus,* and *Pseudochlorococcum* are some microalgae among the many that are used for the production of high-value microalgal biopolymeric product (Jagadevan et al. 2018). Further, several genera and species of cyanobacteria, such as *Dunaliella tertiolecta*, *Aulosira fertilissima*, *Nostoc muscorum*, *Spirulina subsalsa*, *Synechocystis* sp.,

Spirulina platensis, and *Synechococcus* sp., are used for the production of biopolymers (Martins et al. 2017). Cyanobacteria are emerging as an alternative host system due to their minimal nutrient requirements and photoautotrophic nature due to short generation time and minimal and simple requirements of micro and macronutrients for growth and proliferation (Samantaray and Mallick 2012).

8.2.3.1 Polysaccharides from Microalgae

Polysaccharides are macromolecules based on glycosidically linked combinations of up to 40 different monosaccharides, sometimes substituted by non-sugar groups such as alcohols, organic acids, or sulfates. Microalgae is basically known to produce hetero polysaccharide with the exception of *Gyrodinium impudicum* and *Chlorella vulgaris* that produce homopolymer polysaccharides of galactose and β-(1,3)-glucan, respectively. The microalgae are rich source of polysaccharides and may contain up to 46% of the dry weight (DW) of their cells. These polysaccharides are polymers of galactose, β-(1,3)-glucan, xylose, rhamnose, fucose, fructose, and methyl sugars linked with the help of glycosidic bond. The wild-type strains are basically used to produce polysaccharides that are endogeneous to their cell metabolism whereas transgenic microalgae can be designed to produce polysaccharide of choice. Microalgae is being developed to produce algal polysaccharide because of their industrial, pharmaceutical, and medical applications *Chlorella stigmatophora, C. vulgaris, Dunaliella salina, Rhodella reticulate, cyanobacteria,* and *Arthrospira platensis* are some of the algal species known to produce polysaccharide naturally (Chanda, Merghoub, and EL Arroussi 2019). Gaignard et al. (2018) produced galactoxylan (a EPS) from *Flintiella sanguinaria* – a species of red marine algae. Galactoxylan was composed of xylose (47%, molar ratios), galactose (21%), glucuronic acid (14%), rhamnose (10%), glucose (6%), and arabinose (2%). Depending on microalgae strains, many different monosaccharides can be found, such as galactose, glucose, rhamnose, mannose, arabinose, xylose, galacturonic, and glucuronic acids (Gaignard et al. 2018).

8.2.3.2 Polyester

The microalgae do not produce polyester naturally except the microalgae genera belonging to cyanobacteria. PHA/PHBs are polyesters which can be produced by cyanobacteria. Being secondary metabolites, the growth normally takes place in the stationary phase of cellular growth. Martins et al. (2017) showed that *Cyanobium* sp., *Nostoc ellipsosporum*, *Spirulina* sp. LEB 18, and *Synechococcus nidulans* belonging to cyanobacteria genera were able to produce PHA. *However, N. ellipsosporum* and *Spirulina* sp. LEB 18 were the superior producers with high yield and less fermentation duration (Martins et al. 2017). Samantaray and Mallick (2012) produced PHB using photoautotrophic N_2-fixing cyanobacterium, *Au. fertilissima* (Samantaray and Mallick 2012). Though a lot of research has been dedicated toward increasing fermentative productivity using traditional route but

none of them has yielded the desired result in reducing the cost of fermentation. The advent of modern biotechnological tools has extended capability to create transgenic cyanobacteria by introducing genes or modulating bacterial pathways inside cyanobacteria for increasing the productivity of PHA. Hempel et al. (2011) introduced the bacterial Poly-(R)-3-hydroxybutyrate (PHB) pathway of *R. eutropha* H16 into the diatom *Phaeodactylum tricornutum*. The genes for the three bacterial enzymes PhaA (ketothiolase), PhaB (acetoacetyl-CoA reductase), and PhaC (PHB synthase) were expressed in the cytosol of the diatom *P. tricornutum* for PHB production *which* accumulated bioplastic in granule-like structures in the cytosol of the cells (Hempel et al. 2011). Carpine et al. (2017) carried out the genetic engineering of *Synechocystis* sp. PCC6803 with an aim to enhance the acetyl-CoA level directly from CO_2 to rewire carbon flux toward the native PHB biosynthesis route. This study resulted in the production of PHB with 232 mg/l, ~12% of dry cell weight (dcw), and a productivity of 0.3 mg/l/h(Carpine et al. 2017).

8.2.3.3 Polyamides

Among the three polyamides that exist in nature, only cyanophycin (multi-L-arginyl-poly-L-aspartic acid) can be produced inside the cyanobacterial strain. It is a non-ribosomal polypeptide consisting of equimolar amounts of aspartate and arginine. It serves as a nitrogen/carbon reserve polymer in many cyanobacterial strains as well as in a few heterotrophic bacteria (Watzer and Forchhammers 2018). Due to its biotechnological and medical applications, in particular as a source of polyaspartic acid – a biodegradable material that is functionally equivalent to petroleum-based nonbiodegradable polyacrylates, much attention is given for its large-scale production. Cyanophycin is synthesized by cyanophycin synthetase that adds both aspartate to the aspartate backbone of the polymer and arginine to the β-carboxyl group of aspartate residues in the backbone. The gene coding for cyanophycin synthase is cphA (Burnat, Herrero, and Flores 2014). Synechocystis sp. strain PCC 6803, *Anabaena* sp. strain PCC 7120, *Acinetobacter* sp., and *Trichodesmium* sp. are some examples of cyanobacteria that are capable of producing cyanophycin (Watzer and Forchhammers 2018).

8.3 Microbial Production Pathways for Various Types of Biopolymers

8.3.1 Polysaccharide Production Pathways in Bacteria

There are three distinct classes of microbial polysaccharides based on localization, viz. intracellular, capsular, and extracellular polysaccharides (EPSs). Intracellular polysaccharides are produced inside the bacterial cells and act as energy source

for microbial cells. Capsular polysaccharides are the constituent of cell wall and are difficult to harvest. Peptidoglycans, lipopolysaccharides, and teichoic acids are some examples of capsular polysaccharides. EPS are secreted out by microbial cells and can be easily harvested. Some examples of the EPS are Xanthan, levan, alginate, cellulose, dextran, and curdlan. EPS have wide commercial applications ranging from pharmaceutical to food-processing, extended to detoxification, bioremediation, paints, biotechnology, and petrochemicals. In this chapter, we will concentrate our study on exopolysaccharide production and strategies to increase their production during fermentation.

EPS is categorized into two categories: (i) homo polysaccharide – composed of only one type of monosaccharide and (ii) hetero polysaccharide – composed of two or more different types of monosaccharide. These EPS are decorated by noncarbohydrate substituents, such as acetyl, pyruvyl, or succinyl groups, which confer anionic properties to the polysaccharide. Moreover, these decorations also contribute to the enormous structural diversity of polysaccharides and properties which can be exploited for many industrial, food, and medical sectors (Zeidan et al. 2017). The biosynthesis of EPS occurs in multiple steps using activated precursors. The enzymes involved in EPS synthesis are located at different cellular regions and can be categorized into four groups based on their involvement in the last three of the four steps of bacterial EPS synthesis. The first group includes intracellular enzymes such as hexokinase which phosphorylates glucose (Glc) to glucose-6-phosphate (Glc-6-P), to form UDP-glucose and dTDP-glucose sugar intermediates. The second group stage of synthesis is the catalysis of sugar nucleotides, Uridine-5′-diphosphate (UDP)-glucose pyrophosphorylase converts Glc-1-P to UDP-Glc, one of the vital molecules in EPS biosynthesis. The third group stage includes the action of glycosyltransferases (GTFs) which are localized in the periplasm. GTFs transfer the sugar nucleotides to a repeating unit linked to glycosyl carrier lipid, identified as isoprenoid alcohol. The polysaccharides are then modified through such enzymatic activities as acylation, acetylation, sulphation, and methylation, and exported to the extracellular surface with the help of hydrophobic enzymes like flippase, permease, or ABC transporters in the fourth stage of the synthesis (Osemwegie et al. 2020).

Currently, four types of distinctive mechanisms are known for EPS synthesis in bacteria: (i) Wzx/Wzy-dependent pathway; (ii) the ATP-binding cassette (ABC) transporter-dependent pathway; (iii) the synthase-dependent pathway; and (iv) the extracellular synthesis by use of a single sucrase protein (Schmid, Sieber, and Rehm 2015). Figure 8.1 gives an overview of all these biosynthetic pathways.

Wzx (flippase)/Wzy (polymerase)-dependent pathway is involved in the synthesis of numerous cell surface sugar polymers, including lipopolysaccharide (LPS), heteropolymeric O antigen (O-Ag), enterobacterial common antigen (ECA), EPS and capsular polysaccharides in a wide range of gram-negative and

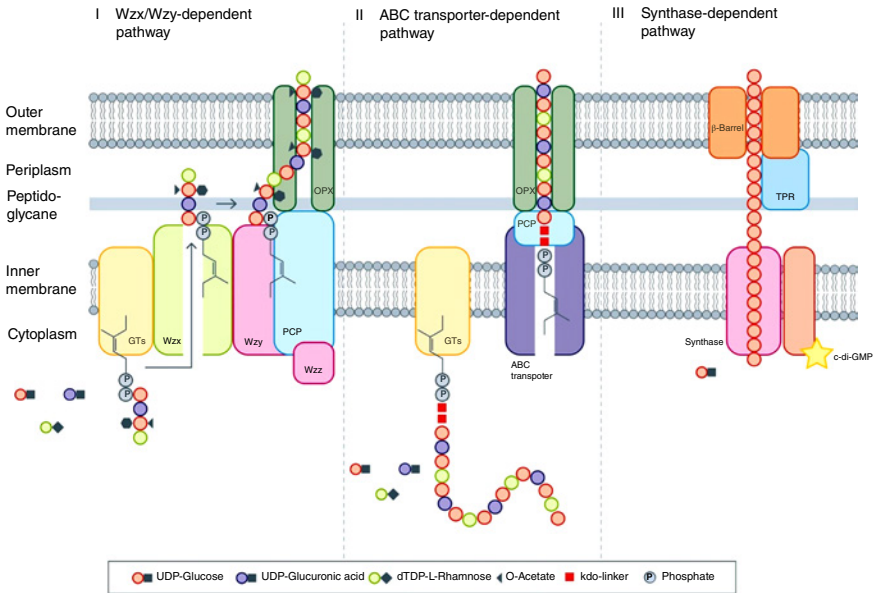

Figure 8.1 Overview of different polysaccharide biosynthetic pathways in bacteria. *Source:* Schmid et al. (2015). Licensed under CC BY-4.0. © Frontiers Media S.A.

gram-positive bacteria. The synthesis of EPS via the Wzx/Wzy-dependent pathway takes place in the cytoplasm with the synthesis of lipid-linked polysaccharide repeat units. The first sugar is added by an initiating glycosyltransferase to the polyisoprenoid lipid carrier molecule undecaprenyl phosphate (UndP, $C_{55}P$) in the form of an activated nucleotide precursor at the inner leaflet of the inner membrane (IM), resulting in a pyrophosphate linkage with the carrier (undecaprenyl pyrophosphate, UndPP), followed by translocation of UndPP-linked repeat units from the inner to the outer leaflet of the IM by the flippase Wzx, where they are polymerized by Wzy protein in periplasmic space before being exported out via a putative catch-and-release mechanism. O-unit addition occurs at the reducing terminus of the growing chain, the length of which is regulated by the polysaccharide co-polymerase Wzz, resulting in organism-specific preferred modal lengths (Islam and Lam 2013). All polysaccharides assembled by the Wzx/Wzy pathway have a highly diverse sugar pattern (up to four or five types of sugar within their chemical structure are common) and are therefore classified as heteropolymers (e.g. xanthan). All strains using this pathway carry the genes for the flippase (Wzx) and the polymerase (Wzy) within their EPS operons (Schmid, Sieber, and Rehm 2015).

The second pathway of bacterial EPS biosynthesis is the ABC transporter-dependent pathway which is mainly present in capsular polysaccharide (CPS) biosynthesis. These polysaccharides do not really represent EPS, since they are still linked to the cell surface (Schmid, Sieber, and Rehm 2015). These CPS glycans are assembled at the cytoplasm–membrane interface on a conserved glycolipid consisting of ~5 to 9 β-linked 3-deoxy-β-D-*manno*-oct-2-ulosonic acid (Kdo) residues attached to a reducing terminal (lyso) phosphatidylglycerol (*lyso*PG). Glyco-ABC transporters are composed of two copies each of a transmembrane domain (TMD) and a nucleotide-binding domain (NBD). ATP binding and hydrolysis in the NBD dimer drives conformational changes in the TMD dimer that power CPS secretion across the IM. Translocation of CPS across the periplasm and outer membrane employs a hetero oligomeric complex composed of polysaccharide copolymerase (PCP) and outer membrane polysaccharide export (OPX) proteins (Liston et al. 2018).

The third pathway does not depend upon flippase for translocation of repeat unit of polysaccharide. This pathway is also known as synthase-dependent pathways which secrete complete polymer strands across the membranes and the cell wall. Synthase-dependent EPS secretion can occur in the presence or absence of a lipid acceptor molecule. Depending on the polysaccharides, a membrane-embedded glycosyltransferase can facilitate simultaneous polymer formation and translocation across the IM. Once the polymer reaches the periplasm, a tetra tricopeptide repeat (TPR)-containing scaffold protein is thought to protect it from degradation before it is exported across the outer membrane through a β-barrel porin (Whitney and Howell 2013). Synthase-dependent pathways are often

utilized for the assembly of homopolymers requiring only one type of sugar precursor, e.g. in case of curdlan biosynthesis, only β-(1-3)-linked glucose is found in the polymer. Another example of a strict homopolymer is bacterial cellulose, consisting only of β-(1-4)-linked glucose units. In the case of alginates, the preliminary polymer is synthesized as polymannuronic acid, which is processed by different epimerases and further modifying enzymes to glucuronic/mannuronic acid block-polymers, which can differ in the ratio and sequence of G/M building blocks (Schmid, Sieber, and Rehm 2015).

8.3.2 Mechanism of Fungal Polysaccharides Synthesis

Most of the EPS produced by fungi are highly hygroscopic β-glucans. Depending upon the linkage, glucans can be divided into two groups: α- and β-glucans. Most glucans are water-insoluble linear polymers made of glucose units joined through 1,3 bonds. However, the most important glucans in fungi are 1,3-linked polysaccharides. Synthesis of these polymers made up of β-1,3 chains with a variable degree of β-1,6 branching involves several reactions: initiation, chain elongation, and branching. Elongation of chain is the most important step in synthesis and is catalyzed by glucan synthetases which utilize uridine-diphosphoglucose as sugar donor (Ruiz-Herrera 1991). The biosynthesis of fungal EPS seems to follow the same mechanism as involved in the synthesis of bacterial EPS. For example, the synthesis of sceleroglucan, a fungal EPS composed of a (1,3)-β-D-glucopyranosyl backbone with (1,6)-β-D-glucopyranosyl residues as branches, is composed of three major steps: substrate uptake, intracellular formation, and extrusion from the cell. Uptake of glucose into the cell is mediated by glucose transporter(s), followed by phosphorylation of glucose to glucose-6-phosphate via a hexokinase reaction. After interconversion of glucose-6-phosphate to glucose-1-phosphate by phospho-glucomutase, a UTP-glucose-1-phosphate uridylyltransferase activates glucose-1-phosphate to UDP-glucose. A (1 → 3)-β-glucan synthase polymerizes the backbone chain using UDP-glucose as monomeric precursor. The last step yielding to the (1 → 6)-β branching at every third glucose molecule is supposed to be catalyzed by trans-D-glucosidases. The (1 → 3)-β chain of sceleroglucan is elongated toward the nonreducing terminus and that (1 → 6)-β-linked glycosyl side residues are incorporated simultaneously as the (1 → 3)-β-glucan backbone is elongated (Schmid et al. 2010).

8.3.3 Mechanism of Synthesis of Polyester in Bacteria

Bacteria accumulate polyesters such as PHA or polyhydroxybutyrate in response to growth limitation due to depletion of non-carbon nutrient sources such as nitrogen, phosphorous, or oxygen. The PHB synthesis starts from metabolism of glucose producing acetyl-coenzyme-A (acetyl-coA), which is

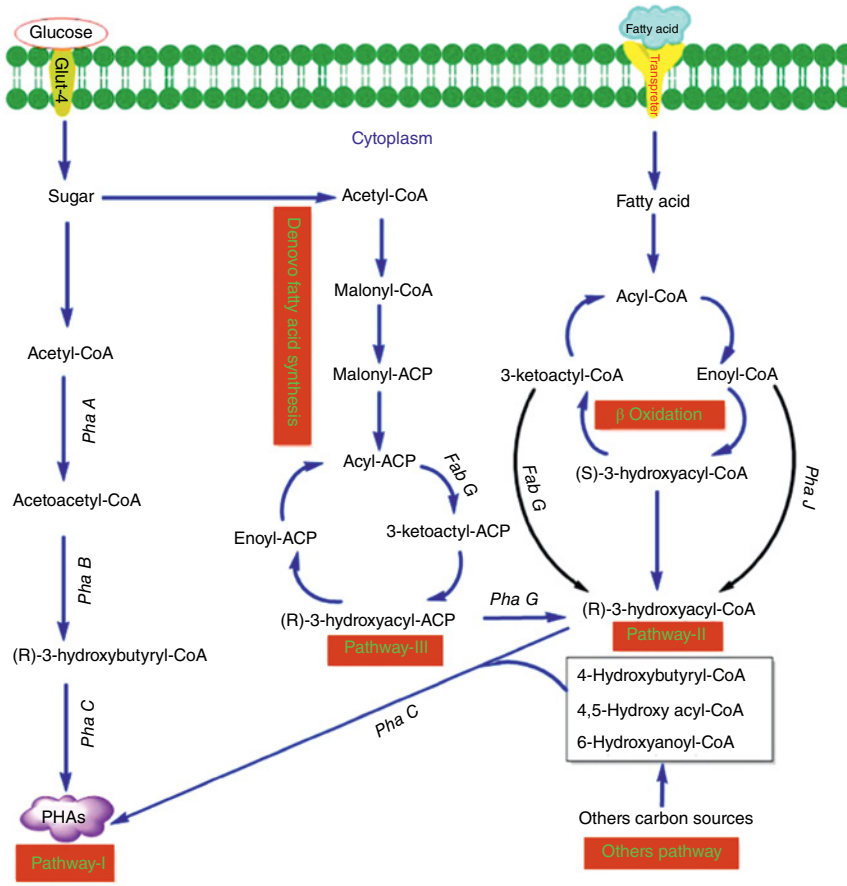

Figure 8.2 Metabolic pathways for synthesis of poly hydroxy alkanoates (PHas) in bacteria. *Source:* Mohapatra et al. (2017). Licensed under CC BY-4.0. © Elsevier.

converted into PHB by three biosynthetic enzymes. In the first step, 3-ketothiolase (PhaA) combines two molecules of acetyl-CoA to form acetoacetyl-CoA. Acetoacethyl-CoA reductase (PhaB) allows the reduction of acetoacetyl-CoA by NADH to 3-hydroxybutyryl-CoA. Finally, PHB synthase (PhaC) polymerizes 3-hydroxybutyryl-CoA to PHB, coenzyme-A being liberated. Only (*R*)-isomers are accepted as substrates for the polymerizing enzyme. Carbon sources in bacteria are metabolized differentially. So far, three pathways for the synthesis of monomers of PHAs in bacteria have been well-studied. Pathway I utilizes sugars like glucose and fructose to yield PHB homopolymer. Copolymers are produced through pathways II and III (Mohapatra et al. 2017) as outlined in Figure 8.2.

8.3.4 Mechanism of Synthesis of Polyamide in Bacteria

The mechanism of polyamide in bacteria can be understood by taking an example of extracellular synthesis of PGA and ε-poly-L-lysine (PL). PGA and PL are the only two polyamides synthesized in ribosomal-independent fashion. In bacteria, the synthesis of PGA starts from precursor L-glutamic acid derived endogeneously using α-ketoglutaric as a direct precursor derived from central tricarboxylic acid pathways. The biosynthesis pathways for PGA are best understood in bacterium *B. subtilis*. In general, the complete biosynthesis of PGA can be divided into four distinct stages: racemization, polymerization, regulation, and degradation. Racemization involves conversion of L-glutamate to D-glutamate with the glutamate racemase gene (*rac*E/*glr* and *yrp*C), and *glr* is essential for converting L-glutamate into D-glutamate for the synthesis of γ-PGA. The polymerization steps involves poly glutamate synthase (pgs), encoded by four genes (*pgs*B, C, A, and E) and their homologs (*yws*C, *ywt*AB, and *cap*BCA) in *Bacillus* species. This machinery is solely responsible for polymerizing γ-PGA at the active site of the synthase complex (PgsBCA) in an ATP-dependent reaction. PgsB and PgsC form the main parts of the catalytic site, whereas PgsA removes the elongated chain from the active site, which is necessary for addition of the next monomer and transporting γ-PGA through the compact cell membrane. The regulation steps involve two signal transduction systems: the ComP-ComA regulator, and the two-part DegS-DegU, DegQ, and SwrA system. DegSU, DegQ, and ComPA appear to be involved in transcriptional regulation in response to quorum sensing, osmolarity, and phase variation signals, while SwrA appears to act at a post-transcriptional level. There are two enzymes capable of degrading γ-PGA in *Bacilli*: endo-γ-glutamyl peptidase and exo-γ-glutamyl peptidase. In *B. subtilis*, the genes encoding endo-γ-glutamyl peptidase are *ywt*D, *dep*, or *pgd*S. These enzymes cleave PGA chain attached to the membrane surface and helps in release of PGA extracellularly in medium (Luo et al. 2016). Similarly, in gram-negative bacteria *Fusobacterium nucleatum PGA* synthesis was proposed to be based on the amide ligase mechanism in which one glutamate residue is added to carboxyl terminus of phosphoryalted oligo-(γ-glutamate) or glutamate primer. PgdS, a γ-glutamyl hydrolase, might hydrolyze the nascent PgA, inducing release of the polymer and controlling its molecular mass (Rehm 2010).

8.4 Tools and Technologies Available for Metabolic Engineering

The field of metabolic engineering has come a long way since it was first described 20 years ago. Initially, the modification/optimization of metabolic pathways for increased production of desired biochemical were hit and trial method based on

past experiences, random genetic modification, and were intuitive in nature. This makes the selection and optimization of suitable strain laborious, time-consuming due to repetition of same cycle of experiment over and over unless a desired modification has been identified. The advancement in genetic engineering especially post-genomic technologies, high-throughput biology, and computational biology has enabled the newfound ability to make accurate prediction of entire cellular function and ability to design metabolic pathways and introduce into bacteria using recombinant DNA technology approaches. Nowadays, system metabolic engineering approach is employed for the modification of pathways, which combines systems biology, synthetic biology, and evolutionary engineering with traditional metabolic engineering. The aim of these strategies is to increase the competitiveness of bioprocess in terms of titer (product concentration), yield, and productivity (Choi et al. 2019).

Once the product and suitable strain has been identified for increasing productivity, the next step is the metabolic pathway reconstruction in non-native producer of the desired product. For native producer, directly metabolic flux analysis can be performed for optimization of metabolic pathway toward high productivity of selected products. The next sections will describe various tools and techniques available for metabolic pathway reconstruction, genome analysis, metabolic flux analysis and optimization, metabolic control analysis (MCA), and Omics analysis.

8.4.1 Metabolic Pathway Reconstruction

Metabolic reconstruction assumes central importance when the chosen bacterial strain does not have metabolic pathway for production of the desired product. In such a scenario, metabolic pathway capable of producing the desired chemical in host non-native producer strain should be constructed. Several approaches can be used for reconstruction of metabolic pathways: (i) by mapping a group of gene and protein sequences of an organism to known reference pathways according to sequence homology and using the matched reference metabolic pathways as templates to position the genes and proteins (e.g. enzymes) to construct the metabolic pathways that they participate in. (ii) Ab initio methods that do not use known reference pathways, aims at inferring metabolic pathways from gene expression data or other data sources, (iii) metabolic network with ordinary differential equations (ODEs) that capture the dynamics of chemical concentrations in a metabolic system, and (iv) by using reaction rules generated from known biochemical reaction (Hadadi et al. 2016; Qi, Li, and Cheng 2014). Various metabolic pathway databases containing known metabolic reaction/pathway like Kyoto Encyclopedia of Genes and Genomes (KEGG), MetaCyc, BRENDA, BNICE.ch, and ATLAS are available for pathway reconstruction.

KEGG: Kyoto Encyclopedia of Genes and Genomes (KEGG) is a knowledge base for systematic analysis of gene functions in terms of the networks of genes and molecules. KEGG acts as catalogue for storing information about genes, pathways, and chemical compound. KEGG contains three different types of databases viz (i) GENES database for storing genomic information, (ii) PATHWAY database, which includes graphical representations of cellular processes, such as metabolism, membrane transport, signal transduction, and cell cycle, and (iii) the third database in KEGG is LIGAND for the information about chemical compounds, enzyme molecules, and enzymatic reactions (web reference).

Metacyc: MetaCyc is a curated database of experimentally elucidated metabolic pathways from all domains of life. MetaCyc contains 2749 pathways from 3161 different organisms. MetaCyc contains pathways involved in both primary and secondary metabolism, as well as associated metabolites, reactions, enzymes, and genes (web reference). It contains data about chemical compounds, reactions, enzymes, and metabolic pathways that have been experimentally validated and reported in the scientific literature and cover both small molecule metabolism and macromolecular metabolism (e.g. protein modification). The goal of MetaCyc is to catalog the universe of metabolism by storing a representative sample of each experimentally elucidated pathway.

MetaCyc applications include:

- Online encyclopedia of metabolism.
- Predict metabolic pathways in sequenced genomes.
- Support metabolic engineering via enzyme database.
- Metabolite database aids metabolomics research.

BRENDA: BRaunschweig ENzyme Database (BRENDA) represents a protein function database, containing comprehensive enzymatic and metabolic data, continuously updated and evaluated from the primary literature. It covers more than 370 000 enzymes from 6 300 enzyme classes. It includes a wealth of enzyme-related information by combining the manually annotated functional data with genomic sequences, enzyme structures, and computed data. The manually annotated data cover ~60 categories grouped into "Nomenclature," "Enzyme-Ligand Interactions," "Functional Parameters," "Organism-related Information," "Enzyme Structure," "Molecular Properties," and "Bibliography/Links/Disease." Each EC class is updated periodically, data from selected new references are annotated and included (Schomburg et al. 2017).

8.4.2 Metabolic Flux Analysis

Metabolic flux analysis is stoichiometry-based static model to quantify intracellular metabolic fluxes as a consequence of all catalytic and transcriptional interactions. It is used to examine production and consumption rates of metabolites in a

biological system. At an intracellular level, it allows for the quantification of metabolites, thereby elucidating the central metabolism of the cell. It helps in understanding the contribution of metabolic alterations to pathology (Dai and Locasale 2017). ^{13}C-based flux analysis and constraint-based flux analysis have been used generally on the basis of stoichiometry of metabolic reactions and mass balances around intracellular metabolites under pseudo-steady-state (PSS) assumption. Practically, MFA has been applied to generate new knowledge on the biological system, analyze cellular physiology system-wide, and consequently design metabolic engineering strategies at a systems-level. Flux quantification can be performed using a number of approaches, which can be broadly classified into *in silico* and *in vivo*-based metabolic flux analysis (González 2013). SUMOFLUX, METRAN, FLUXML, WUFLX, and OpenFLUX are some of the software packages available for metabolic flux analysis *in silico*.

SUMOFLUX: SUMOFLUX, a generalized method for ^{13}C flux ratio analysis relative (fractional) information on contributions from alternative pathways are calculated from a small subset of ^{13}C-data using predefined analytic formulas. Flux ratio is a number that indicates the relative fraction of a specific metabolite flowing through a chosen reaction or pathway. Flux ratios are estimated based on a stoichiometric model, knowledge of the ^{13}C-configuration of all of the relevant substrates, and the labeling patterns of metabolites as measured by mass spectrometry (or nuclear magnetic resonance). The full SUMOFLUX workflow for flux ratio estimation consists of five steps as underlined (Kogadeeva and Zamboni 2016).

i) A reference dataset of several thousand flux-maps is sampled from that space of flux-maps that fulfills certain stoichiometric constraints (mass balances) of the metabolic network.
ii) The ^{13}C-labeling patterns of the metabolites included in the network are simulated independently for each reference data point.
iii) To capture measurement data, only those ^{13}C features are selected that are analytically accessible, and then superimpose noise values corresponding to those measured.
iv) Flux ratios of interest are calculated for all of the data points within the reference dataset, as the dependent variable in regression analyses.
v) Divide the reference dataset into independent training and test subsets, using the former to train a random forest with which to predict the calculated flux ratios from simulated ^{13}C data.

The predictor's performance is then assessed by applying mean absolute error (MAE) or suitable error measurement techniques. MAE < 0.05 is considered as good predictor's performance.

FluxML: FluxML is a universal modeling language for metabolic flux analysis designed to provide a standard document format for creating, validating, and

reliable exchanging of arbitrary metabolic models across different simulation tools. Its hierarchically structure based on widely used XML (eX-tensible Markup Language) and specified all essential information required to enable performing both isotopically stationary and nonstationary ^{13}C metabolic flux analysis including the structure of the metabolic network, atom transitions, experimental data, model constraints, and parameters (web reference). FluxML is organized into three major releases, termed Levels. Level 1 is dedicated to isotopically stationary ^{13}C MFA while Level 2 covers isotopically nonstationary ^{13}C MFA in addition. With Level 3, the general case of multiple, isotopically stationary and nonstationary isotopic tracer experiments are supported.

8.4.3 Metabolic Control Analysis

MCA determines the degree of control that a given enzyme exerts on flux and the concentration of metabolites quantitatively. It makes use of a set of sensitivity parameters called control coefficients that quantify the fractional change in a specified property of a metabolic pathway in response to a small fractional change in the activity of an enzyme (Moreno-Sánchez et al. 2008). MCA can be used to elucidate the control structure of a metabolic network. This is achieved through the calculation of flux and concentration control coefficients. Such an analysis can identify reactions that control the carbon flux through the pathway and hence genes/proteins that need to be manipulated to increase the flux toward the desired product (González 2013). In short, the whole purpose of MCA analysis is to find the rate-limiting enzymes of metabolic pathway and increase the activity of enzyme. Several methods are available for MCA analysis. A mathematical modeling-based method, called ORACLE (optimization and risk analysis of complex living entities), can be used for the construction of large-scale kinetic models and can be used as guidance for formulating alternative metabolic engineering strategies as well. Experimental approaches for identification of rate-limiting steps include inspection of the metabolic pathway architecture, determination of nonequilibrium reactions, identification of the steps with the lowest maximal rates in cellular extracts, identifying enzymes with sigmoidal kinetics, comparing the intermediary concentrations between a basal and an active steady-state pathway flux, and the shape of the metabolic flux inhibition curve (Moreno-Sánchez et al. 2008).

8.4.4 Omics Analysis

Omics refers to the collective technologies used to explore the roles, relationships, and actions of the various types of molecules that make up the cells of an organism. These technologies with respect to metabolic engineering are genomics,

Table 8.1 Computational and experimental tools available for metabolic engineering.

Functional genomics tools	Description/usage	References
DNA microarray	Allows differential gene expression in response to different experimental condition or genetic perturbations.	(González 2013)
Proteomics	Understanding cellular metabolism based on the protein expression profiles and revealing the role of post-translational modifications.	(Choi et al. 2019)
2-D gel electrophoresis + Mass spectrometry, LC-MS, Protein isotope labeling	The large-scale quantification and identification of proteins can be achieved by one- or two-dimensional gel electrophoresis, followed by software-aided quantification tools and mass spectrometry techniques.	(González 2013)
Comparative proteomic analyses	Detect differentially expressed proteins by variations in the intensities of labeled protein spots in the gel, under different genetic or environmental conditions.	(González 2013)
Nuclear Magnetic Resonance (NMR), and Gas or Liquid Chromatography coupled toMass Spectrometry (GC-MS and LC-MS)	It is used for the quantification of intra- and extracellular small molecular-weight metabolites.	(González 2013)
Biobrick assembly, Gibson assembly, Golden gate assembly, Ligase cycling reaction	DNA assembly tool used in system metabolic engineering.	(Choi et al. 2019)

transcriptomics, proteomics, metabolomics, fluxomics, genomics + transcriptomics, genomics + transcriptomics + phenomics, and proteomics + metabolomics. Omics technologies provide the tools needed to look at the differences in DNA, RNA, proteins, and other cellular molecules between species and among individuals of a species. These technologies enable us to determine the biological function of each gene and elucidate the metabolic and regulatory networks that operate in a biological system. ME model, ssbio, and thermodynamics-based flux analysis are some of the omics-integrated genome-scale metabolic modeling. Various functional genomics tools are available to supplement the generation and analysis of omics data as listed in Table 8.1.

The next step after designing and reconstruction of metabolic pathway is the introduction of the designed pathway in the suitable producer host strain. Various genetic engineering tools facilitate this process. Nowadays, preformed building blocks are available to design and assemble larger synthetic biological circuits from individual parts and combinations of these parts with defined functions and consequent expression of the assembled metabolic pathway genes. Some of the commonly used DNA assembly building blocks are Biobrick, Novel such as DNA assembly tools, such as BioBrick assembly, Gibson assembly, Golden Gate assembly, ligase cycling reaction, single strand assembly, transformation-associated recombination(TAR) cloning, and uracil specific excision reagent (USER) cloning (Choi et al. 2019).

8.5 Dynamic Metabolic Flux Analysis and its Role in Metabolic Engineering

Metabolic flux analysis which was used earlier to measure intracellular fluxes suffers from certain disadvantages such as the requirement of PSS, restricted to only continuous culture and the mid-exponential growth phase of batch cultures and is suitable only for determined or overdetermined system. It fails miserably when it comes to the dynamic or transient system or when the requirement of system demands flux analysis for the whole duration of batch culture or undetermined system. To circumvent this problem, the concept of dynamic metabolic flux analysis (DMFA) has been put forward which is nothing but an evolved form of traditional metabolic flux analysis. DMFA is based on time-series data analysis and is useful for process monitoring and estimation of the transient *in vivo* fluxes (Antoniewicz 2013; Martínez et al. 2015). DMFA combines metabolic network analysis based on the PSS assumption for intracellular metabolism with dynamic models of extracellular environment. Since, DMFA being used for transient system such as batch and fed batch culture, it should factor rapidly changing environment or dynamics of system. The system dynamics can be captured by following two different approaches: (i) MFA is combined with kinetic model to predict external rates (and possibly a few internal rates) based on simulated extracellular environment and (ii) MFA is combined with techniques for analyzing time-series data from cell culture experiments, i.e. data-driven approach. The advantage of kinetic-driven approach is that it can be used for dynamic simulations of fermentations to evaluate alternative bioprocessing conditions and identify potentially useful genetic manipulations. Kinetic models provide a valuable framework for simulating bioprocesses and generating hypotheses regarding optimal process conditions, optimal control, and potentially useful genetic

manipulations that can improve the overall process performance. On the other hand, the data-driven approach provides a framework for monitoring and visualizing transient intracellular fluxes with applications in process monitoring and control. This approach is used for monitoring intracellular dynamics and generating flux information that is needed for constructing kinetic models (Antoniewicz 2013).

8.6 Production of Biopolymers from Metabolically Engineered Microbes

Commercial application of biopolymers has driven the quest for more efficient producer strain, optimized and economical processes, and high production capacity. Metabolic engineering in combination with modern computational techniques has minimized the effort and time to achieve desired strain or process, which otherwise is too laborious and time-consuming. Metabolic engineering approaches to engineer metabolic pathways in microbes are (i) improving nucleotide-precursor availability, (ii) recombinant expression in suitable microbial cell factories, (iii) optimizing substrate utilization, (iv) attenuation of competing central carbon fluxes (Schilling et al. 2020), (v) disruption of pathways that compete with biopolymer precursor synthesis, and (vi) to overexpress the genes involved in biopolymer assembly.

8.6.1 Metabolic Modification of Pathway for Synthesis of Polysaccharides

Steffens et al. (2016) constructed a mutant strain of *xanthomonas campestris* pv. *Campestris* B100 (XCC) for increased production of anionic polysaccharide xanthan using metabolic engineering strategy of improving nucleotide-precursor availability. The nucleotide sugars GDP-mannose, UDP-glucose, and UDP-glucuronic acid are the direct precursors for xanthan production. Similarly, lipopolysacharide (LPS) also feed on the same nucleotide precursor for biosynthesis in the outer leaflet of gram-negative bacterium such as *Xanthomonas* species. LPS consists of three subunits Lipid A, core oligosaccharide, and O-antigen. O-antigen is the largest part of lipolysaccharide and is supposed to be nonessential part of bacterial cell wall and has no role in maintaining structural stability and survivability in bacteria. Therefore, O-antigen can be eliminated to optimize the nucleotide precursor availability toward xanthan biosynthesis. The author achieved this task by identifying genes located in *wxc* gene cluster responsible for the LPS O-antigen production in Xcc, followed by targeting the region 3 of gene cluster to inhibit the biosynthesis of the O-antigen

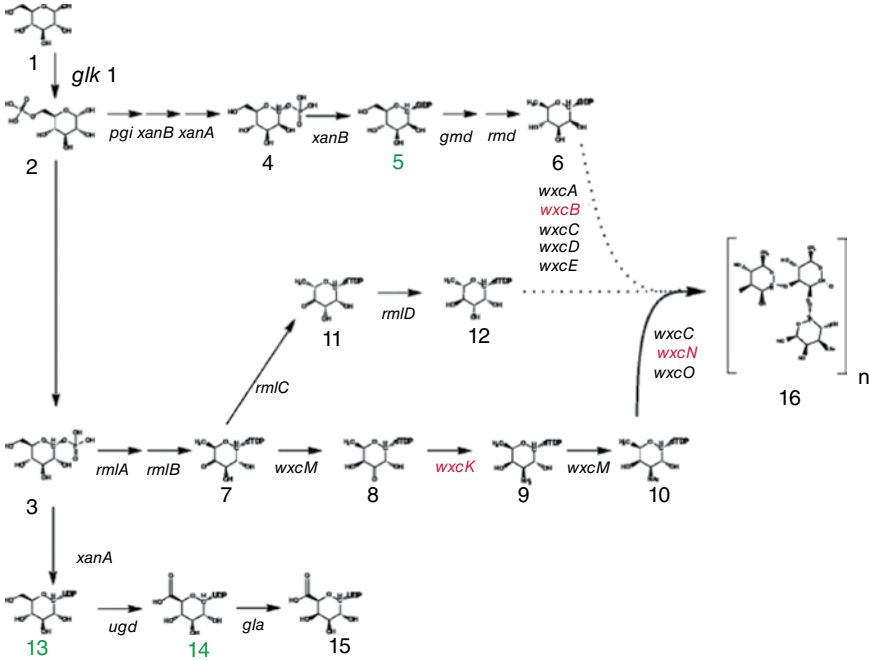

Figure 8.3 Redirection of metabolic flux to the ε-PL biosynthesis in *S. albulus* 11012B Δ*ttm* mutant. "X" symbol indicates "inactivation of pathway" and dotted arrow indicates "redirection" of metabolic flux (ATP). *Source:* Steffens et al. (2016). © Springer Nature.

branches. Thus, mutant strain Xcc H21012 was which carried mutation in gene *wxcB*. Inactivation of *wxcB* resulted in a complete loss of the O-antigen and increased production (6.3 g xanthan per gram of biomass) compared to wild-type strain (5.0 g xanthan per gram of biomass) (Steffens et al. 2016). The diversion of metabolic flux in biosynthetic pathway in *xanthomonas campestris pv. campestris* B 100 due to inactivation of gene is outlined in Figure 8.3.

Dai et al. (2019) used a combined approach of recombinant expression and disruption of metabolic pathway to construct *Xanthomonas campestris* CGMCC15155 strain for production of white xanthan gum. Xanthomonadian is a key component for xanthan synthesis in the pathway. However, due to its pigment causing ability, it gives yellow color thereby causing increased use of ethanol for depigmentation which further complicates the purification process. The strategy to address this issue involves insertional inactivation of pigA gene involved in xanthomonadin synthesis by inserting the *Vitreoscilla* globin (*vgb*) gene, under the control of the LacZ promoter. The inactivation of *pigA* gene caused inhibition of

xanthomonadin synthesis responsible for pigmentation of xanthan. The loss of pigmentation agent resulted in less use of ethanol for depigmentation process and cost-effective downstream processing of xanthan (Dai et al. 2019).

8.6.2 Levan

The commercial application of industrially important fructan, levan, in the food, pharmaceutical, and cosmetic industries has driven the metabolic engineering to increase its production during fermentation. Various metabolic engineering strategies have been applied for creating overproducer microbial strains, for example, disruption of extracellular sucrase gene (*sacC*) in *Zymomonas mobilis* led to the 50-fold increase in activity of levansucrase enzyme produced by SacB. SacB, lebvansucrase polymerizes fructose units to form levan in sucrose-containing medium. The mutant strain showed increased levan titer compared to wild-type strain from 15.5 to 21.2 g/l (Senthilkumar et al. 2004). This strategy of metabolic engineering can be categorized as optimization of substrate utilization (sucrose) by increasing the activity of enzyme (levansucrase) through deletion of adjacent gene.

In a recent study, Feng et al. 2015 constructed a mutant strain of *B. amyloliquefaciens* NK-Q-7 strain by deleting TasA gene (responsible for biofilm formation), and six extracellular protease genes (*bpr*, *epr*, *mpr*, *vpr*, *nprE,* and *aprE*), together with the *pgsBCA* cluster (responsible for γ-PGA synthesis), in the *B. amyloliquefaciens* NK-1 strain. The advantage of this metabolically engineered strain was that it has higher secretion of levansucrase enzyme responsible for levan production due to the absence of biofilm at its outer surface; lack of protease enzyme makes the enzyme stable and the channeling of precursor metabolite toward levan production instead of γ-PGA, thereby simplifying the downstream processing. The highest levan production (31.1 g/l) was obtained from the NK-Q-7 strain which was 103% higher than that of the NK-ΔLP strain (15.3 g/l) (Feng et al. 2015). This strategy combined two metabolic engineering strategies highlighted above viz increasing availability of precursor metabolite (by blocking/disrupting gene responsible for PGA synthesis) toward levan synthesis and optimization of substrate utilization by increasing the extracellular secretion of levansucrase.

8.6.3 Metabolic Modification of Pathway for Synthesis of Polyester

A lot has been discussed about naturally occurring polyester polyhydroxy alkanoates/PHBs. So, we will be focusing on metabolic engineering strategy of other naturally occurring polyester like polymalic acid. PMA is a water-soluble biopolymer composed of l-malic acid (MA) monomers and is mainly produced by the yeast-like fungus *Aureobasidium pullulans* and has prospective application as

drug carrier, nano-imaging agent for safe and noninvasive diagnosis in the clinical setting, biopolymeric materials, and carriers in the drug, food, and biomedical fields. Acid hydrolysis of PMA yields malic acid, a C-4 dicarboxylic acid widely used as flavor enhancer and acidulant in food and beverage industries. It is also used in metal cleaning, textile finishing, fabric dying, and water treatment, and is a potential C4 platform chemical (Feng et al. 2018). In a recent study, Feng et al. (2018) used *in silico* simulation of a genome-scale metabolic model (*i*ZX637) to identify overexpression of pyruvate carboxylase gene (pyc) which can enhance PMA synthesis via reductive tricarboylic acid cycle. Genome-scale metabolic network (*i*ZX637) of *Aureobasidium pullulans*, containing 1347 reactions and 1133 metabolites, was reconstructed and was used for constraint-based flux analyses to determine the most effective route for maximizing PMA production minimizing the influence of biomass accumulation. Since PMA synthesis is directly related to malic acid, three routes of MA synthesis, the oxidative branches of the TCA cycle, the reductive branches of the TCA cycle, and the glyoxylate shunt, were examined followed by plotting of specific PMA synthesis rate at different flux values of the cell growth rate. The basic tools used for the model analysis were flux balance analysis (FBA). Once, the Pyc gene was identified, it was cloned in *Aureobasidium pullulans* and grown in fed batch bioreactor on sugarcane molasses medium for production of PMA. The engineered strain increased the PMA titer by 15.1% compared to wild-type strain (Feng et al. 2018). This metabolic engineering strategy can be categorized as attenuation of competing central carbon fluxes for enhanced production of targeted metabolite.

8.6.4 Metabolic Modification of Pathway for Synthesis of Polyamides

ε-poly lysine (PL) is a secondary metabolite of *S. albulus* best used as preservative food, cosmetics, health care, and other industries. In addition, ε-PL can also be used as gene carrier, drug carrier, weight loss and health care products, water absorbent material, biochip, and bioelectronic coating agent (Wang et al. 2020). Recently, Yamanaka et al. (2020) modified the metabolic pathway of *S. albulus* NBRC14147 by deleting gene cluster responsible for production of five antifungal polyene secondary metabolite —tetramycin A and B, tetrin A and B, and a trace amount of nystatin A1. These polyene antibiotics draw away the metabolic flux from biosynthesis ε-PL pathways leading to decreased production during fermentation. Gene cluster responsible for production of polyene antibiotics, type-I polyketide synthase (PKS) gene was identified through genome analysis using antiSMASH. Targeted inactivation of the biosynthetic gene cluster for tetramycins and tetrins (*ttm*) in a nystatin A1 production-deficient mutant completely abolished the production of polyene macrolides by redirecting

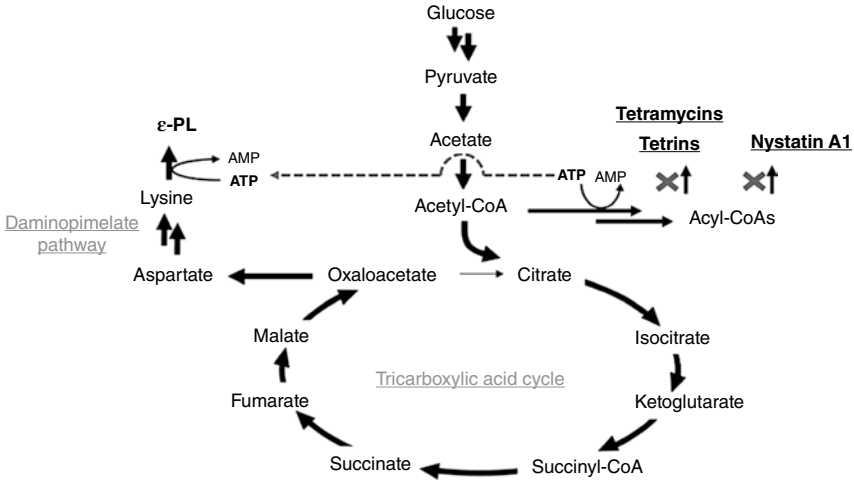

Figure 8.4 *Xanthomonas campestris pv. campestris* sugar nucleotide metabolism producing precursors for the synthesis of the LPS O-antigen and the exopolysaccharide xanthan. Enzymatic reactions are indicated by arrows and names of the involved genes. Mutated genes of the strains Xcc H21012 (wxcB), Xcc H20110 (wxcN), and Xcc H28110 (wxcK) are indicated in bold letters at their respective position within the metabolic pathway. *Source:* Yamanaka et al. (2020). © Elsevier.

metabolic flux, probably intracellular flux of ATP, in *S. albulus* 11012B to the ε-PL production by simply shutting down the concomitant polyene macrolide biosynthesis. This led to an approximately 20% improvement in ε-PL production. The biosynthetic flux for ε-PL was thus successfully enhanced by inactivation of the concomitant secondary metabolite biosynthetic pathways. Production of single metabolite by eliminating competing metabolite (say ε-PL) allows simpler purification after fermentation production of biopolymers (Yamanaka, Hamano, and Oikawa 2020). This metabolic strategy for modification of pathway is a combination of attenuation of competing central carbon fluxes and disruption of pathways that compete with biopolymer precursor synthesis. Figure 8.4 outlines the diversion of metabolic flux toward ε-PL biosynthetic pathway.

8.6.5 Culture of Metabolically Engineered Microbes in Fermentation or Bioreactor for Production of Biopolymer

The environment of a commercial-scale bioreactor is drastically different from that of laboratory-scale cultures such as shake flasks. It has long been recognized that most genetically engineered strains do not perform the same way in the two scenarios. The common problems while growing genetically engineered strain in

fluctuating bioreactor conditions are genotypic drift during long-term cultivation, differences in metabolite levels, protein stability changes, oxidative damage, and gene-expression perturbation which undermines the effort undertaken in the modification of pathway in producer strains. The large-scale bioreactors are operated with a large volume of the medium which exerts various types of physical, mechanical, and chemical stress on engineered producer strain. For example, the increase in volume creates a hydrostatic pressure gradient in bioreactor. This pressure gradient can affect biological properties, including enzyme activity and cell membrane permeability or other colloidal or thermodynamic properties, such as broth bulk viscosity, emulsion stability, and product or biomass settling. Chemical stresses commonly encountered during cultivation are related to the dissolution of gases in media such as O_2, CO_2, and other factors such as pH, temperature, nutrient concentration, and accumulation of inhibitory level of toxic product (Wehrs et al. 2019) or product inhibition. One of the significant considerations while producing biopolymer is the viscosity of the medium. Biopolymer production increases the viscosity of medium over time, which causes inefficient stirring and low O_2 transfer, thereby affecting molecular weight and final quality of biopolymer.

Various strategies have been adopted to encounter these problems faced during the growth and production of biopolymer using engineered strain. The problem of inefficient O_2 transfer during highly viscous production of xanthan was encountered by inserting *Vitreoscilla* hemoglobin (VHb) gene in the *Xanthomonas campestris* CGMCC15155 producer strain. The product of VHb gene promoted oxygen diffusion and metabolism in the host resulting in increased xanthan production (Dai et al. 2019). Another major problem encountered with engineered metabolic pathway containing microbial strain is tolerance. The genetically engineered bacteria are fragile/weak compared with their wild-type strain and respond to even minor environmental fluctuation in bioreactors such as pH or inhibitory by-product or end product. The tolerance of engineered bacteria can be increased by physically adapting to the toxic substance or end product expected to encounter during production by growing engineered strain in the graded concentration of toxic product followed by selection. Another strategy is the genetic modification at the level of chaperones, membrane-modifying enzymes, redox enzymes, transport pumps, and transcriptional factors to increase the robustness of engineered strain for efficient production of targeted biopolymer (Mukhopadhyay 2015). Batch, fed-batch, and continuous culture system can be used for the production of biopolymers. The problem of product-inhibited growth or nutrient limitation can be solved by adapting fed-batch or two-stage fermentation system. Biopolymer productions are preferably carried out in two-stage fed batch or

single-stage fed-batch process. Fed-batch process has the advantages of extending exponential phase till desired for achieving high productivity of biopolymer. For example, the PHA can be produced in high concentration by adapting two-stage fed-batch system. First stage is suitable for biomass growth thereby eliminating the problem of nitrogen limitation followed by second-stage fermentation suitable for PHA production (Verlinden et al. 2007).

8.7 Recovery and Purification of Biopolymers from Fermentation Broth

Down streaming cost of biopolymers is 40–60% of total cost of biopolymer. Therefore, effective recovery and purification of biopolymer from fermented broth are essential to remain competitive in the market in terms of price. Further, most of the biopolymers produced today are also used for pharmaceutical and biomedical application and require a high grade of purity by regulatory authorities for their use in humans. Biopolymers recovery and purification are governed by rheology of biopolymer (viscosity and flow behavior), enzymatic degradation of biopolymer, and production of mixed biopolymer product during fermentation. Mixed production of biopolymer is often problematic with respect to product recovery and purification. Various metabolic strategies for diverting metabolic flux toward single biopolymer production have already been outlined above. The following sections highlight purification strategy adopted for recovery and purification from fermented broth. These strategies will be understood by taking xanthan and ε-PL as an example and it can be applied to any biopolymer with slight modifications depending upon the requirement of process.

8.7.1 Separation and Purification of Xanthan

Xanthan produced from gram-negative bacterium *Xathomonas campestris* is known for its too high viscosity in fermented broth. The main steps of the recovery of xanthan are outlined in Figure 8.5. Following are the main process conditions that must be considered for recovery and purification (Palaniraj and Jayaraman 2011):

i) Viscosity of xanthan containing fermented broth.
ii) Precipitation of xanthan from broth.
iii) Depigmentation.
iv) Degradation.

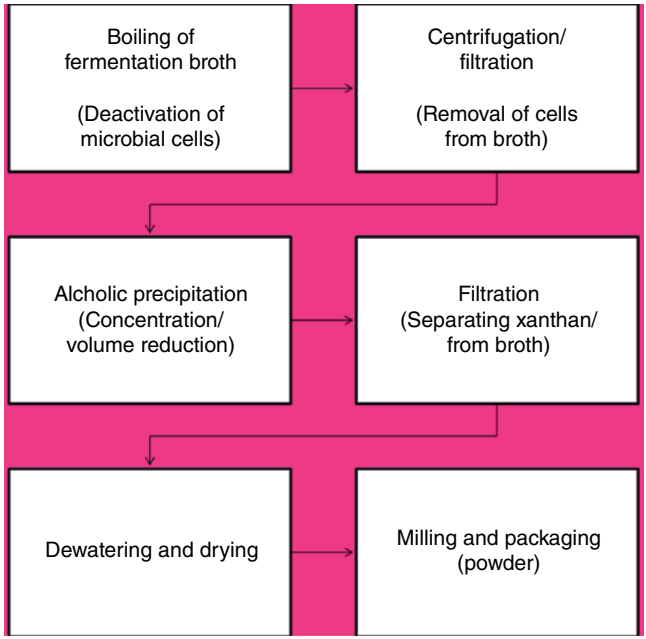

Figure 8.5 Steps involved in recovery and purification of xanthan from fermented broth.

(i) Viscosity consideration

Highly viscose nature of fermented broth poses serious challenge during centrifugation/filtration (separation step). Highly viscose fermented broth should be diluted with water, alcohol, mixture of alcohol and salts prior to filtration/centrifugation to ease handling. Heating can also be applied to reduce the viscosity. Heating also helps in release of xanthan from microbial cells at 80–130 °C, 10–20 minutes, pH 6.3–6.9. Heating also serves to dissolve microbial cells during enzymatic solubilization of microbial cells in extremely high viscose fermented broth.

(ii) Precipitation consideration

Precipitation can be achieved by decreasing the solubility of the dissolved colloid using methods such as addition of salts, water-miscible non-solvents, and concentration by evaporation. Commonly used solvents are methanol, ethanol, isopropanol, and acetone. These are added in V:3V ratio, i.e. one volume of xanthan containing broth and three volume of alcohol. Precipitation can also be caused by addition of salts which reduces the water affinity of polymer or coacervation due to ion binding of the cations of the added salt to the ionized groups on the polyanion. Polyvalent cations are calcium, aluminum, and quaternary ammonium salts.

(iii) Depigmentation

Crude xanthan in broth appears yellow due to the presence of xanthomonadin pigment. The xanthan can be depigmented using water-miscible non-solvent.

A direct approach of xanthan recovery and purification is Ultrafiltration. This technique can be applied directly after initial separation of cells from fermented broth to yield xanthan with high purity. Afterward, xanthan is dewatered, dried, and milled in powder.

8.7.2 Separation of Poly-L-lysine

ε-Poly-L-lysine (ε-PL) is composed of 25–35 L-lysine residues that are linked by adjacent ε-amino and α-carboxyl groups, and can be produced by various *Streptomycetaceae*, such as *S. albulus*, a few filamentous fungi, and some *Bacilli* as a secondary metabolite. It has strong antibacterial effect and is widely used as natural preservative. Since ε-PL is secreted extracellularly and is a polyamino acid with charged nature, its separation involves general downstreaming process starting with filtration. The recovery and purification is a combination of solid–liquid separation and ion-exchange chromatography due to its charged nature. Bankar et al. (2014) applied combination of different techniques to purify poly-L-lysine, which is outlined in Figure 8.6 (Bankar, Chaudhary, and Singhal 2014).

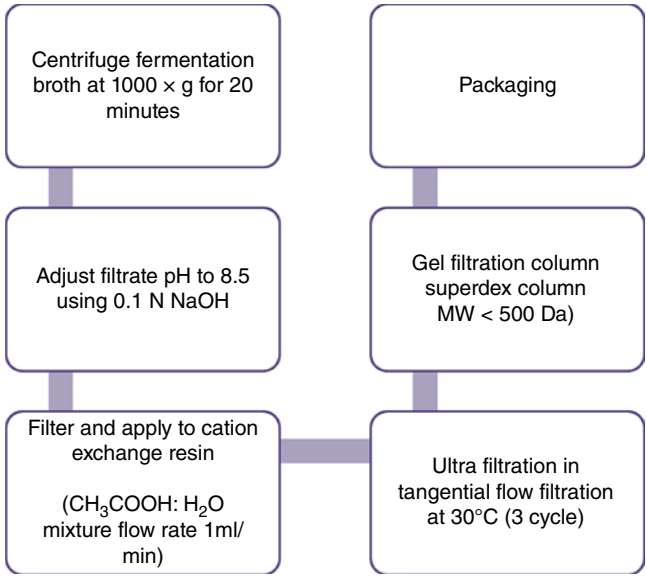

Figure 8.6 Steps involved in ε-Poly-L-lysine separation and recovery from fermentation broth as outlined by Bankar et al. (2014). *Source:* Based on Bankar et al. (2014). © John Wiley & Sons.

Chen et al. (2020) developed an eco-friendly aqueous two-phase system for purification of ε-PL from fermentation broth of *S. albulus M-Z18*. An alcohol/salt solvent system of ethanol/ammonium sulphate extraction system was combined with ultrafiltration system to achieve final ε-PL product of 92.39% purity and 87.72% recovery. The ε-PL was confined to top phase of two immiscible phase of ethanol/ammonium sulphate system. Actual separation occurred during partitioning between the phase and ultrafiltration was done to remove ammonium salt from the solution (Chen et al. 2020).

8.8 Conclusion and Future Challenges

Metabolic engineering, in combination with system biology and synthetic biology, presents an inclusive tool which takes a holistic approach toward increasing product concentration, yield, and productivity of targeted biopolymer. Its approach is not only restricted to strain engineering or cellular pathway modification but in doing so, it accounts for the whole process from fermentation to recovery and purification of the desired product. The advent of new genetic engineering tools, computational tools, and understanding of metabolic pathways has encouraged more and more researchers to make a transition from traditional approaches. Metabolic flux and metabolic control analysis are the two crucial components of metabolic engineering to determine intracellular carbon fluxes across the pathway, which helps in a suitably optimized diversion of pathway resources to targeted biopolymer/product. Though metabolic engineering is an inevitable tool of the coming future, there still exist many challenges that need to be addressed to enable the translation of developed producer strain and process in large number commercially. First limitation is the lack of complete understanding of the corresponding biosynthetic pathways/enzymes and generally long/complex biosynthetic pathways involving some difficult-to-express genes of plant or animal origin. To address this problem, protein and pathway design tools that facilitate the development of a suitable enzyme and pathway are required for the production of natural and non-natural chemicals. Secondly, low *in silico* metabolic simulation predictability restricts the development of efficient producer strain. An efficient computational tool will solve this problem.

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9

Bioplastics Production

What Have We Achieved?

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9.1 Introduction

Plastics have transformed everyday life; this petroleum-based plastic has been in use for years in a variety of industrial and day-to-day applications owing to their versatility and durability. Since its introduction in the 1950s, estimated 8300 million metric tons (Mt) of virgin plastics had been produced to date. As of 2015, approximately 6300 Mt of plastic waste had been generated, around 9% of which was recycled, 12% was incinerated, and 79% was accumulated in landfills or the natural environment (Geyer, Jambeck, & Law, 2017). There have been several negative attributes associated with polymers ending up in landfills, including (i) diminishing land resources fit for other uses of higher societal value; (ii) dioxins and carcinogenic polycyclic aromatic hydrocarbons (PAHs) toxicity after incineration; (iii) deleterious effects on marine wildlife and the aesthetic qualities of cities and forest; besides being (iv) acting as a potential site for a lot of greenhouse gas, i.e. methane generation (Geyer et al., 2017; Law, 2017; North & Halden, 2013). In response to the problems associated with the synthetic polymers, the public tendency, the scientific interest, and the governments' determination have worked in unison over the past two decades to support the development of a new class of plastics from alternative sources. Bioplastics (also called bio-based plastics) made

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from plant or other biological material (wholly or in part) instead of petroleum is becoming a popular substitute. Their development is linked to several factors such as the conservation of the petrochemical resources, high oil prices, waste management, and increased greenhouse gas emissions. And, with the benefits of biologically sourced plastics well understood, bioplastics have been considered relevant for offering sustainability solutions.

As such, bioplastic is a broad term covering many polymers with widely varying properties. Their subcategories include (i) bio-based (or partially bio-based) durable plastics: polyethylene (PE), polyethylene terephthalate (PET); (ii) bio-based biodegradable and compostable plastics: polylactic acid (PLA), polyhydroxyalkanoate (PHA), polybutylene succinate (PBS), and starch blends; (iii) fossil-based and biodegradable plastics: polybutylene adipate terephthalate (PBAT) and polycaprolactone (PCL) (Europeanbioplasics, 2016). These innovative materials are technically equivalent to their fossil counterparts; yet, they can help to reduce a product's carbon footprint by as much as 40%, greenhouse gas emission by 200%, and fossil energy use by 95% (DiGregorio, 2009). Bioplastics elimination from the biosphere is environment friendly. Moreover, these can be conventionally managed and recycled in the existing recycling streams. Over the last several years, the production of these "eco-friendly" biopolymers from renewable resources is increasing in this market to better the earth's environment, and their production has shown significant growth.

According to the latest market data, the global production capacity of bioplastics is predicted to grow by 20% in the medium term, from around 2.04 million tons in 2017 to approximately 2.44 million tons in 2022 (Europeanbioplastics, 2017a). There are several leading companies viz. Metabolix Inc. (USA), Shenzhen Ecomann Technology Co. Ltd. (China), Tianjin GreenBio Materials Co. Ltd. (China), Meredian Inc. (USA), and Biomer (Germany), which have initiated and industrialized production of bioplastics. Today, bioplastics have already become a necessity in many industrial applications such as agriculture and horticulture, electrical and electronics, food packaging, composting bags, and other consumer products. Also, their use in biomedical, automotive, structural, and gastronomy, among others, is increasing. Some recent high-profile applications of bioplastics are the world's first bioplastic refrigerator (by Electolux) and LEGO bricks (by LEGO group) (Thryft, 2018). True to say, the properties profile and applications of bioplastics are on the rise. However, despite all these positive attributes, bioplastics still have a limited market (1% of the total plastics market) (PlasticsInsight, 2017). They have some disadvantages in terms of high cost, lack of legislation, and most of all, the raw materials. Unfortunately, the bioplastics currently on the market are mainly made from flour or starch from corn, wheat, or other grains, and only a few bioplastics are derived from agricultural residues or food. As the production capacities of these bio-based bioplastics would increase, it could have a negative impact on

food availability and cause price increases in food. Hence, switching to biodegradables is a step in the right direction, but it is not the final solution that we require. With an increasing demand for global plastic consumption, much research is being dedicated to exploring green materials and new ways to process them (Ayres, 2019).

9.2 Current Trends

For several decades, bio-based plastics have remained more or less in the research phase. With crude oil remaining relatively inexpensive, it was challenging for any bio-based plastics to become established in the market. This century has seen some dramatic changes that have favored further research and development of these new plastics, and more applications and new molecules are now emerging. Today, a bioplastic alternative exists for almost every conventional plastic material and the corresponding use. While internal strengths for bioplastics appear to be their performance, which is equal to that of synthetic plastics (Kourmentza et al., 2017). The external drivers forcing manufacturers to adopt more green packaging labels are search for a stable source of raw material, increasing environmental awareness, and preference of consumers toward eco-friendly packaging, along with the regulatory tide that has been turning in the favor of bioplastics (Smith, 2008). Table 9.1 lists the types of policies that have been applied to support this nascent industry. Even some companies have mandated increased use of renewably sourced materials in their products for marketing differentiation, reduced environmental footprint, or weight reduction such as in automotive. Example – Toyota has set the goal of using renewable or recycled material in 20% of its plastic resin parts by 2015 (Rossi, Griffith, Gearhart, & Juska, 2005). Companies such as Coca-Cola, Danone, Ikea, Samsung, Procter & Gamble, Heinz, and others already have some of their products and/or their packaging made of bioplastic. LEGO started production of parts for their famous kits from bioplastics, and they plan to have it all produced from bioplastics by 2030. Supermarkets Iceland design only nonplastic packaging for their brands and also supermarkets Tesco would like to use only recyclable packaging (Resinex, 2019).

Undoubtedly, due to implementation of these legislations and changes, the trend toward alternative to conventional plastics will significantly accelerate. Besides, the demand for bioplastics and even recycled plastics will help attain a series of advantages that contribute to United Nations sustainability goals by reducing resource use, degradation, and pollution along the whole life cycle while increasing quality of life (Karan, Funk, Grabert, Oey, & Hankamer, 2019). Because of the fact that these bio-based plastics are produced from agricultural crop-based feedstocks (carbohydrates and plant materials), there is a good diversity of these bioplastics, available in the market.

Table 9.1 Countries with enacted plastic bag legislations.

China	In January 2015, China's ban on plastic bags and food service items in Jilin province created an unprecedented surge for bioplastics manufacturing in China. Also, China announced that it would no longer accept international plastic waste for recycling from 31 December 2017
France	In September 2016, France became first country to ban plastic cups and plates. A new French law will require all disposable tableware to be made from 50% biologically sourced materials that can be composted at home by January 2020. That number will rise to 60% by January 2025
Germany	The German markets have been seriously hampered by the country's unfavorable legislation on the waste generated that can be efficiently utilized for energy generation
India	In March 2016, central government issued a notification containing new plastic waste management rules. The new rules increase minimum thickness for carry bags from 40 (as regulated under Plastic Waste Management Rules 2011) to 50 μm in size and stipulates minimum 50 μm for plastics sheets; these are to be implemented across the country within six months after preparing relevant by-laws The rules also stipulate that manufacture and use of nonrecyclable multilayered plastic bags will be phased out in two years Major Indian states with a little but significant growth in the use of biodegradable alternatives due to an effective plastic ban – Karnataka, Uttar Pradesh
Italy	In 2015, when Europe enacted legislation to reduce the number of single-use carrier bags, Italy became the first country to promote the use of compostable plastic bags. January 2018, Italian lawmakers enacted a measure banning the use of plastic bags for fruits, vegetables, and baked goods in favor of eco-friendly biodegradable and compostable alternatives
Japan	Japan has set the objective to reach 20% bioplastics in the plastics market by 2020
Singapore	The Government has implemented a government waste management program. Eventually, this may impact the bioplastics market
Thailand	The country has emerged as a leading producer of feedstocks for the bioplastics industry
United States	The growth of bioplastics in the US regions have been influenced by some of the popular legal frameworks in the following regions. Several US states require plastic six-pack beverage rings to be photodegradable, and many new federal procurement programs have designed bioplastics as their preferred choice in areas such as adhesives, insulating foam, construction panels, films, carpets, glass cleaners, greases, and metalworking fluids New York – Plastic Bag Reduction, Reuse and Recycling Act; retailers of stores are to establish in-store recycling programs that provide an opportunity for the customer to return clean plastic bags to be recycled. District of Columbia – Ban on the use of disposable nonrecyclable plastic carryout bags and an imposition of fees on all other disposable carryout bags provided by certain retail stores California – As of 1 July 2015, certain large stores are prohibited from providing a single-use plastic carryout bag to a customer, unless the retailer makes that bag available for US $0.10 and certain conditions are met

9.3 Different Types of Bioplastics

This section presents a brief summary to all the kinds of bioplastics available in the market (Figure 9.1).

9.3.1 Bio-based Polyethylene (Bio-PE)

Bio-polyethylene is a bioplastic produced from sugarcane, using a fermentation process. Physically and chemically, it is a drop-in equivalent for traditional polyethylene – it does not biodegrade but can be recycled using established recycle streams, which makes implementation of bio-PE fairly easy (Jeffrey, 2013). Besides, Bio-PE are efficient to reduce global warming and greenhouse gas effects. The Bio-PE Life Cycle Assessment shows the capture of 2.0–2.5 tons of carbondioxide per ton of polymer versus the release of 2.5 tons of carbondioxide per ton of fossil polyethylene produced (Leonara, 2010). Braskem in Brazil is the world leader in the production of Bio-PE (annual production capacity of 200 000 tons), with the Green Polyethylene – "I'm green"™, a thermoplastic resin produced from ethylene made from sugarcane ethanol (Braskem, 2019). Recently, US-based Only Natural Pet, America's leading brand of natural pet supplies, launched its pet food line (Mindful Meals) in sustainable bio-based packaging, which has been developed using Braskem's sugarcane sourced I'm green™ biopolymer

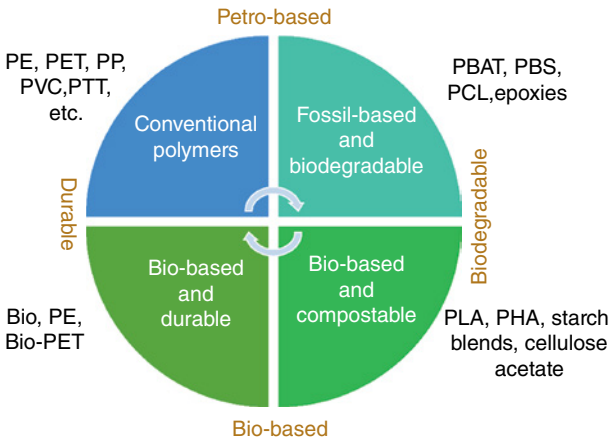

Figure 9.1 Polymers in market. *Source:* Modified from Europeanbioplastics (2017a). PE, polyethylene; PET, poly (ethylene terephthalate); PP, polypropylene; PBS, poly (butylene succinate); PBAT, polybutylene adipate terephthalate; PCL, Polycaprolactone; PLA, polylactic acid; PHA, polyhydroxyalkanoate.

(Philadelphia & Boulder, 2018). Applications like these are ideal to further the sustainability goals and satisfy the ongoing commitments to environmental sustainability and carbon reduction. Business wise, bio-based PE has been on the market for a few years, but its production capacity has hitherto remained the same. Further developments have been slowed down because of the shale gas boom (Aeschelmann & Carus, 2019).

9.3.2 Bio-based PET

The global bio-PET market is one that is growing fast. Due to eco-friendly nature and superior properties than PET such as high versatility, strength, and recyclability, bio-PET is witnessing huge demand from various end-use industries such as food and beverages, cosmetics and pharmaceuticals, automotive, textile, and so on. In 2017, beverages containers accounted for the largest share, followed by films and sheets and food containers (Jose, 2018). Geographically, Asia-Pacific followed by North America dominated the market in 2017 by value and volume, with majority of the demand coming from economies such as India and China owing to booming food and beverages, textile, and automotive industries, rising middle class population with high disposable income and rapid economic growth. Globally, bio-based PET market expected to grow at the compound annual growth rate (CAGR) of 20.8% from 2018 to 2026, aided by technical innovation, rising consumer awareness about the use of green products, and strict government and environment regulation for reducing GHG emission market (Jose, 2018).

Traditional bio-PET was a resin manufactured from bio-based mono-ethylene glycol (MEG), which is obtained from plants such as sugar cane and sugar beet by thermochemical process, and petroleum-based terephthalic acid (PTA), in a ratio of 30 : 70. With the aim of development of 100% bio-based PET, many companies including Avantium (Netherlands), Toyota Tsusho Corporation (Japan), Toray Industries (Japan), and Gevo Inc. (USA) are investing in the pilot-scale production of bio-MEG (Bioplastics, 2018). Also, bio-based PET can be used in all existing application of conventional PET, from beverage containers, food containers, non-food containers to films and sheets, molded parts and components, and fabrics. In 2015, Coca-Cola launched the world's first PET plastic bottle made 100% from plant materials, in its alliance with Virent, Gevo, and Avantium, under its Plant PET Technology Collaborative initiative (Guzman, 2015). Due to this initiative, bio-based PET production has witnessed the most dynamic development in the ongoing decade, with production capacity being around 600 000 tons in 2013 and projected to reach about 7 000 000 tons by 2020 (Aeschelmann & Carus, 2019) (Figure 9.2).

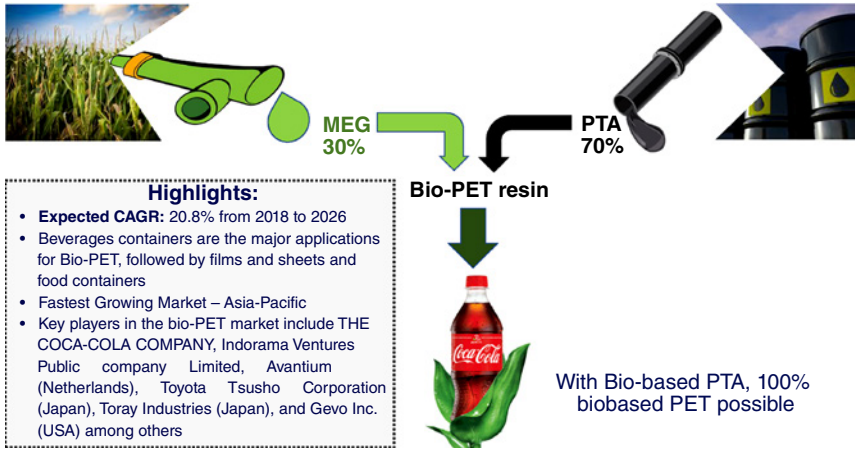

Figure 9.2 Summary of Bio-PET business and current trend. MEG, mono-ethylene glycol; PTA, petroleum-based terephthalic acid.

9.3.3 Polylactic Acid

PLA (polylactic acid or polylactide) is by far the most promising bioplastic for the near future. It is a thermoplastic aliphatic polyester derived from renewable resources, such as corn starch (in the United States), tapioca roots, chips, or sugarcane (mostly in Asia), or sugarcane (in the rest of the world). But, characteristically, PLA resembles conventional fossil fuel-based plastics such as PE, PP, and PET. Since PLA can easily be processed on manufacturing facilities that already exists for the production of common petrochemical-based plastics, and no further industrial investments are required, the market for bio-PLA is expected to register a CAGR of ~20.49% during the forecast period of 2019–2024 (PortNews24, 2019). Commercially, Nature works (USA) that manufactures PLA Ingeo™, by polymerizing lactic acid obtained by fermentation of maize plant sugars, leads the market. Since PLA has properties typical of semicrystalline thermoplastic polymers, they can be used in a wide variety of applications (Figure 9.3).

In applicability, PLA usage in the packaging industry dominated the market in 2018 for the production of plastic cutlery, kitchen trays or 3D filaments (Resinex, 2019). In addition, PLA and PLA copolymer blends are also been used successfully for medical and pharmaceutical purposes such as the manufacturing of medical implants that can be resorbed by the body, and as drug carrier or MRI contrast agents. Example, in 2016, Abbott made Adsorb – a stent based on PLA, which gets dissolved in two to three years leading to fewer complications for the patients (Ang et al., 2017). PLA is also the most common type of plastic filament

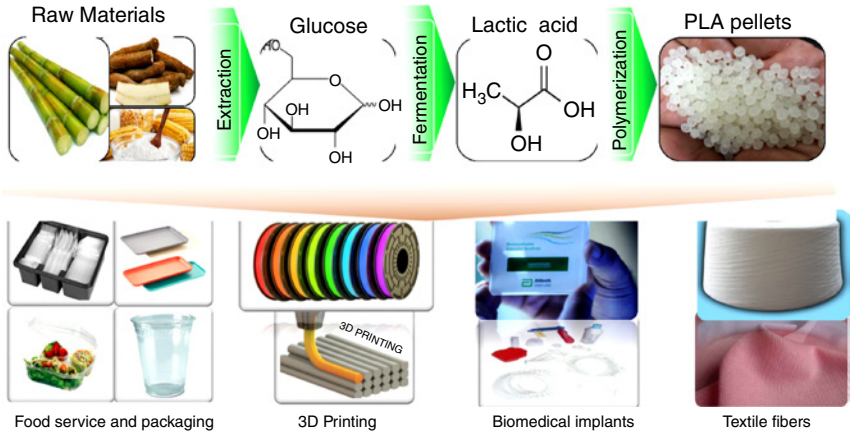

Figure 9.3 Polylactic acid (PLA) polymer in the market.

used for home-fused deposition modeling. Further, the growing production of genetically modified corn and the growing usage of bio-PLA in 3D printing are likely to act as opportunities in the future (PortNews24, 2019).

9.3.4 Starch Blends

Thermoplastic starch, accounting for about 18.8% of the 2.05 million tons of bioplastics produced in 2017 worldwide, is yet another, the most significant, and widely used bioplastic. To prepare them, the starch granules are plasticized using different plasticizers, such as sorbitol and glycerine, under heating and shearing, giving rise to a continuous phase in the form of a viscous melt, which is then processed using traditional plastic processing techniques, such as injection molding and extrusion (Kaseem, Hamad, & Deri, 2012).

Typically, use of starch helps in cost reduction and rising biodegradability of resultant composite. But, poor water resistance, processability, dimensional stability, and low strength for its end products are limiting factors for the use of materials manufactured only from starch. Hence, the modification of starch is often achieved by blending water insoluble and biologically degradable polymers like polyester, polyesteramids, polyesterurethanes, or polyvinylalcohols to form a waterproof starch plastic and improve its cost competitiveness whilst maintaining other properties at an acceptable level(Lu, Xiao, & Xu, 2009; Wang, Yang, & Wang, 2003). The commonly used polyesters are PHA, obtained by microbial synthesis, and polylactide (PLA) or poly(ε-caprolactone) (PCL), derived from chemical polymerization. The use of cheap natural polysaccharides like cellulose and chitosan is also being investigated as a blend with starch. Also, starch composites can incorporate recycled

plastics. For example, recently, BioLogiQ (Idaho Falls, Idaho; www.biologiq.com) has introduced NuPlastiq, a biodegradable plastic resin produced from waste starch at potato-cutting facilities (for French fries and potato chips), and they blend it with conventional polyethylene resins (PE) from Dow, Midland, Michigan, to form thinner films with the same strength (Jenkins, 2019; Sherman, 2019).

In terms of applications, with the introduction of improved resin grades, their ability to blend with other biopolymers, increasing number of suppliers, along with their compostibility and high strength, the use of starch-based plastics is prevalent. Since long they have been used in packaging industries, as food service disposables, and as compostable yard and kitchen bags. Besides, they also find applications in the medical industry because of their biocompatibility, low toxicity, degradation properties, and mechanical properties. Of late, the Green Dot Bioplastics team has successfully developed cell phone cases from their compostable, starch-based bioplastic composite "Terratek SC 50 and SC 65." The Terratek SC line is a proprietary blend of wheat starch and polypropylene made up of 65% renewable material and is ideal for virtually unlimited applications in the production of both pliable and hard plastic products, including tableware, golf tees, credit cards, and more (Greendot, 2019).

9.3.5 Polyhydroxyalkanoate

One of the most exciting developments in biopolymers of late is PHA. Derived from renewable feedstocks by a fermentation process involving microbes instead of crude oil, PHAs are 100% bio-based, biodegradable, and compostable polyesters not only in industrial composting plants but also in other environments, such as marine waters. Also, PHAs and their copolymers such as P(3HB-co-3HV) feature a great variety of thermoplastic, crystallization, elastic behavior, and mechanical resistance properties (Kourmentza et al., 2017). Overall, PHAs have the ability to get blended with various natural and synthetic materials, changing their composition and molecular arrangement, and hence some of their basic properties like plasticity, temperature characteristics, and physical strength (Raza, Riaz, & Banat, 2018). This versatility has today posed them as high impactful and attractive biomaterials, suitable for widened end-user applications and products in everything from automotive, healthcare, biomedical, consumer goods, and packaging segment to electronics and 3D printing.

An annual market data update, presented at the 12th European Bioplastics Conference in Berlin, 2017, figures that the global PHA production capacity is predicted to grow by 20% over the next five years from around 2.05 million tons in 2017 to approximately 2.44 million tons in 2022, with a CAGR of 22.4% (Europeanbioplastics, 2017b). Moreover, the fact that PHAs are a microbial product leaves ample space to develop PHAs with more unique monomer compositions

economically through metabolic engineering approaches. Hence, the current market for PHAs is characterized by a dynamic growth rate, and it is hoped that PHAs will be able to replace petrochemical plastics in these areas as production processes improve. However, their economic sustainability on an industrial scale is being questioned, as the cost of production is currently prohibitive to all applications except higher-value medical uses. While the price of polymers such as PP and PE is around US $0.60–0.87/lb., PHA biopolymer cost is estimated to be 3–4 times higher, ranging between US $2.25–2.75/lb. (Kourmentza et al., 2017).

Presently, one of the major bottlenecks is the cost of high-purity carbon substrates, comprising of pure carbohydrates (glucose, sucrose, maltose, starch), fatty acids, and its derivatives, which have been estimated to be 28–50% of the total production process (Aslan, Ali, Morad, & Tamunaidu, 2016; Kourmentza et al., 2017; MdDin et al., 2012; Reddy, Ghai, Rashmi., & Kalia, 2003). These sugars from first-generation food crops such as corn starch, sugarcane juice, and vegetable oils can be extracted with little effort. But, utilizing these carbon sources competes with food supply production, besides raising concerns over the sustainability of PHA production in terms of their impact on food prices (Jiang et al., 2016). Consequently, it is essential to exploit non-food-based carbon sources for sustainable production of PHA.

Next, in terms of applicability, plastic packaging is the single largest market for PHA polymer resins. PHA can be incorporated into packaging components such as coatings, laminations, and biodegradable printing inks and can thus be used for articles of everyday use like single-use flatware, cups and extruded vials, jars, bottles, containers and boxes for shampoos, lotions, etc. (Muhammadi, Shabina, Afzal, & Hameed, 2015). Copolymer blends within the family of PHAs have been used to produce waterproof barrier coatings for paper and cardboard, product packaging film, foamed articles, as well as injection-molded articles for packaging. For example, Biopol by ICI/Zeneca, a copolymer of polyhydroxybutyrate – polyhydroxyvalerate (PHB-PHV) is used to produce back of diaper sheets (Nanda, Sasmal, Panigrah, & Nayak, 2009). Similarly, Metabolix's amorphous Mirel® PHA has been in use as a performance modifier for PLA-based clear, bio-based, compostable "clam shell" thermoform containers that provide excellent surface for printing and is suitable for a broad range of applications in food and consumer packaging. In a joint effort with the U.S. Army Natick Soldier Research, Development and Engineering Center is developing Mirel-brand PHA-based latex coating to improve the moisture resistance of paper articles used in the production of water-resistant shipping containers and waste bags for naval vessels (Lingle, 2015).

Findings recommend that PHA is an apt candidate for the designing of resorbable medical frameworks such as in sutures, meshes, implants, and tissue-engineering scaffolds, due to their diverse and ascendant mechanical, biodegradable, and tissue compatible properties (Koller, 2018). The hydrolytic degradation of PHA results in the formation of 3-hydroxy butyric acid, a usual component of blood, and thus PHAs are nontoxic to the cells and tissues (Zhang,

Shishatskaya, Volova, da Silva, & Chen, 2018). Notably, a number of in vivo experiments that have been carried out to date with PHA-based materials and not a single evidence for carcinogenic effects had been evidenced (Koller, 2018). This biocompatibility enables development of new applications for PHAs in the medical field. Furthermore, the biocompatibility and FDA approval have supported the investigation of PHAs in the packaging and delivery of pharmaceutical agents and cells for medical applications. PHAs can be turned into microcapsules, microspheres, and nanoparticles and therefore used potentially as intracellular-sustained drug-release vectors (Hartley Yee & Ray Foster, 2015).

Production of agricultural mulches, seeding strips, and tapes made from biodegradable polymers is another strong potential market (Ashter, 2016). The tape biodegrades in the soil as the seeds germinate and take root. Plastic mulch films are then used to give the new seedlings a head start in the spring; the mulch helps reduce evaporation and conserve moisture, increases soil temperature, and keeps control of the weeds. Compostable seed belts and active component capsulations made from bioplastics have also proven to be beneficial. Some research groups have also evaluated the use of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) as a degradable matrix enabling controlled release of pesticides and herbicides (Prudnikova, Boyandin, Kalacheva, & Sinskey, 2013) (Figure 9.4).

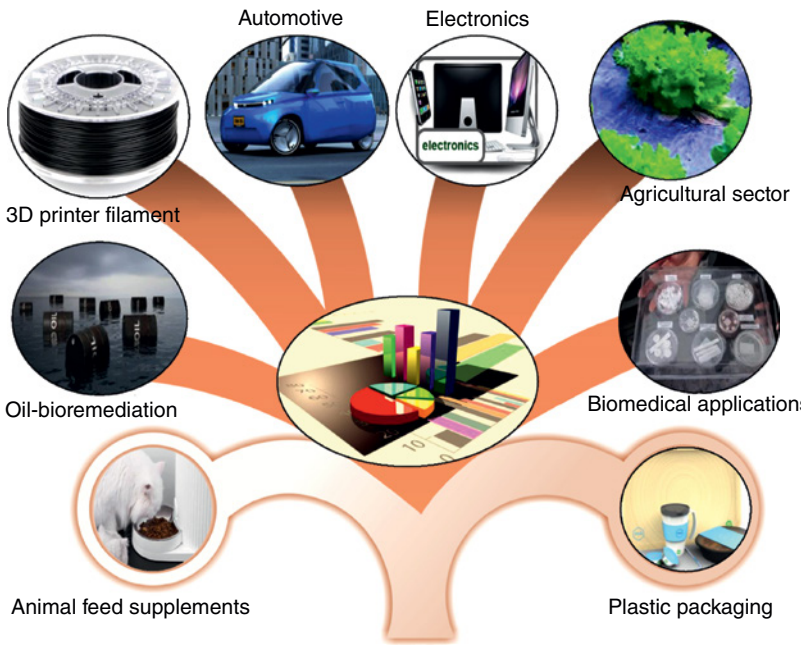

Figure 9.4 Polyhydroxyalkanoate (PHA) market applications.

Furthermore, technological developments have expanded the range of PHAs applications to include keyboard elements, mobile phone covers, and selected automobile components, which have now assumed significant magnitudes. Mitubishi's Durabio, a bio-based highly transparent isosorbide-based engineered PHA, is highly resistant to heat and humidity and has been used by the company in touch panels on automobiles, TV, and smartphone screens (BioplasticsNews, 2014). The use of bioplastics in 3D printing has also been investigated, and Biome 3D is one such material made from plant starches. Biome3D is a biodegradable plastic that combines easy processing and a superior print finish, while offering much higher print speeds, reducing overall job times. They are a great example of how sustainable alternatives can gain market share based on their performance, rather than just their "green credentials" (BiomeBioplastics, 2015). Another emerging application for PHA-based tools is to mitigate the health and environmental challenges (elevated radionucleotide concentrations) during hydrofracking process in shale gas industry (Ashter, 2016). And, with Minerv Biorecovery, a Bio-on's patented technology, which enables hydrocarbon sea pollution to be eliminated within approximately three weeks, it extends the unprecedented application of PHA even for environmental recovery and bioremediation of hydrocarbon pollution (oil-bioremediation) (Bio-on, 2017).

Overall, PHAs have the ability to get blended with various natural and synthetic materials, changing their composition and molecular arrangement, and hence some of their basic properties like plasticity, temperature characteristics, and physical strength (Raza et al., 2018). Moreover, the fact that PHAs are a microbial product leaves ample space to develop PHAs with more unique monomer compositions economically through metabolic engineering approaches. And as an add on, stringent government regulations on conventional plastics globally, accompanied with increased funding in R&D for the development of environment-friendly products, are also driving the PHA market high. This versatility has today posed them as high impactful and attractive biomaterials, with highly varied and evolving industrial applications and products manufactured using them.

9.3.6 Polybutylene Succinate

PBS is one of the newest biodegradable aliphatic polyester made by polycondensation of succinic acid and 1-4 butanediol (BDO), with properties that are comparable to polypropylene. Earlier, PBS was exclusively derived from fossil raw materials but can now be 100% bio-based depending on whether succinic acid and BDO are fossil based or derived biologically. Both succinic acid and BDO can be produced by fermentation (Myriant and BioAmber, USA) based on renewable feedstocks (such as glucose, sucrose, and bio-based glycerol) and provide an improved material carbon footprint compared to petrochemically derived succinic acid and BDO (Succinity, 2019). Also, use of bio-based succinic acid and BDO enables PBS to be

of lower cost and 100% renewable, which would expectedly improve PBS growth and profit. There are already a few producers with existing commercial capacities for the production of bio-PBS, like Succinity (Germany), Mitsubishi Chemicals (Japan), and Genomica-Novamont (USA), and additional dedicated and non-dedicated capacities are expected to start up in the coming years.

Technically, PBS has a better melt processability and thermal and chemical resistance. Also, PBS has high compatibility with a fiber (Succinity, 2019) and is much more flexible and thus does not require plasticizers, compared to other biodegradability resins. With many interesting properties, including biodegradability, bio-PBS has found its way into applications where composability is important, for instance in agricultural mulch films, plant pots, hygiene products, fishing nets, and fishing lines. It is possible to use PBS in electronics and other consumer goods applications, as well as various (food) packaging applications such as teacups, plates, and bowls. Another interesting application for PBS is in combination with PLA for 3D printing (PPD, 2019).

9.3.7 Polybutylene Adipate Terephthalate

PBAT is a semi-aromatic, biodegradable thermoplastic copolyester, with the precursors of PBAT, the same as PBS with the addition of adipic acid. This makes PBS and PBAT relatively similar. But PBAT can theoretically be up to 50% bio-based since bio-based adipic acid is not available yet. It is still at the research stage (Aeschelmann & Carus, 2019). Commercially, PBAT has mostly been produced by one big company, BASF (Ecoflex), but a new player, Jinhui Zhaolong High Technology, entered the market and another one, Samsung Fine Chemicals, which has a relatively small production capacity at the moment, is planning to extend its production capacity (Aeschelmann & Carus, 2019).

Technically, PBAT's properties are very similar to polyethylene, for example it has relative high elongation at break (30–40%), as well as moderate high impact and puncture toughness, but only moderate tensile strength and low stiffness (Shahlari & Lee, 2012). It thus can be used for similar applications in food packaging and agricultural film applications. Furthermore, it is fully biodegradable (compostable) and can be processed on conventional blown film equipment used for polyethylene. PBAT has also been investigated as a toughening agent for PLA (PPD, 2019). Blending with PLA would also reduce both the cost of the ultimate blend and PBAT dependence on nonrenewable resources.

9.3.8 Polycaprolactone

Prepared by catalyst-driven ring-opening polymerization of ε-caprolactone, PCL is a semicrystalline, hydrophobic polymer with a low melting point of 63 °C, a glass-transition temperature of 60 °C, and superior viscoelastic properties (Saad &

Suter, 2001). This makes PCL easy to be processed and manipulated into varied shapes and sizes. Also, due to its ester linkage, it is hydrolysable under physiological conditions (such as in the human body). Hence, PCL is considered bioresorbable, as well as biodegradable, and has therefore received a great deal of attention for use as an implantable biomaterial and microparticles for drug delivery (Chang, Lee, Park, Kim, & Jeong, 2018).

In particular, it is especially interesting for the preparation of long-term implantable devices and controlled release of therapeutic molecules (e.g. drug, antibiotics, protein, gene) in target tissues over a period of time, owing to its degradation, which is slower than other biodegradable polyesters in physiological conditions. However, when it comes to tissue engineering, PCL suffers from some shortcomings. Its poor surface wetting and interaction with biological fluids on account of its hydrophobicity leads to poor cell adhesion, proliferation, and mechanical strength. Hence, it is blended with other synthetic/natural polymers. The incorporation of chitosan (Shao et al., 2012), calcium-phosphate-based ceramics, and bioactive glasses (Hajiali, Tajbakhsh, & Shojaei, 2018) into PCL has yielded a class of hybrid biomaterials with remarkably improved mechanical properties, controllable degradation rates, and enhanced bioactivity that are suitable for bone tissue engineering. PCL is used in the rapidly growing field of human esthetics following the recent introduction of a PCL-based microsphere dermal filler belonging to the collagen stimulator class (Ellansé) (Moers-Carpi & Sherwood, 2013). With incorporation of collagen, PCL-based products are also thought to be able to correct facial ageing signs such as volume loss and contour laxity (Kim & Van Abel, 2015). A number of review articles provides an insight into the recent developments and challenges of PCL-based biomaterials as a critical component of new therapeutic strategies for many diseases, covering the studies performed in the last decades (Malikmammadov, Tanir, Kiziltay, Hasirci, & Hasirci, 2018).

9.3.9 Epoxies

Epoxies are approximately 30% bio-based and are produced out of bio-based epichlorohydrin. The market is well established and is not expected to grow much since epoxies have already long been partly bio-based (Aeschelmann & Carus, 2019).

9.3.10 Cellulose Acetate

Cellulose acetate (CA) is 50% bio-based. This market is similar to that of epoxies: well established, for example cigarette filters are made from CA, with small growth.

9.4 Challenges Facing the Bioplastics Industry

Although bioplastics have many advantages, it is equally important to consider that the manufacturing of bioplastics reveals potential drawbacks. Bioplastics production is still a complicated process with a small scale of productions in discontinuous batch and fed-batch cultivation modes accompanied by low efficiency and high production cost. On the other hand, the industrial processes for making petrochemical plastic have been in place for decades, so the production chain is very efficient (Wang, Yin, & Chen, 2014); Second, plentiful shale gas promises are keeping the price of conventional plastics low, and this situation makes bioplastics less competitive compared with low-cost petroleum-based plastics (Goldsberry, 2018); Third, high cost of refined substrates, such as corn starch, cane sugar, or cellulose (currently the major raw materials for bioplastics production), can account for 30–50% of the total production cost (Kourmentza et al., 2017; Reddy et al., 2003; Zulkafli, 2014). Moreover, a dependency on the use of refined sugars and polysaccharides, derived from first generation (sugar-based) feedstocks as feedstock in polymer industries could directly interfere with food and feed chain and raises food prices by diverting edible crops; Fourth, bioplastics require large amounts of fertilizers, pesticides, and land to manufacture the agricultural crops, which some sections believe can, in future, lead to conversion of land to agriculture (Grabianowski, 2018). Fourth, usage of large amount of solvents and/or labor during their downstream processing, further elevates the cost, besides compromising with the green tag associated with bioplastics (Choi & Lee, 1999). And last but not the least, the land required for bioplastics competes with food production because the crops that produce bioplastics can also be used to feed people. The Plastic Pollution Coalition projects that to meet the growing global demand for bioplastics, more than 3.4 million acres of land – an area larger than Belgium, the Netherlands, and Denmark combined – will be needed to grow the crops for bioplastics (Cho, 2017). This has created an ongoing public, political, and industrial debate, with wide-reaching implications, on the competition between food, animal feeds, and industrial markets for agricultural raw materials.

9.5 Misconceptions and Negative Impacts

Hearing the term bioplastics, we universally believe that they all are biodegradable. But biodegradability and recycling are problems for bioplastics too. Some bioplastics biodegrade but do not compost: these bioplastics biodegrade when exposed to oxygen and ultraviolet radiation (naturally under sunlight). But they do not completely decompose and keep on releasing toxic chemicals and gases including

methane when dumped. Other plastics are designed to biodegrade when composted. However, these require high temperatures to degrade, and most cities lack the infrastructure for proper bioplastic composting, and unfortunately, require industrial composting processes (Grabianowski, 2018). Hence, bioplastics can be a part of the solution, but they are not the golden bullet everyone is making out to be. Being biodegradable, they can break down completely through the action of active microorganisms, although they make no promises about not leaving a toxic residue behind. Bioplastics often end up in landfills where, deprived of oxygen, they may release methane, a greenhouse gas 23 times more potent than carbondioxide.

Moreover, recycling of bioplastics is another issue worth considering. Bioplastic material might actually contaminate the recycling process if not separated from conventional plastics. Example, small accidental mix of PLA plastic with PET plastics in the recycling stream lessen the quality and value of the resulting recycled plastic products. Moreover, with chief difficulty in differentiating bioplastics from regular plastics via naked eyes in manual sorting plants, end of different kinds of bioplastics in a recycling plant, means more of plastic and garbage. Hence, being just bio-based does not resolve the litter issue and being biodegradable or biomass based is not synonymous with being environmentally friendly or sustainable (PROEUROPE, 2009).

9.6 Take Home Message and Future Directions

Despite all the exciting research and breakthroughs in the field, the market for bioplastics remains small. It is valued at around US $1.6 trillion currently, accounting for even less than 1% of the entire plastics market. However, this percentage is rapidly increasing and estimates predict it could be more than double by 2030 if nothing is done to prevent this. But there are three reasons why bioplastics have not gone mainstream yet, namely (United Biopolymers, 2017). Lack of functionality of existing bioplastics; higher costs compared to PE when used for commodity applications (Bioplastics are 2.5× to 10× more expensive than normal plastics); and small and unreliable supply chain (bioplastics are only available in small quantities from single suppliers). Process economics have revealed that the use of inexpensive and renewable carbon substrates as biopolymers carbon feedstock can result in as much as 40–50% reduction in the overall production cost (Akaraonye Everest, Keshavarz Tajalli, & Roy Ipsita, 2010). While the engineering of platform organisms broadens the spectrum of possible substrates, the efficiency of the process could be increased by the use of organisms naturally capable of degrading lignocellulose-derived sugars. Hence, there is also a major interest in utilizing non-food crops, such as lignocellulosic, for production of drop-in polymers or new dedicated bioplastics (Brodin et al., 2017). From the economic point of view,

lignocellulosic biomass can be produced quickly and at lower cost than other agriculturally important biofuel feedstocks such as corn starch, soybeans, and sugar cane. It is also significantly cheaper than crude oil. The major component of lignocellulosic biomass, cellulose is considered to be the strongest potential candidate for the substitution of petroleum-based polymers owing to its eco-friendly properties like renewability, biocompatibility, and biodegradability. Furthermore, lignocellulosic feedstocks have crucial advantages over other biomass supplies in this manner because they are the nonedible portion of the plant, and therefore, they do not interfere with food supplies. Moreover, forestry, agricultural, and agro-industrial lignocellulosic wastes are accumulated every year in large quantities and are much readily available. Disposal of these wastes to the soil or landfill causes serious environmental problems; however, they could be utilized for the production of a number of value-added products, and this can be a real aid towards cost-effective production of bioplastics at an economic rate from the production side.

From the side of consumption, there is a clear need for a regulated communication on both the labels and sorting instructions, as well as their sustainability. The different labeling on bioplastics packages, such as "biodegradable," "home compostable," "compostable," and "degradable," should be made more descriptive, with whether and how consumers should sort them, and which are the most sustainable waste management options to treat them. Even users must be educated about bioplastics and proper recycling techniques to discern which bioplastics are biodegradable and which are compostable. Add on, material producers and retailers using these new materials have a responsibility for introducing them in a responsible and coordinated manner so that previous education efforts for waste prevention and recycling are not ruined. Besides, controlled recycling streams that can detect bioplastics as "Non-PET," and separate 100% bioplastics with PET plastics, could enable see a significant reduction in the environmental impact of plastic materials.

In all, biodegradable polymers may not be a one-stop solution to all environmental problems created by plastics, but it is a step in the right direction as time is of essence for biodegradable polymer development as society's current views on environmental responsibility make this an ideal time for further growth of biopolymers.

9.7 Questions for Thought

1) Do bioplastics really work as substitute for conventional plastics?
2) Can lignocellulosic biomasses really act sustainable materials for producing cost-competitive bioplastics?
3) Which biodegradable materials will be replaced in the future by plastic that is being phased out of production?

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Conflict of Interest

The authors declare no conflict of interest.

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10

Conversion of Lignocellulosic Biomass to Ethanol: Recent Advances

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10.1 Introduction

The current energy for transportation is mainly fossil fuel. The increasing global population and urbanization resulted in increasing demand for transportation energy worldwide. But the continuous burning of conventional fossil liquid fuels (petrol and diesel) emits large amount of green-house gases (GHG) to the environment. This is evident from the recent global warming and climate change. Bioethanol produced from plant materials attracted importance as an alternative fuel to replace petrol due to economic and environmental benefits. Bioethanol minimizes the environmental accumulation of GHG, since its production and combustion cycle result in net zero carbon emission to environment. The sugarcane juice and starch grains are the first-generation feedstocks used for bioethanol production. The ethanol production from these feedstocks is simple. The sugar is directly fermented to ethanol, and starch is hydrolyzed to simple sugars and subsequently fermented. But the long-term use of first-generation feedstock leads to conflict with food supply. Therefore, the second-generation feedstocks comprising nonedible LCB attracted attention for bioethanol production. In contrast to first-generation feedstocks, lignocellulosic biomass (LCB) contains a recalcitrant network of lignin-carbohydrate complex (LCC). Hence, the conversion of the cellulosic polysaccharides to ethanol is challenging (Raj and Krishnan, 2020). The conventional biochemical processing of LCB comprises three steps, viz., pretreatment, enzymatic hydrolysis, and fermentation. The major hurdle hindering the commercial viability of the process is the economical conversion of LCB into

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concentrated fermentable sugar syrup using an appropriate pretreatment and efficient enzymatic hydrolysis. The production of high concentration of sugar is important to achieve high ethanol titer in the subsequent fermentation step. The concentration of ethanol in the final fermentation broth should be >4% to minimize the energy utilization in distillation (Qin et al., 2018). The processing of LCB at high solids loading (HSL) (>20%) is required to achieve desirable concentration of fermentable sugars and ethanol (Chen and Liu, 2017). However, the heterogeneous reactions of pretreatment and enzymatic hydrolysis often encounter various problems. This chapter describes the various aspects of LCB and its biochemical processing to ethanol.

10.2 LCB: Structure, Composition, and Recalcitrance

LCB is composed mainly of plant cell wall. Figure 10.1a shows hypothetical depiction of the structure of plant cell wall. The plant cell wall contains three strata or layers, viz., primary cell wall, secondary cell wall, and middle lamella (Zeng et al., 2017). The thin and flexible primary cell wall forms during the cell growth. The thick layer of secondary cell wall forms beneath the primary cell wall. The outermost layer, middle lamella, surrounds the primary cell wall and occurs in between two adjacent cells. While primary and secondary layers contain high amount of structural polysaccharides, middle lamella contains high level of lignin. The polysaccharides and lignin account for about 60–70% and 20–30% of dry weight of cell wall, respectively (Figure 10.1b). The other components in the

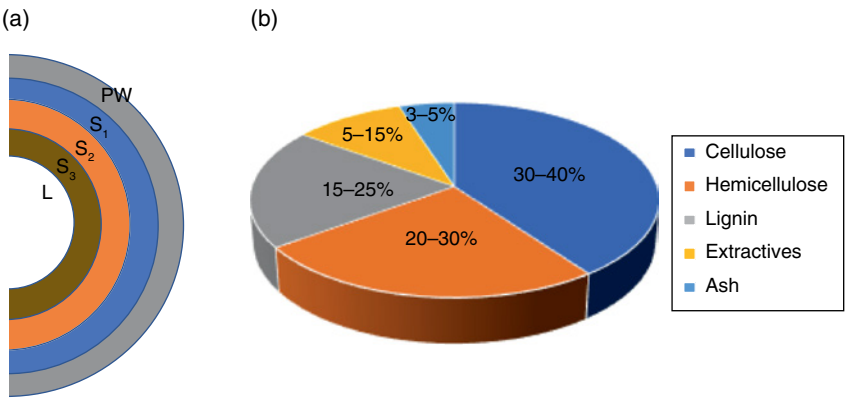

Figure 10.1 (a) Layers in plant cell wall. L – Lumen, PW – Primary wall, S_1, S_2, S_3 – Secondary wall layers, (b) approximate composition of biomass.

cell wall include proteins, ash, and extractives. Food crop residues and energy crops are considered as the abundant and promising LCB for cellulosic ethanol production. The compositions of some of the conventional crop residues and energy crops are given in Table 10.1.

Cellulose and hemicellulose are the major polysaccharides in LCB. Cellulose is made up of glucose monomers linked by β-1,4-glucosidic bonds (Figure 10.2a). Hemicelluloses are heterogeneous polymers comprising mainly pentosans with substitutions. Xylan, a polymer of xylose (pentose) linked by β-1,4-xylosidic bonds, is the main component of hemicelluloses in most LCB (Figure 10.2b). The xylan polymer is usually substituted with acetate, arabinose, glucuronic acid, and methyl glucuronic acid. The substitutions in the xylan polymer are involved in the formation of LCC network through covalent bonds. Lignin is a phenolic polymer. The structure of monomeric units forming lignin polymer are given in Figure 10.2c. Because of the LCC formation between the cell-wall components, the LCB is highly recalcitrant to biocatalytic degradation (Raj and Krishnan, 2020).

10.3 LCB to Ethanol: Bioprocess Strategies

The biochemical processing of LCB employs a pretreatment to break the LCC network. The biochemical processing of LCB to ethanol involves three major steps, viz., pretreatment, enzymatic hydrolysis, and fermentation (Figure 10.3). These three steps are conducted separately in a process called separate hydrolysis and fermentation (SHF). An improvised process combines enzymatic hydrolysis and fermentation into one step, known as simultaneous saccharification and fermentation (SSF). Both SHF and SSF require addition of external saccharifying enzymes. In an advance consolidated bioprocessing (CBP), a single ethanologenic microbe-secreting cellulases performs saccharification and fermentation in one step without the addition of external enzymes.

10.4 Pretreatment of LCB

Pretreatment disintegrates the LCB's recalcitrant structure and exposes the cellulose chains for cellulase's action. Different pretreatment methods use hot water, acid, alkali, and solvent to alter the structure and composition of LCB. The recovery of the polysaccharides and their subsequent conversion to ethanol determines the pretreatment efficiency. Though high pretreatment severity effectively disintegrates LCC network, some amount of the polysaccharides also degrade to toxic chemicals inhibiting the biocatalysts. A desirable pretreatment should completely recover the polysaccharides with less toxic compounds and facilitate further

Table 10.1 Composition of crop residues and energy crops.

S. No.	Biomass	Glucan (%)	Xylan (%)	Acid-insoluble lignin (%)	Acid-soluble lignin (%)	Extractives (%)	Ash (%)	References
1	Rice straw	35.3	23.8	13.1	4.4	14	11.3	de Assis Castro et al. (2017)
2	Wheat straw	33.7	19.1	16	3.8	10.3	9.7	Zheng et al. (2018)
3	Sugarcane bagasse	42.2	27.6	18.1	3.5	5.6	2.8	de Moraes et al. (2015)
4	Sorghum bagasse	41.5	27	26	1.5	10.2	1.8	Khalili and Amirri (2020)
5	Corn stover	39.6	26.7	17.7	0.7	12	7.2	Qin et al. (2018)
7	Napier grass	41.8	23.2	23.4	1.6	8.1	1	Tsai et al., (2018)
8	Miscanthus	43.1	23.6	19.5	4.2	7.7	1.9	Bergs et al., (2020)

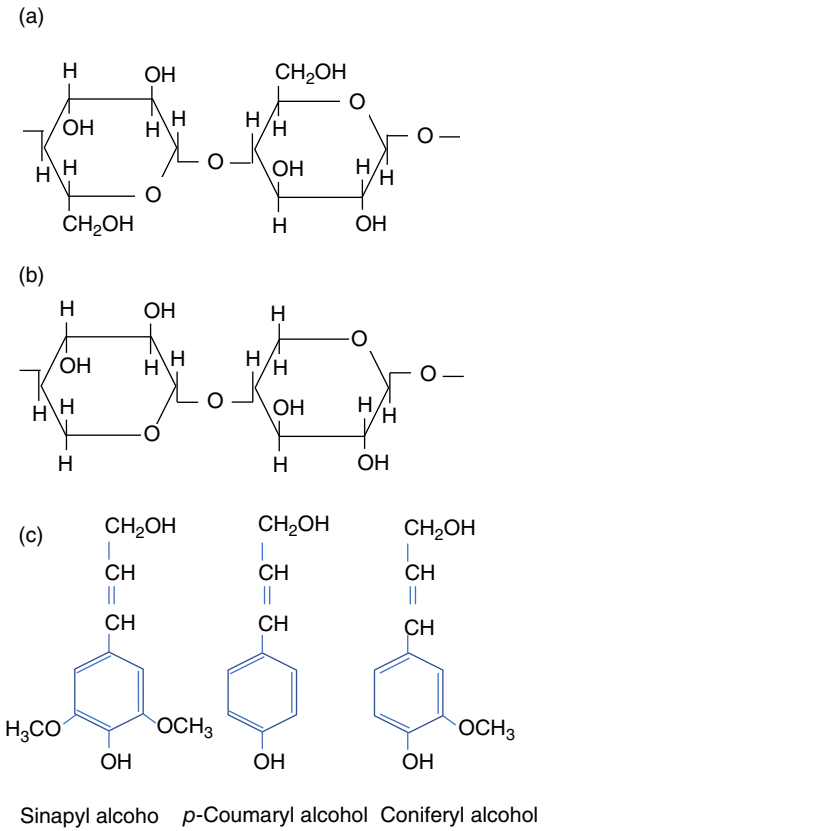

Figure 10.2 Structure of (a) cellulose, (b) xylan polymer, and (c) lignin monomers.

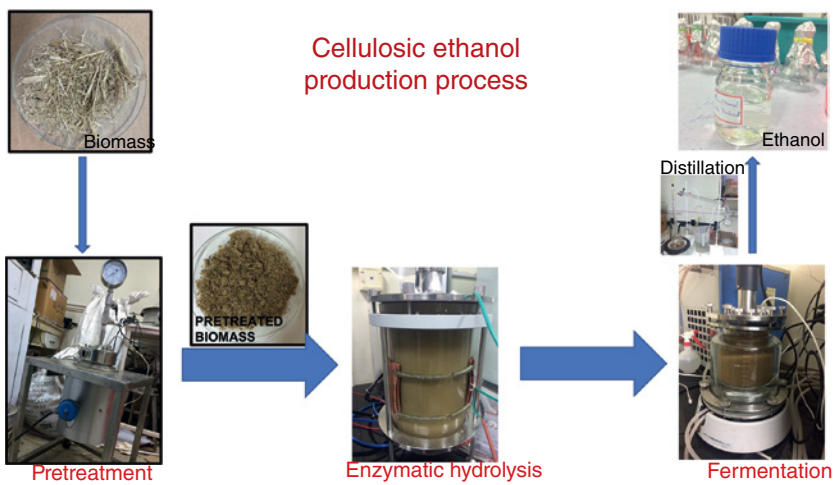

Figure 10.3 Biochemical processing of LCB to produce cellulosic ethanol.

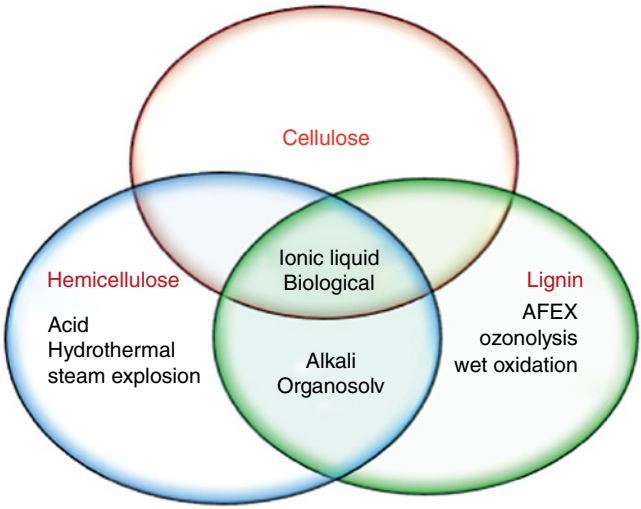

Figure 10.4 Effect of different pretreatment types on the degradation of lignocellulosic components of biomass.

biocatalytic processes. The other common characteristics of an ideal pretreatment include low capital cost, less heat and power requirement, noncorrosive, and easy operation (Alvira et al., 2010). The method and conditions of pretreatments differ with the nature of the LCB due to the variation in the composition and recalcitrance of biomass. For example, the degradation of LCC structure is more difficult for wood than crop residues (Alio et al., 2019). Different pretreatments have been evaluated for the disintegration of softwood and hardwood biomass. Bhalla et al. (2019) have reported higher enzymatic hydrolysis efficiency for the commercial hardwoods (*Eucalyptus* ssp. and hybrid poplar) pretreated using solvents, Cu-catalyzed alkaline hydroxide, and ionic liquids. The pretreatment methods are broadly classified into physical, physicochemical, chemical, and biological. Figure 10.4 shows the overall pretreatment's effect on the major lignocellulosic components. The pretreatment selection depends on the removal of desired component in the biochemical process. The effects of different pretreatments on different LCB are given in Table 10.2.

10.4.1 Physical Pretreatment

The physical pretreatments comprise mechanical, irradiation, and ultrasound treatments. The mechanical pretreatments like grinding, milling, and extrusion reduce the particle size and cellulose crystallinity. The pretreated solid's larger surface area increases the exposure of the cellulose fibers for enzymatic digestibility. Ball milling

Table 10.2 Comparison of pretreatment of LCB by different methods.

Pretreatment method (Conditions)	Biomass size	Inhibitors	Recovery (%)		Delignification (%)	Enzymatic hydrolysis (%)		References
			Glucan	Xylan		Glucan	Xylan	
Steam explosion (180–240°C for 5–20 mins)	<20 cm	HMF, furfural formic acid, levulinic acid	80–100	40–76	0–20	54–95	—	Chen and Liu, (2015)
Liquid hot water (160–240°C, 20 min)	<0.5 mm	—	100	2–20	22–31	—	—	Wang et al., (2016b)
AFEX (1:1 to 2:1 NH_3:Solids, 60°C–110°C, 15–30 min)	100–2000 µm	Oligosaccharides, organic acids, phenolic acids and aldehydes	>95	85	0	70–95	50–80	Lau and Dale (2009)
Twin-screw extrusion (80–350 rpm, 75–230°C)	2–10 mm	—	—	—	—	48–95	24–84	Zheng and Rehmann, (2014)
Microwave (500–900 W, 15–30 min)	0.5–1 mm	Acetic acid	—	76	63–70	81	37	Ethaib et al., (2015)
Electron beam (0–1200 kGy)	—	HMF, furfural	70–95	20–80	1–10	70–75		Fei et al., (2020)
Nitrogen explosive decompression (150–200°C)	<3 mm	Organic acids and furfural	81	20	0	46	—	Raud et al., (2019)
Dil H_2SO_4 (0.1–5% H_2SO_4, 100–250°C, 1.7–100 min)	0.1–0.4 mm	HMF, furfural, acetic acid, formic acid, phenolics	80–90	28	10	35–85	8.8–88	Li et al., (2014b)

(*Continued*)

Table 10.2 (Continued)

Pretreatment method (Conditions)	Biomass size	Inhibitors	Recovery (%)		Delignification (%)	Enzymatic hydrolysis (%)		References
			Glucan	Xylan		Glucan	Xylan	
NaOH (1–10%, 60–180°C, 5–60 min)	6 mm	—	95	50	60–80	60–70	6–17	Kucharska et al., (2018)
NH_4OH (5–30%, 30–210°C, 5–60 min)	9–35 mesh	—	100	50–90	0–80	70–90	–	Kim et al., (2016)
Ethanol (50–75%, 150–210°C, 18–240 min)	<10 mm	HMF, furfural	>90	12	37–75	70–97	28–90	Zhang et al., (2016)
Glycerol (66%, 220°C, 2 h)	—	HMF, furfural	91	30	70	90	–	Sun et al., (2016)
Ionic liquids (0.5–20% biomass loading, 0.5–24 h, 30–90°C)	<0.2 mm	—	99	>85	13–85	75–96	93–98	Usmani et al., (2020)
Deep eutectic solvents (60°C, 0.01–48 h)	—	HMF, fufural, levulinic acid, formic acid	99	79	80	>85	>85	Ai et al., (2020)

of corn stover biomass at low temperature of 80 °C for 30 minutes disrupted its cell structure and increased the glucose yield (Gu et al., 2018). The twin-screw-extruded biomass showed significant increase of glucose yield in enzymatic hydrolysis (Zheng and Rehman, 2014). The pretreatment of wheat bran and soybean hulls by extrusion at 7 Hz/150 °C improved the sugar yield by 60–73% in enzymatic hydrolysis (Lamsal et al., 2010). The size reduction of LCB by mechanical methods is commonly used as a prior requisite for most other types of pretreatment to improve its effectiveness. But the mechanical processing is energy intensive, and the energy requirements depend on the lignocellulosic composition, moisture content, and crystallinity of the LCB. Irradiation pretreatment uses high-energy beams like ultrasound, electron beam, and microwave to disrupt the structure of LCB. The process requires less energy and is more selective (Ethaib et al., 2015). The pretreatment using ion beam irradiation attracted attention due to its simplicity, shorter treatment time, and chemical-free operation (Fei et al., 2020). Similar to other irradiation methods, it also attacks crystalline cellulose and improves the subsequent enzymatic hydrolysis efficiency. Pretreatment of biomass with γ-irradiation at 800 KGy improved the biomass loading up to 30–40% and production of higher concentration of glucose (235 g/l) (Liu et al., 2017). The irradiation can be combined with mechanical method to enhance the enzymatic hydrolysis efficiency. The pretreatment of rice hulls by passing through twin-screw extruder followed by ultrasonic irradiation improved the enzymatic hydrolysis efficiency (Zhang et al., 2020).

Recently, plasma treatment of LCB has attracted interest due to the advantages associated with this method. It uses electricity to ionize the gases like nitrogen/air, argon, and ozone to cause chemical and physical changes in the biomass (Vanneste et al., 2017). The plasma pretreatment breaks the LCC network in biomass without forming inhibitors. The plasma pretreatment alters the biomass characteristics favorable for cellulose digestibility. The plasma pretreatment of water hyacinth has been reported to improve the sugar yield by 125% (Gao et al., 2014). Glow discharge plasma pretreatment of waste cotton and cardboard has been reported to enhance the enzymatic hydrolysis by removal of 68% lignin and 22% hemicellulose (Ramamoorthy et al., 2020). In another approach, cold plasma treatment of cellulolytic thermophilic *Geobacillus* sp. strain WSUCF1 has been performed to enhance the microbial degradation of lignocellulose and substrate utilization (Rathinam et al., 2019, 2020). The plasma pretreatment is an emerging green technology in the biochemical conversion of LCB to fuels and chemicals.

10.4.2 Physicochemical Pretreatment

The physicochemical pretreatment methods are commonly employed for a variety of LCB. These methods are based on the combination of heat, pressure, and chemical. The major effect of these pretreatments is the disintegration of the

physical structure of LCB with variable degradation of hemicellulose and lignin. The well-known physicochemical methods are steam explosion and liquid hot water pretreatment.

10.4.2.1 Steam Explosion

Steam explosion pretreatment (SEP) is the most widely used technique for breaking a variety of feedstocks. The SEP is environmental benign, since it does not use toxic chemicals. In SEP, high-pressure steam enters the biomass cell by vapor diffusion. The rapid decrease in the reactor pressure to atmospheric pressure disintegrates the LCB's structure and partially solubilizes the hemicellulose. The hydronium ions formed from water catalyzes the cleavage of glycosidic bonds in hemicelluloses by auto-hydrolysis. The acetic acid released from hemicellulose also catalyzes the auto-hydrolysis (Li et al., 2014b). The cellulose is left intact in solid fraction with little degradation. The SEP does not remove significant amount of lignin. The lignin fragments formed during SEP condense to reform polymer with change in properties like hydrophobicity (Chen and Liu, 2015). The SEP enhances the enzymatic digestibility of cellulose due to the partial removal of hemicellulose and change in the crystallinity of cellulose. But the lignin fragments and the repolymerized lignin reduce the digestibility of cellulose through unproductive binding of cellulases. Besides, the lignin fragments bind to the cellulose surface and reduces the accessibility of cellulose microfibers to enzyme action. The SEP is also performed in the presence of acids like sulfuric acid to improve the rate of catalysis and reduce the residence time. Similar to SEP, nitrogen explosive decompression (NED)-pretreated barley straw showed higher enzymatic digestibility (Raud et al., 2019). SEP is combined with the other pretreatment method as hybrid pretreatments to enhance the enzymatic hydrolysis efficiency. A hybrid method combining alkaline pre-extraction followed by SEP enhanced the sugar recovery and ethanol production (Yuan et al., 2018). Similarly, a sequential pretreatment of olive stones with dilute acid followed by SEP significantly improved the enzymatic hydrolysis efficiency (Padilla-Rascon et al., 2020).

10.4.2.2 Liquid Hot Water

Similar to SEP, the liquid hot water pretreatment (LHWP) employs only water without the addition of toxic chemicals. During the pretreatment, water is maintained in liquid state at high temperature (160–240 °C) and high pressure (5–40 bar) (Wang et al., 2016b). The LHWP disintegrates the structure of LCB and degrades hemicellulose by auto-hydrolysis. After the pretreatment, the cellulose is retained in solid fraction and hemicellulose is solubilized in the liquid fraction. Since chemicals are not used, the equipment corrosion is minimal, and hence, the cost of maintenance is lower than the other thermochemical

methods. But the energy requirement is high for this pretreatment. Most recently, a sequential approach has been followed for pretreating eucalyptus using LHWP and extrusion to improve cellulose hydrolysis (Tian et al., 2019). A modified LHWP in the presence of alkali has been employed to disrupt the structure of bamboo with the removal of hemicellulose and lignin (Yang et al., 2019). The alkaline LHWP enhances the enzymatic hydrolysis by increasing the accessibility of cellulose.

10.4.2.3 Ammonia Fiber Explosion

The ammonia fiber explosion pretreatment (AFEXP) is similar to steam explosion, but the aqueous medium is replaced with anhydrous ammonia. LCB is treated with anhydrous ammonia under high pressure and moderate temperature. A rapid depressurization results in disintegration of the LCB structure (Lau et al., 2009). AFEXP is a dry process, since ammonia is absorbed by the LCB without forming liquid phase. Hence, after the pretreatment, there is no generation of liquid stream. The temperature in AFEXP is moderate (<90 °C), and the residual ammonia after the pretreatment can be recovered and reused. In addition to disintegration of the LCB structure by explosion like SEP, the AFEXP efficiently degrades lignin by the action of ammonia at high pH. But, the degraded lignin fragments bind on the surface of cellulose fibers and inhibits the enzymatic hydrolysis (Li and Zheng, 2017). Combination of anhydrous ammonia along with hydrogen peroxide (H-AFEX) has been recently explored. H-AFEX pretreated Miscanthus showed maximum delignification with low hydrogen peroxide dosage (Zhao et al., 2017). The pretreatment of LCB with anhydrous ammonia is also performed at low moisture (LMAA) and higher temperature. It is further combined with hot water pretreatment to increase the glucan digestibility (Cayetano and Kim, 2018).

10.4.3 Chemical Pretreatment

Chemicals like acid, alkali, solvents, and ionic liquids are used to break the recalcitrance of the LCB. The variation of pH has significant effect on the modification of LCB composition. Therefore, based on the objective of the process, the pH of the pretreatment is selected. Low pH using acid is preferred if the process involves separation of hemicellulose sugars from cellulose. The pretreatment at acidic pH significantly degrades hemicellulose. If the removal of lignin is desired, then high pH using alkali is preferred. The pretreatment at alkaline pH dissolves lignin selectively without affecting the polysaccharides. However, high severe conditions would have detrimental effect on the LCB composition. The pretreatments using solvents at neutral pH are not subjected to pH effect.

10.4.3.1 Dilute Acid Pretreatment (DAP)

DAP is the most widely used pretreatment technique for cellulosic ethanol production from lignocellulosic materials. It uses inorganic and organic acids to degrade the hemicellulose with little effect on lignin and cellulose (Momayez et al., 2017). Various acids such as H_2SO_4 and HCl are used at low concentrations ranging from 0.1 to 5% and high temperatures (100–250 °C). It produces derivatives of sugars and phenolic compounds that are toxic to biocatalysts (Jonsson and Martín, 2016). Pentose and hexose sugars are derivatized into furfural and hydroxymethyl furfural (HMF), respectively. These compounds are further converted into levulinic acid. Degradation of lignin forms phenolic acids and phenolic compounds. These sugar and lignin-derived inhibitors reduce the efficiency of cellulases and ethanologenic microbes in hydrolysis and fermentation, respectively. The corrosive nature of acid corrodes equipment, hence anticorrosive materials are desirable for fabrication of reactors. Since the acid pretreatment does not affect lignin significantly, feedstock with low lignin are more suitable than high-lignin biomass. The cellulases efficiently digest dilute-acid-pretreated feedstock to soluble sugars. To achieve high ethanol yield from dilute-acid-pretreated feedstock, the pretreatment conditions should be appropriate for minimizing the formation of inhibitors and maximizing the digestibility of cellulose. The DAP is combined with other pretreatments to further enhance the cellulose digestibility. Hydrogen peroxide pretreatment combined with acetic acid (HPAC) has been employed for low- and high-lignin LCB such as maize, sugarcane bagasse, and eucalyptus. The HPAC pretreatment removed both lignin and hemicellulose and increased the enzymatic hydrolysis efficiency by 20-folds (Mota et al., 2019). The combination of sulfuric acid and microwave pretreatments for wheat and rye stillage formed minimum inhibitors and increased the sugar yield in hydrolysis (Mikulski and Kłosowski, 2020). The lignin-rich residue from the acid pretreatment of LCB has been valorized through hydrothermal liquefaction to make the LCB-based biorefinery more economical (Jaswal et al., 2019).

10.4.3.2 Alkali Pretreatment

The hydroxides of calcium, sodium, ammonium, and potassium are most commonly used for alkali pretreatment (Kim et al., 2016) at concentration ranging from 0.5 to 30%. Alkali degrades lignin and causes swelling of the biomass, thereby improving the saccharification efficiency. The alkaline pretreatment is conducted at 60–210 °C. The alkali cleaves ester bonds between lignin and hemicellulose and solubilizes lignin (Kucharska et al., 2018). The mechanical ball milling in the presence of low concentration of alkali followed by hydrothermal pretreatment of sugarcane bagasse improved the glucose yield in the enzymatic hydrolysis (Huang et al., 2019). Steam explosion of alkali-impregnated biomass at higher temperature of 204 °C for 10 minutes enhanced the enzymatic hydrolysis of sugarcane trash and aspen wood (Mihiretu et al., 2019).

10.4.3.3 Organosolv

Organosolv pretreatment (OSP) utilizes organic solvents to selectively solubilize the components in LCB. The OSP uses common low boiling point solvents like ethanol, methanol, and acetone and high boiling point solvents like ethylene glycol and glycerol (Sun et al., 2016; Zhang et al., 2016). The solvents break down hemicellulose and lignin with high recovery of intact cellulose. The solvents cleave β-O-aryl ether bonds in lignin and solubilize lignin. The efficiency of OSP increases in the presence of acidic or alkali catalysts. A novel organosolv pretreatment, OxiOrganosolv, uses oxygen gas to delignify biomass along with the desired solvent system. The process removed 97% of lignin with complete cellulose recovery and enhanced the enzymatic hydrolysis efficiency (Kalogiannis et al., 2020). The OSP using glycerol with $FeCl_3$ catalyst increased the sugar yield from water hyacinth (Santana et al., 2020). Microwave-assisted ethanol pretreatment of wood yielded pure cellulose for efficient hydrolysis (Alio et al., 2019). A modified electro-assisted organosolv pretreatment of eucalyptus using gamma-valerolactone and 1-butyl-3-methylimidazolium acetate (BmimOAc) as binary solvent system assisted by electrical energy solubilized cellulose and lignin (Sun and Othman, 2019). Cellulose dissolution happened when the capacitance was less than 2317F and lignin dissolved when it was higher. The hemicellulose remained intact in the process indicating the selective fraction of LCB by this method.

10.4.3.4 Ionic Liquid (IL) and Deep Eutectic Solvent (DES)

ILs are salts in liquid state with melting point <100 °C. The ILs are nonvolatile, nontoxic, biodegradable, and noncorrosive. They have an organic cation part and an anionic part. Some of the commonly used organic cations in ILs are imidazolium ($[(C_3N_2)Xn]^+$), ammonium $[NX_4]^+$, pyrrolidinium ($[C_4N)Xn]^+$, pyridinium ($[(C_5N)Xn]^+$), sulfonium $[SO_3]^+$, phosphonium $[PX_4]^+$, and choline (Usmani et al., 2020). The structure of some of the ionic liquids are shown in Figure 10.5. The IL pretreatment (ILP) is carried out at low-moderate temperatures in the range of 30–90 °C. The ILs solubilize lignin by breaking hydrogen bonds and reducing the cellulose crystallinity. The porosity of the biomass is also increased by the ILP. These alterations in the biomass increases the enzymatic digestibility of cellulose. Low-temperature ILP on spruce and oak dust using 1-ethyl-3-methylimidazolium acetate [Emim][OAc] at 45 °C improved ethanol yield by 3.9 times (Alayoubi et al., 2020). Extrusion of willow using the same solvent [Emim][OAc] showed glucose yield of 99% (Han et al., 2020).

DES are green solvents and are considered cheaper analogues of ionic liquids. DES is a mixture of two or more constituents, which freezes at a temperature lower than that of its constituents. The pretreatment of sorghum bagasse by the combination of twin-screw extruder and neutral pH DES (choline chloride:glycerol) supported high solids enzymatic hydrolysis at 50% solids loading (Ai et al., 2020).

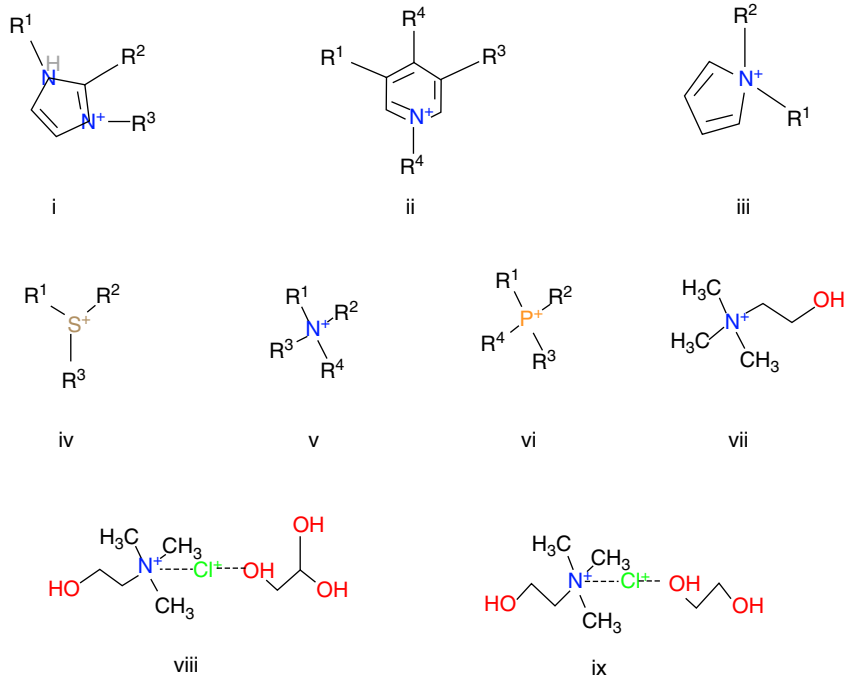

Figure 10.5 Structure of the common cations used for ionic liquids: (i) imidazolium, (ii) pyridinium, (iii) pyrrolidinium, (iv) sulfonium, (v) ammonium, (vi) phosphoniu, and (vii) choline, and deep eutectic solvents: (viii) choline chloride and ethylene glycol and (ix) choline chloride and glycerol.

Ultrafast pretreatment process was developed using DES solvent, choline chloride:lactic acid, with microwave heating at 800 W. The pretreatment carried out for 45 seconds removed maximum lignin and xylan with the retention of cellulose. It increased the enzymatic hydrolysis yield by two- to fivefold (Chen and Wan, 2018).

10.4.3.5 Supercritical Fluid Pretreatment

Supercritical fluids show properties in between those of liquids and gases above their critical temperature and pressure. The most commonly used supercritical fluids for pretreating LCB are supercritical water and CO_2. Supercritical CO_2 in the presence of water forms carbonic acid, which creates an acidic condition resulting in hemicellulose hydrolysis and in the presence of solvents it results in delignification by breaking the LCC network (Serna et al., 2016). Thus, the supercritical fluid pretreatment improves the enzymatic saccharification of biomass. Supercritical CO_2 pretreatment of oil palm trunk at temperature range of

80–120 °C has been reported to improve cellulose digestibility (Sohni et al., 2020). Mild pretreatment of sugarcane bagasse at 60 °C using supercritical CO_2 showed 72% glucose yield (Santos et al., 2011).

10.4.4 Biological Pretreatment

10.4.4.1 Bacterial Pretreatment

Biological pretreatment uses microbial or enzyme biocatalysts for treating the LCB. The biocatalysts do not form inhibitors like chemical pretreatments. The microbes or enzymes selectively degrade lignin and xylan and alter the biomass structure by increasing the pore size. Rice straw was treated with cellulase-free xylanase producing *Bacillus firmus* K-1, which removed xylan, thereby increasing the porosity and exposing cellulose fibers for further enzymatic hydrolysis (Baramee et al., 2020). The pretreatment significantly increased the enzymatic cellulose hydrolysis efficiency. The microbial treatment combined with microbubble ozonolysis (MMO-M) of wheat straw facilitated significant glucose yield in enzymatic hydrolysis (Mulakhudair et al., 2017). Strategy of combining biological pretreatment with simultaneous production of cellulase enzyme has been reported using the microbe *Piptoporus betulinus*. It produced cellulase in solid-state fermentation on rice straw with the degradation of hemicellulose (Li et al., 2019). *Piptoporus betulinus* growth showed weakening of the lignin-hemicellulose bonds and little cellulose loss. The enzymatic hydrolysis of pre-treated solids increased with 2.8 times higher sugar yield at lower cellulase loading. Bioelectrochemical approach using thermophilic cellulolytic *Geobacillus* sp. strain WSUCF1 has been reported to enhance the degradation of LCB (Rathinam et al., 2020). The electrobiosynthesis strategy improves the ability of electroactive microbes to utilize substrate with high cell biomass production. The electrobiosynthetic approach is promising for the exploration of lignolytic microbes-mediated selective degradation of lignin in LCB. Thermotolerant microbes have also been studied for CPB-type processes. The coculturing of xylanolytic thermophilic *Geobacillus* sp. strain DUSELR13 with *Geobacillus thermoglucosidasius* produced ethanol from prairie grass and corn stover (Bibra et al., 2018). Similarly, one pot process for conversion of food waste to ethanol has been reported by coculturing thermotolerant *G. thermoglucosidasius* and *Thermoanaerobacter ethanolicus* in a 40 l reactor (Bibra et al., 2020). But the major disadvantage with the microbial treatment is longer process time.

10.4.4.2 Fungal Pretreatment

Fungi are potential biocatalysts for disintegrating the LCB at ambient temperature and low moisture. Fungi belonging to white rot, brown rot, and soft rot, producing lignolytic enzymes like laccase, lignin peroxidase, and manganese peroxidase,

remove lignin by oxidation (Wan and Li, 2012). The most commonly used fungi for biomass pretreatment are *Phanerochaete chrysosporium, Ceriporiopsis subvermispora, Ceriporia lacerata, Cyathus stercolerus, Pycnoporus cinnarbarinus, Pleurotus ostreaus, Phlebia subserialis, Pleurotus streatus, Postia placenta, Gloeophyllum trabeum*, and *Echindodontium taxodii* (Baruah et al., 2018). Fungal pretreatment of corn stover using *Pleurotus sajorcaju* showed 38% lignin degradation and 71% sugar yield in the enzymatic hydrolysis (Ding et al., 2019). The fungal pretreatment has been combined with physicochemical pretreatment like steam explosion, acid, alkali, and organosolv pretreatment to reduce the pretreatment severity, process cost, and inhibitor formation (Shirkavand et al., 2016). Engineered fungi have been developed to reduce the pretreatment time, increase the lignin degradability, limit carbohydrate loss, increase the temperature tolerance, and improve the growth rate (Vasco-Correa and Shah, 2019).

10.4.4.3 Enzymatic Pretreatment

Laccase is the major enzyme useful for pretreatment of LCB by delignification. Laccase produced by halophilic *Aquisalibacillus elongatus* showed 45% delignification of peanut shell waste (Rezaie et al., 2017). Similarly, a laccase produced by the thermophile *Thermus* sp. showed delignification of steam-exploded eucalyptus in the presence of 1-hydroxybenzotriazol (HBT) as the mediator (Navas et al., 2019). A recombinant thermostable laccase of *Geobacillus* sp. strain WSUCF1 added to cellulase cocktail has been reported to improve the efficiency of saccharification of alkali and acid-pretreated corn stover and bagasse by 1.3–2 folds (Rai et al., 2019). Similarly, the mixture of commercial laccase and cellulase cocktail has been employed for simultaneous delignification and hydrolysis of aqueous-ammonia-pretreated bagasse (Raj and Krishnan, 2019). The combination of laccase with xylanase has also been reported for efficient delignification of various feedstocks (Woolridge, 2014). The laccase is also used in combination with other physical methods. The laccase-assisted hydrodynamic cavitation pretreatment of corncob increased the saccharification efficiency (Ganesan et al., 2020).

10.4.5 Optimization of Pretreatment Process

Optimization of the pretreatment conditions enhance the sugar yield in the subsequent enzymatic hydrolysis. The factors for optimization vary with the type of pretreatment. The catalyst concentration, retention time, temperature, and biomass particle size are among the factors that most influence the thermochemical pretreatment efficiency. The other pretreatment-specific parameters include screw speed and moisture content for extrusion process. Sweet sorghum bagasse

extruded at 100 °C and 100 rpm with 30% moisture improved the sugar yield to 70% (Heredia-Olea et al., 2015). Response surface methodology, full or fractional factorial design, artificial neural network, and genetic algorithms have been employed to optimize multiple pretreatment factors. The pretreatment of yellow poplar under optimized oxalic acid concentration (82 mM) and pretreatment time (58 minutes) maximized the sugar yield in enzymatic hydrolysis. Similarly, the alkali pretreatment under the optimized conditions (2 hours, 76.21 °C) maximized both the sugar yield and lignin removal (21.4%) (Rambo et al., 2020). Five variables (milling time, temperature, double treatment, chemical concentration, and pretreatment time) optimization using the fractional factorial design showed that the acid pretreatment was more efficient on combining with alkali than organosolv (Rezende et al., 2018). The ultrasonic-assisted organosolv pretreatment of empty fruit bunches under the conditions (48.2 °C, 30 minutes, and 55% (192.5 W) sonication power) optimized using artificial neural network (ANN) showed maximum sugar yield (Lee et al., 2020). The combination Taguchi design and ANN enabled optimization of acid pretreatment (1.5% HCl, 130 soaking, and 121 °C) of Napier grass (Mohapatra et al., 2017).

10.5 Enzymatic Hydrolysis

The enzymatic saccharification requires LCB pretreatment, since the native biomass is recalcitrant to enzymatic hydrolysis. The pretreated LCB is hydrolyzed to simple sugars using an enzyme cocktail comprising different catalytic activities. The composition of enzyme cocktail varies with the method of pretreatment of LCB. The dilute-acid-pretreated LCB contains mainly cellulose; hence, a cocktail of cellulases is appropriate for hydrolysis. When LCB is pretreated with alkali to selectively remove lignin, the pretreated LCB contains both cellulose and xylan. In this case, the enzyme cocktail comprising cellulases and xylanases is required for complete hydrolysis. The glycosyl hydrolases (GH) depolymerizing cellulose and hemicelluloses are the core enzymes in the enzyme cocktail used for biomass hydrolysis. The other xylan debranching enzymes and auxiliary activities are used to enhance the hydrolysis efficiency.

10.5.1 Cellulose Hydrolysis

The cellulose in the LCB is hydrolyzed by a combination of cellulases with different activities. The cellulases cocktail is considered as the core enzymes since cellulose is the major polysaccharide in LCB and is highly recalcitrant to enzymatic hydrolysis. The main enzymes in the cellulases cocktail are endoglucanases (EG) (β-1,4-D-glucan glucanohydrolase, EC 3.2.1.4), cellobiohydrolase I (CBH I) (β-1,4-D-glucan cellobiohydrolase, EC 3.2.1.176), cellobiohydrolase II (CBH II)

(β-1,4-D-glucan cellobiohydrolase, EC 3.2.1.91), and β-glucosidases (βG) (β-1,4-D-glucoside glucohydrolase, EC 3.2.1.21) (Lynd et al., 2002). Figure 10.6a shows the mode of action of cellulases. The EG cleaves the internal β-1,4-D-glucosidic bonds in cellulose chains. It mainly acts on the amorphous region of the cellulose chain. The action of EG is random, resulting in the formation of oligosaccharides with different DP. The CBH I and CBH II act at the ends of cellulose or oligosaccharide chains and release cellobiose. CBH I and CBH II release cellobiose processively from reducing and nonreducing ends, respectively. CBHs play a major role in the overall hydrolysis of cellulose. The βG hydrolyzes cellobiose to glucose units, the monomers of cellulose polymers.

10.5.2 Xylan Hydrolysis

Xylan is the major hemicellulosic polysaccharide in crop residues, which are the primary feedstocks for cellulosic ethanol production. A mixture of GH enzymes with different activities hydrolyze xylan. The sites of action of xylanolytic enzymes are shown in Figure 10.6b. Endoxylanases (EX) (endo-β-1,4-D-xylan xylanohydrolase; EC 3.2.1.8) depolymerizes the xylan backbone and forms xylooligosaccharides. The EX randomly cleaves the internal β-1,4-D-xylosidic linkages in the xylan polymer. β-Xylosidases (β-X) (β-1,4-xyloside xylohydrolase; EC 3.2.1.37) break the β-1,4-D-xylosidic linkages in the xylooligosaccharides forming xylose (Collins et al., 2005). Due to the substitutions in the xylan polymer, some of the oligosaccharides formed by the EX are substituted. Therefore, the xylose formed from the substituted xylooligosaccharides contains substitutions. The fermentability of the substituted xylose depends on the combination of xylanolytic enzymes expressed in the ethanologen. Besides, the nature of substitutions in xylan affects the efficiency of EX catalysis. The hydrolysis of substituted xylan to xylose requires debranching enzymes acting at the branching linkages in the xylan (Collins et al., 2005). α-L-Arabinofuranosidase (EC 3.2.1.55), α-(4-*O*-methyl)-D-glucuronidase (EC 3.2.1.139), and acetylxylan esterase (EC 3.1.1.72) remove the substitutions, viz., arabinosyl, glucuronyl, methylglucuronyl, and acetyl residues, respectively. Feruloyl esterases (EC 3.1.1.73) break the ester bond linking lignin with xylan. Therefore, a mixture of these xylanolytic enzymes completely hydrolyze heteroxylan to xylose.

10.5.3 Accessory Enzymes

The core enzyme cocktail for biomass hydrolysis to fermentable sugars comprises EG, CBHs, βG, EX, and βX. These enzymes act on the glucan and xylan backbone polymers in cellulose and xylan. The core enzyme cocktail significantly hydrolyzes the biomass polysaccharides to simple sugars, but the rate and conversion efficiency are low. The debranching enzymes acting on xylan branching points play important roles in enhancing the degradation of the cellulose. Because, these

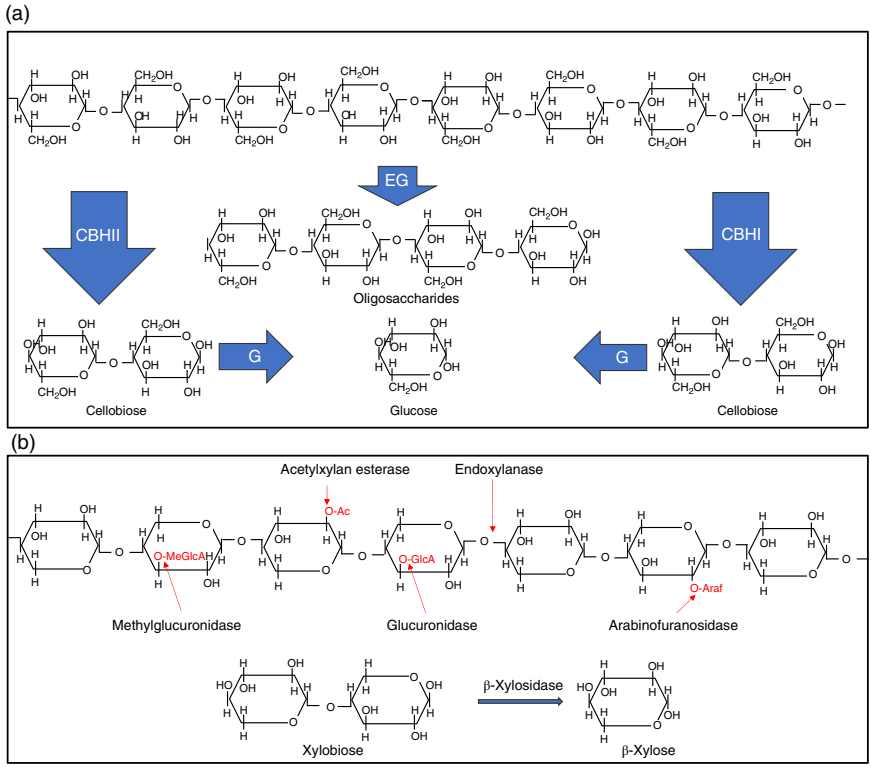

Figure 10.6 Mode of action of (a) cellulases on cellulose and (b) xylanases on xylan.

debranching enzymes increase the hydrolysis of xylan covering the cellulose microfibrils. Therefore, the debranching xylanolytic enzymes are employed as accessory enzymes to enhance the hydrolysis of biomass polysaccharides. Based on the type of heteroxylan in the biomass, appropriate mixture of accessory enzymes are added to the core enzyme cocktail to improve the hydrolysis efficiency. There are many reports on the addition of accessory enzymes to increase the hydrolysis yield (Laothanachareon et al., 2015). The crude enzyme preparations are used to reduce the enzyme cost. The mixing of crude enzymes from different microbial sources at appropriate amounts is the best strategy for cost-effective complete saccharification of biomass (Soleimani and Ranaei-Siadat, 2017). Since pectin is associated with the cell wall matrix, the degradation of pectin also increases the accessibility of cellulose to cellulases. Thus, an enzyme cocktail comprising GH hydrolases acting on the polymeric chains of cellulose and hemicelluloses and accessory enzymes is vital for efficient saccharification of biomass.

10.5.4 Auxiliary Activity and Non-Hydrolytic Enzymes

The efficiency of cellulose hydrolysis is enhanced by auxiliary activities (AA) of non-hydrolytic enzymes such as lytic polysaccharide monooxygenase (LPMO) (β-1,4-D-glucan oxidoreductase EC 1.14.99.56). The LPMOs are oxidoreductases that cleave β-1,4-glucosidic bonds in the cellulose chain by oxidative mechanisms involving molecular oxygen and a reducing agent (Kim et al., 2014). The AA9 oxidizes the C1 and C4 of β-1,4-glucosidic bonds in cellulose forming aldonolactone and ketoaldose as one of the products. The LPMO contains Type 2 Cu center and cleaves the β-1,4-glucosidic bonds in cellulose through the formation of Cu-superoxyl and Cu-oxyl intermediates. The LPMO, also known as auxiliary activity enzyme (AA9), not only degrades the cellulose chain, it also degrades hemicellulose by oxidation. The addition of AA9 to the core enzyme cocktail enhances the overall hydrolysis efficiency by decreasing the cellulase loading up to five- to sixfolds and reduce the saccharification time (Karnaouri et al., 2016). The AA9 level in the enzyme cocktail varies with the nature of biomass. However, the overdose of AA9 reduces the hydrolysis yield. The advanced cellulases cocktail employed in industries contain LPMOs and expansin-like proteins to improve the rate of biomass hydrolysis (Muller et al., 2018). The other non-catalytic proteins such as expansin also plays a significant role in enhancing the cellulose hydrolysis. The expansin-like proteins weaken the intermolecular hydrogen bonding in crystalline cellulose. Swollonin acts similar to expansin and improves the hydrolysis of cellulose (Eibinger et al., 2016). Non-catalytic protein-like swollenin or expansin in cocktail preparation removes the crystallinity and loosens the microfibrils of cellulose polymer by disrupting the hydrogen bonds. The non-catalytic proteins increase the cellulase activity by >5 folds with higher sugar yield (Morrison et al., 2016).

10.5.5 Enzyme Cocktail for Biomass Hydrolysis

The high cost of enzyme is the one of the impediments in achieving the economic processing of biomass to cellulosic ethanol. The microbial strains of fungi or bacteria growing on LCB utilize cellulose as the carbon source for their survival. The strain secretes all necessary enzymes required for deconstruction of polymeric sugar to monomers. The cellulolytic enzymes produced by these fungi are used for in vitro degradation of LCB. However, a mixture of cellulases secreted by one fungal strain shows incomplete degradation of biomass to simple sugars due to the suboptimal levels of the enzyme activities. A good enzyme cocktail should show complete saccharification of biomass at shorter retention time, less enzyme loading, and high substrate loading (Soleimani and Ranaei-Siadat, 2017). Yet, a single microbial strain that produces all components of enzymes required for complete hydrolysis of biomass has not been discovered. As each biomass varies in composition and the pretreatment of biomass alters the structure and composition, each biomass requires separate enzyme cocktail for maximum saccharification efficiency. Hence, an ideal cellulase cocktail should be the one that is efficient for a variety of biomass types subjected to different pretreatment methods.

10.5.5.1 Cocktail Development

Fungi are the most preferred systems for the core enzyme preparation because it secretes copious amount of extracellular enzymes than bacteria. The most commonly used fungal species for industrial cellulase production are *Aspergillus* sp., *Penicillium* sp., and *Trichoderma* sp. (Wilson, 2009). The enzymatic saccharification of LCB with only base enzyme needs longer duration and high enzyme concentration, which are not suitable for commercial purposes. The addition of other accessory enzymes to the base enzyme increases the hydrolysis efficiency, decreases the reaction time, and lowers the enzyme loading. The organism used for the base enzyme preparation is either native or mutant and should secrete maximal level of the core enzymes. *Trichoderma reesei* is the most explored species for developing enzyme cocktail for biomass hydrolysis. *Trichoderma reesei* produces most of the cellulose-degrading enzymes. However, *T. reesei* does not synthesize all the enzyme components required for efficient enzyme cocktail. For example, βG synthesis in *T. reesei* is not at optimal level for biomass hydrolysis. In addition, the hemicellulases synthesis in *T. reesei* is also minimal. Therefore, βG and hemicellulases produced by other microbial strains are added to the enzyme mixture produced by *T. reesei*. There are numerous reports on enhancement of biomass hydrolysis efficiency by combination of *T. reesei* enzymes and other bacterial and fungal enzymes (Laothanachareon et al., 2015). Addition of xylanases in the cocktail preparation depends on the method of biomass pretreatment. It is also vital to check whether increase in saccharification efficiency on xylanase

supplementation to the core enzymes mixture is due to cellulase activity in the xylanase preparation. There are reports using xylanase supplementation with celluclast 1.5l in sugarcane bagasse. Li et al. (2014a) have shown an increase in the saccharification efficiency by the addition of 20% xylanases. Apart from cellulase and xylanase, addition of oxidative enzyme also improves the saccharification of biomass. The enzyme such as LPMO helps in rapid biomass liquefication and thus makes easy accessibility of cellulose for cellulolytic enzymes. The simple approach for enzyme cocktail preparation involves the following steps, viz., (i) a base cellulases cocktail production from fungus like *T. reesei*, (ii) identification of cellulolytic and hemicellulolytic enzymes present at suboptimal level in the base cellulases cocktail, (iii) production of required enzymes from other fungi or bacteria, (iv) addition of appropriate amount of hemicellulases and accessory enzymes to the base cellulases cocktail, and (v) addition of oxidative enzymes with auxiliary activities to the cellulases–hemicellulases mixture. For example, *T. reesei* cellulases is used as the base enzyme and supplemented with β-glucosidase from *Aspergillus* sp., hemicellulases and auxiliary activities from other microbial sources. Alternative method is the coculture of two different fungal strains producing cellulases and hemicellulases. Both the strain should be able to grow in same growth condition because dominant strain can inhibit the growth of other strain (Adsul et al., 2014). The modern strategy is to use recombinant technology, where genes encoding the desired accessory enzymes and auxiliary activities are expressed in the strain producing core cellulase enzymes. When core and the other accessory enzymes are produced separately and mixed, statistical method is a good option for optimizing the composition of enzyme cocktail. The fractional factorial design is used to identify the most significant enzyme components (Zhou et al., 2009). By this method, the crude enzyme of *T. reesei* has been supplemented with optimized ratios of multiple accessory enzymes such as expansin protein, acetyl xylan esterase, β-glucosidase, and α-L-arabinofuranosidase. The research on enzyme cocktail development undergoes for many years and still it needs more improvements for cost-effective cocktail. Invention of novel strain and deep excretome profiles of cellulolytic microbes would help to formulate efficient enzyme cocktail for biomass saccharification. As the composition and structure of pretreated biomass depends on its nature and method of pretreatment, these two factors are important criteria to be considered for development of efficient cocktails. For example, the spruce wood containing more mannan requires the addition of mannanase enzyme to the cocktail for high saccharification efficiency (Xin et al., 2017). The cocktail showing maximum hydrolysis efficiency for corncob has been shown to be less effective for the other feedstocks like corn stalk and wheat straw. Table 10.3 shows the improvement in hydrolysis efficiency using the enzyme cocktail mixtures from different organisms in different lignocellulosic substrates.

Table 10.3 Improvement of saccharification yield using enzyme cocktail mixture.

Enzyme cocktail	Source	Biomass	Sugar yield	References
Cellulase Xylanase	*Trichoderma reesei* *Clostridium thermocellum*	Peracetic-acid-pretreated bagasse	Threefold increase in total sugar	Goncalves et al., (2015)
Cellulase Cellobiase Xylanase, Arabinofuranosidase	*Trichoderma reesei ATCC26921* *Aspergillus niger* *Trichoderma viride* *Pleurotus ostreatus*	Ammonia-pretreated corn corb	Xylose conversion increased from 69% to 85%	Marcolongo et al., (2014)
Accellerase, Novozyme188 arabinofuranosidase, xylanase	*Clostridium thermocellum,* *Geobacillus thermodenitrificans* *Geobacillus stearothermophilus*	AFEX-pretreated corn stover	40–80% of total sugar	Gao et al., (2011)
Arabinofuranosidase, Mannanase, Xylanase	*Clostridium cellulovorans*	Lime-pretreated sugarcane bagasse	Sixfold increase in total sugar	Beukes and Pletschke, (2010)
Cellulase β-glucosidase Accellerase	*Chaetomium globosum* Commercial enzymes	Alkali-pretreated rice straw	2.9-fold increase in total sugar	Wanmolee et al., (2016)

(*Continued*)

Table 10.3 (Continued)

Enzyme cocktail	Source	Biomass	Sugar yield	References
Cellulase Xylanase β-glucosidase	*Thermoascus aurantiacus* *Acremonium thermophilum*	Hydrothermally pretreated corn stover	Twofold increase in total sugar	Zhang and Viilari, (2014)
Cellulase Xylanase Arabinofuranosidase	*Aspergillus foetidus* MTCC 4898 *A. niger* ADH-11	Alkali-pretreated maize stover	2.34-fold increase in total sugar	Patel et al., (2015)
Cellulase Xylanase Arabinofuranosidase	*Trichoderma reesei* *Aspergillus aculeatus*	Alkali-pretreated rice straw	47.3% increase in glucose yield	Laothanachareon et al., (2015)
Cellulase Xylanase β-glucosidase	Cellic Ctec Cellic Htec Novozyme188	Steam-pretreated sorghum bagasse	47% increase of total sugar	Sun et al., (2015)

10.6 High Solids Loading Enzymatic Hydrolysis (HSLEH)

The hydrolysis of pretreated biomass at HSL is required to obtain high concentration of sugars. But the efficiency of enzymatic hydrolysis is negatively influenced by HSL. The saccharification of biomass carried out above 15% (w/w) solids loading is termed as high solids enzymatic hydrolysis due to the profound changes in the rheology of the slurry. The viscosity and yield stress increase tremendously at HSL (Weiss et al., 2019). Hence, efficient mixing of the reaction mixture is a challenging task. With the increase in solids loading above 20% (w/w), the slurry becomes inconsistent, and there is a shift toward non-Newtonian behavior. Even though there are limitations associated with high solids enzymatic hydrolysis, the saccharification of the biomass above 20% (w/w) yielding sugar concentrations as high as 10% (w/w) is essential for the production of high ethanol concentrations. The shortcomings in high solids hydrolysis can be surpassed by developing effective strategies like fed-batch addition of solids, SSF, novel impellers, and bioreactor design. The process cost for the enzymatic hydrolysis at HSL is considerably low due to the reduced demand on power requirements for heating and cooling (Chen and Liu, 2017). In addition, the consumption of water is minimized substantially, and there is a significant reduction in the disposal cost. Altogether, the high solids saccharification improves process economy due to critical outputs of the process like high concentrations of sugar and ethanol.

10.6.1 Enzyme Inhibitors and Detoxification

As the thermochemical pretreatment of biomass is associated with the production of inhibitory compounds, the effect of inhibitory compounds becomes more significant with increase in solids loading. To alleviate the inhibition of degradation products on the enzymes, the biomass is detoxified by washing with water, overliming using calcium hydroxide, or bioabatement using microbes (Kumar et al., 2020). Washing of the pretreated biomass is the most commonly applied method for removing the pretreatment inhibitors. In the case of overliming process, the addition of calcium hydroxide is effective for neutralizing the acidic inhibitors like acetic acid, formic acid, levulinic acid, and the sugar degradation products like furfural and HMF. The treatment with calcium hydroxide also brings down the levels of phenolic compounds. However, the presence of lime affects the efficiency of enzymatic hydrolysis and fermentation (Zhang et al., 2018). The bioabatement is a promising strategy for minimizing the usage of water and chemicals, as the detoxification of the biomass is carried out by microbes. Primarily, the fungal strains producing lignin-degrading enzymes are used. Even though the process of bioabatement is considered as environment friendly, there is

nonspecific degradation of sugar polymers present in the pretreated biomass for the microbial growth. As a result, the recovery of sugars in the biomass undergoes significant reduction. Moreover, the duration of bioabatement is longer compared to conventional washing/overliming steps. Therefore, the design of efficient pretreatment process for the maximum recovery of sugars and minimum generation of inhibitory compounds is the most preferable solution for preventing the inhibition of the subsequent high solids hydrolysis and fermentation processes.

10.6.2 Cellulase Feedback Inhibition

The high solids saccharification is ideally oriented toward the production of high sugar concentrations. The increased loading of biomass is proportional to the total sugar concentration, as there is higher availability of sugar polymers for the enzymatic degradation. Yet, there is a decline in the efficiency of saccharification with solids loading. In the process of high solids hydrolysis, the gradual accumulation of the sugars poses challenge for achieving maximum sugar yield due to the end product inhibition on enzymes. The enzymes used for biomass saccharification shows enhanced activity in the initial stage of hydrolysis and the released sugars bind to the enzymes and limit its catalytic efficiency (Hsieh et al., 2014). The sugar feedback inhibition is a prominent effect observed only for high solids hydrolysis, as the minimum sugar concentration for the inhibition of enzyme is attained with increasing solids loading. The sugar feedback inhibition is mostly direct, in which the enzymes breaking down the sugar polymer is inhibited by the sugars released from the polymer. A common example is the inhibition of cellulases by glucose produced from the breakdown of cellulose. However, indirect sugar feedback inhibition is also reported. For instance, the xylo-oligomers arising from the xylan degradation inhibit the endoglucanase acting on cellulose (Xin et al., 2019). Therefore, the development of enzyme cocktails consisting of cellulase, hemicellulases, and other accessory enzymes is necessary to minimize the formation of incompletely hydrolyzed sugar oligomers, thereby eliminating their inhibitory effects on enzymatic hydrolysis. Further to combat the sugar feedback inhibition, the simultaneous conversion of sugars into ethanol is a promising strategy. However, the operating conditions for the saccharification and fermentation differ significantly. Moreover, the SSF process is carried out at single optimum temperature in which there is a compromise between enzymatic activity for hydrolysis and cell viability for the fermentation performance. In contrast, the same process is modified by carrying out initial pre-hydrolysis at the optimum condition for enzymes followed by delayed fermentation for the efficient conversion of sugars to ethanol. Besides, the process combines the advantages of both SHF and SSF, thereby emerging as an attractive solution for minimizing the end product inhibition and improving the productivity of the process.

10.6.3 Rheology

The major limitation associated with high solids hydrolysis is the heterogeneous slurry due to the limited availability of free water for mixing. The consistency of the slurry gradually becomes thick and pastelike for solids loading above 20% (w/w). The hydrothermally pretreated wheat straw showed inefficient hydrolysis at 40% solids loading due to the pastelike consistency of the slurry (Jorgensen et al., 2007). Though the slurry becomes flowable with the progressive digestion of the solids by enzymes, the conversion of biomass to sugars requires efficient diffusion of enzymes from the bulk medium to the substrate and vice versa. Moreover, the water constraint prevalent at high solids hinders the mobilization of enzymes, thereby posing substantial mass transfer limitation (Liu and Chen, 2016). The water present in the biomass exists mainly as primary bound water, and there is reduced availability of free water in the bulk medium to aid the mixing of substrate with enzymes. For overcoming the problem of mixing encountered at high solids, the fed-batch addition of biomass is a preferred method. The fed-batch process is developed to prevent rapid increase in the viscosity and yield stress of the slurry by optimizing the biomass feeding profiles (Hernández-Beltrán and Hernández-Escoto, 2018). The rheology of the slurry is maintained at a steady state without allowing a sharp transition toward non-Newtonian behavior by controlled feeding strategy. More importantly, the water constraint is minimized considerably to facilitate efficient mixing of the slurry. Though fed-batch process is favorable for high solids hydrolysis, the biomass saccharification by enzymes varies with the feeding time. The activity of enzymes decreases gradually during the hydrolysis due to inevitable factors like thermal inactivation of enzymes and the unproductive binding of enzyme to substrate. As a result, the enzymes possess high activity during the initial phase of hydrolysis lasting for 12–24 hour, and there is a decline in the hydrolysis efficiency with further progress in time. Therefore, the feeding time of biomass also requires optimization for enhancing the production of sugars in the fed-batch enzymatic hydrolysis at HSL. The strategy of fed-batch process is less attractive for large-scale operation due to the number of optimization steps required for developing the saccharification process and the costs associated with feeding of biomass (Chen and Liu, 2017). Alternatively, the design of novel bioreactors with versatile impellers is a more commercially viable option for the operation of high solids hydrolysis process on an industrial scale.

10.6.4 Reactors and Impellers

The conventional process of high solids enzymatic hydrolysis has been carried out in a cylindrical reactor using Rushton turbine impellers. The turbine impeller promotes mixing only along the radial direction, which results in the creation of

stagnant zones for viscous slurries. Moreover, the power consumption for mixing at high solids using Rushton impellers is another limitation. Alternative to Rushton impellers, the helical impellers are suitable to mix highly viscous slurries (Zhang et al., 2010a). Other impeller types like plate and frame, double helical, marine blade, and paddle type have also been used for high solids saccharification. The dimensions of the impeller and stirring speed are crucial for enhancing the biomass saccharification at HSL. In the case of novel bioreactors, the horizontal drum reactor facilitating free fall mixing is reported to be more compatible for carrying out enzymatic hydrolysis at solids loading as high as 40% (w/w) (Jorgensen et al., 2007). The rotating drum is efficient for converting the thick and inconsistent paste-like slurry into flowable liquid. More recently, the process of high solids saccharification and fermentation was carried out as an integrated approach in non-isothermal simultaneous solid-state saccharification, fermentation, and separation systems (Chen and Li, 2013). However, the development of miscellaneous reactors is less preferred over specialized impellers due to the cost involved in the construction of the reactors. Overall, the efficiency of high solids hydrolysis is significantly improved by modelling the reactor and impeller designs for achieving high sugar production with minimum power consumption.

10.7 Fermentation

The final step is cellulosic ethanol production is microbial fermentation of sugars formed in enzymatic hydrolysis to ethanol. As described above, the two major sugars formed from the biomass during hydrolysis are glucose and xylose. Based on the biomass type, a third sugar arabinose is also formed at low concentration. Most fermentation methods for biomass hydrolysates target metabolism of glucose and xylose to ethanol. The industrial ethanol-producing yeast *Saccharomyces cerevisiae* is not efficient for fermentation of multiple sugars formed from biomass. Because *S. cerevisiae* metabolizes only glucose to ethanol, and it does not have metabolic pathway for conversion of xylose to ethanol. *Zymomonas mobilis* is another well-known ethanol-producing bacterium. Similar to *S. cerevisiae*, *Z. mobilis* is also not capable of metabolizing xylose to ethanol. Though many other yeasts like *Pichia stipitis*, *Candida tropicalis,* etc. are capable of metabolizing xylose to ethanol, these yeasts are not proven for industrial process. In order to co-metabolize glucose and xylose to ethanol, recombinant technology is employed to construct genetically engineered microorganisms equipped with metabolic pathways for the conversion of glucose and xylose to ethanol. Broadly, two approaches are followed to engineer microbes for co-fermentation of glucose and xylose (Figure 10.7). In the first approach, the native ethanol producers like *S. cerevisiae* and *Z. mobilis* are engineered to co-metabolize

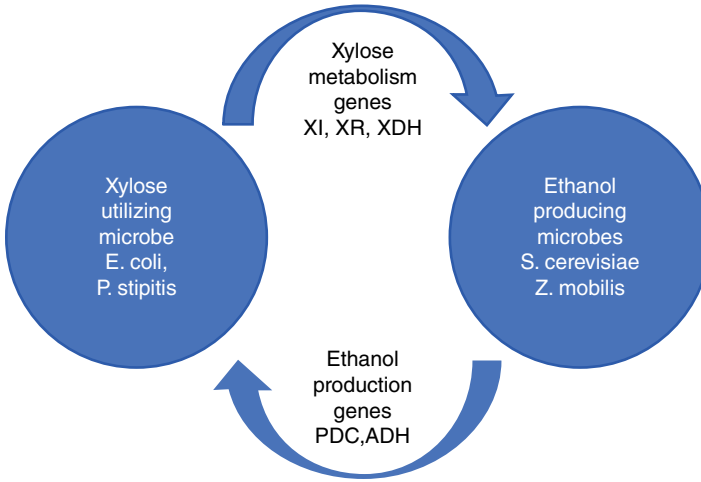

Figure 10.7 Approaches for construction of genetically engineered ethanologens co-fermenting glucose and xylose. XI-Xylose isomerase, XR-Xylose reductase, XDH-Xylitol dehydrogenase, PDC-Pyruvate decarboxylase, ADH-Alcohol dehydrogenase.

xylose. The genes required for xylose metabolism such as xylulose reductase (XR) and xylitol dehydrogenase (XDH) from the yeast *P. stipitis* or xylose isomerase (XI) from bacteria have been transferred to *S. cerevisiae* (Kim et al., 2013). The resulting recombinant *S. cerevisiae* produces ethanol from both glucose and xylose. Similarly, *Z. mobilis* has been genetically engineered to produce ethanol from xylose by transferring xylose-metabolizing genes from *Escherichia coli*. The second approach is to engineer natural multi-substrate metabolizing microbe like *E. coli* to produce ethanol. Based on this approach, ethanol-producing genes pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) from *Z. mobilis* are transferred to *E. coli*, and the resulting recombinant *E. coli* produces ethanol from both glucose and xylose (Wang et al., 2008).These recombinant strains are used to co-ferment glucose and xylose obtained by enzymatic hydrolysis of biomass to ethanol.

The hydrolysis of biomass and fermentation are carried out separately (SHF) in a sequence. Alternate method is simultaneous hydrolysis of biomass and co-fermentation (SSCF). The latter method SSCF is suitable for biomass conversion at HSL, since the high concentration of sugars formed during hydrolysis are rapidly fermented. This prevents accumulation of sugars during hydrolysis and prevents feedback inhibition of cellulases. Jin et al. (2017) carried out fermentation of AFEX-pretreated corn stover at 18% (w/w) solids loading using recombinant xylose fermenting yeast *S. cerevisiae* 424A by simultaneous saccharification and co-fermentation (SSCF) process. Alternatively, the development of coculture

systems involving synergistic action on sugar utilization and ethanol production is a viable strategy. In the coculture systems, the synergy between two fermenting strains improves the overall ethanol titer value due to the complete utilization of sugar mixtures produced during high solids hydrolysis. Moreover, there is enhanced tolerance of mixed culture systems to the process temperature and ethanol concentration (Zhu et al., 2016). In contrast, the problem of by-product formation is prevalent in coculture systems, which introduces additional cost for the separation of ethanol (Jones and Wang, 2018). Even though some of the studies have reported the use of *E. coli* for the fermentation of both glucose and xylose to ethanol, the occurrence of side reactions forming by-products such as acetic acid and glycerol are difficult to be resolved. Moreover, the microbial strains with broad substrate utilization are more susceptible to inhibition by ethanol in the high solids saccharification and fermentation process (Nguyen et al., 2017). In contrast, the tolerance of natural ethanol producers to high ethanol concentration is a major advantage for achieving high process productivity. An exception to the natural ethanol producer is the xylose-fermenting *C. tropicalis* strain. Raj and Krishnan (2019) has reported the production of high ethanol concentration of 72 g/l from low-temperature aqueous-ammonia-pretreated bagasse using *C. tropicalis*. However, the efficient utilization of xylose for ethanol production is limited. Nevertheless, the development of two-stage fermentation with the coproduction of xylitol was observed to improve the economy of the ethanol production process (Raj and Krishnan, 2020). Besides, the shortcoming of single carbon source (glucose) utilization by natural ethanol producers could be overcome by additional steps for the conversion of multiple sugars (pentoses) into value-added products. However, the integration of pretreatment, saccharification, fermentation, coproduct production in one stage for establishing a consolidated bioprocess is still challenging.

To overcome the limitations of high operating cost and increased energy demand in the SHF, high solids saccharification was combined with cofermentation. However, the mismatch between optimum conditions for enzymatic hydrolysis and fermentation is a major limitation. An interesting process strategy was developed by introducing of pre-hydrolysis before fermentation for enhancing the total sugar production at the optimum condition of enzymatic hydrolysis. Pre-hydrolysis is essential for producing high sugar concentration in the fed-batch saccharification at 33% solids loading. Molaverdi et al. (2019) have reported maximum ethanol production for the alkali-pretreated rice straw by the addition of pre-hydrolysis step in solid-state saccharification and fermentation process. Moreover, the SSF process for high solids fermentation is more economical compared to the SHF process. The ethanol production from LCB at HSL are given in Table 10.4. Kadhum et al. (2017) have reported the life cycle assessment for cellulosic ethanol production from pretreated wheat straw at 30% (w/w) solids

Table 10.4 Ethanol production from lignocellulosic biomass at HSLEH and fermentation.

Biomass	Pretreatment method	Solids loading (% w/v)	Process	Ethanol (g/l)	Metabolic ethanol yield (%)	References
Corn stover	Ammonia fiber explosion	18	SSCF	60.3	93	Jin et al., (2017)
	Dilute-acid-pretreatment	30	SSF	81.6	–	Hou et al., (2019)
	Steam explosion	30	SSF	64.6	82.8	Zhang et al., (2010a)
	Steam pretreatment with dilute acid	20	SSF	60	81	Gladis et al., (2015)
Corn cob	Acid and alkali	25	SSF	84.7	79	Zhang et al., (2010b)
Rice straw	Soaking in sodium carbonate	30	SSSF	99.4	89.5	Molaverdi et al., (2019)
	Alkali and acid	24	SSF	52.3	67	Roberto et al., (2020)
Sugarcane bagasse	Low temperature soaking aq. ammonia	40	SHF	72.4	90.9	Raj et al., (2019)
	Alkali pretreatment	30	SSF	68	82.4	Liu et al., (2016)
	Formiline pretreatment	20	SSF	80	82.7	Zhao et al., (2013)
	Steam pretreatment with dilute acid	22	SSF	65	85	Unrean et al., (2016)
Sorghum bagasse	Hydrothermal pretreatment	18	SSF	41.4	70.4	Matsakas and Christakopoulos, (2013)
Wheat straw	Dilute acid pretreatment	45	SHF	115.9	98	Kadhum et al., (2017)
	Hydrothermal pretreatment	40	SSF	47	90	Jorgensen et al., (2007)
	Phosphoric acid and hydrogen peroxide	20	SSF	71.2	85	Qiu et al., (2017)

(*Continued*)

Table 10.4 (Continued)

Biomass	Pretreatment method	Solids loading (% w/v)	Process	Ethanol (g/l)	Metabolic ethanol yield (%)	References
	SO_2 catalyzed steam pretreatment	20	SSCF	57.3	66	Wang et al., (2016a)
Rapeseed straw	Dilute acid pretreatment	22	SSF	53	72.4	Tan et al., (2020)
Barley straw	Hydrothermal pretreatment	25	SSF	52	77	Vargas et al., (2015)
Eucalyptus	Steam explosion	20	SSF	60	95	McIntosh et al., (2017)
Poplar	SPORL pretreatment	20	Semi SSF	41	65	Zhang et al., (2015)
Spruce	SO_2-catalyzed steam pretreatment	20	SSF	40	53	Koppram and Olsson, (2014)
Miscanthus	Alkali pretreatment	20	Partial SSF	81	90	Cha et al., (2014)
Cotton gin trash	Dilute acid pretreatment	45	SSF	41	70	McIntosh et al., (2019)

loading and showed that SSF process was associated with promising attributes such as environmental sustainability and technoeconomic gains for commercialization. Interestingly, the integration of fermentation with high solids saccharification influenced the rheological characteristics of the slurry. Hou et al. (2019) showed a substantial change in the rheological property of the acid pretreated corn stover at 30% solids loading in the simultaneous saccharification and cofermentation process for the production of cellulosic ethanol, L-lactic acid, and L-gluconic acid. Therefore, the development of ethanol tolerant strains and the utilization of multiple sugars in lignocellulosic hydrolysate for the production of ethanol and valuable coproducts in an integrated process approach hold tremendous potential for the commercialization of cellulosic ethanol process.

Solid-state fermentation is an unconventional strategy to integrate the enzyme and ethanol production processes. The cost of the commercial enzyme is a major bottleneck for the successful scale up of the cellulosic ethanol process. However, solid-state fermentation is widely applied for the production of cellulolytic enzymes, thereby minimizing the use of commercial enzymes. More importantly, solid-state fermentation is a consolidated bioprocess, which eliminates multiple steps for saccharification and fermentation processes. Lever et al. (2010) have reported the production of ethanol from wheat straw using crude unprocessed cellulase by solid-state fermentation. Sukumaran et al. (2009) have investigated the enzymatic hydrolysis and ethanol production from rice straw, sugarcane bagasse, and water hyacinth using cellulolytic enzymes obtained from solid-state fermentation on wheat bran. Jain et al. (2013) have demonstrated CPB of switchgrass to ethanol through solid-state fermentation using *Clostridium phytofermentans*. Chu et al. (2012) have reported the combination of SSF and solid-state fermentation for enhancing the ethanol production from steam-exploded corn stover.

10.8 Genetic Engineering in LCB Bioconversion

The processes for the LCB bioconversion into ethanol have been extensively investigated. Among various factors, the major concerns of process development are the biomass recalcitrance and the hemicellulosic pentosans. There are several thermochemical pretreatment methods available for overcoming the LCB recalcitrance. However, the conversion of pentoses to ethanol was challenging until the advent of genetic engineering approach. In brief, genetically engineered microbes, such as *S. cerevisiae, Z. mobiles,* and *E. coli*, efficiently metabolizing pentoses like xylose and arabinose to ethanol are the key developments toward the commercialization of cellulosic ethanol. These were followed by many other engineered ethanologens (Sharma et al., 2016). The other important impact of genetic engineering is its crucial role in developing cellulase cocktails for efficient hydrolysis

of crystalline cellulose to glucose. The cellulase cocktail research is rapidly evolving, and there are more advancements for achieving feasible economic cellulosic ethanol process (Rai et al., 2019). There are many other aspects like high biomass crops, low lignin feedstocks, chimeric cellulases, non-hydrolytic enzymes, etc. are also improved by genetic engineering toward making the cellulosic ethanol competitive with petrol.

10.9 Conclusions

The biochemical processing of LCB to produce cellulosic ethanol has been extensively investigated. Over the past decade, tremendous improvement in the process has been achieved. Among various factors, cellulase enzyme cocktail formulation and pretreatment technology are the important factors determining the economics of the process. Industrial cellulase cocktails supplemented with non-hydrolytic enzymes boosted the enzymatic hydrolysis efficiency with low enzyme loading. The optimization of the process at HSL of biomass is important for achieving the sugar and ethanol concentrations comparable to the first-generation ethanol process.

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11

Advancement in Biogas Technology for Sustainable Energy Production

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11.1 Introduction

The incessant and increasing energy utilization, using up of fossil fuels and rising environmental disquiet, has cumulatively fetched the attention of energy production toward biofuel. The world's population is increasing at an alarming pace. The increase of approximately 10 billion people in population by 2050 will tremendously increase the demand for energy by approximately 24 billion tonnes coal equivalent per year as per United Nations calculations (Schiffer, 2008). Due to increasing environmental issues and fuel prices, the development of alternative sources of energy has become imperative. Hence, great emphasis is being given for the generation of biofuels (ethanol, biodiesel, biogas, etc.) from renewable energy sources.

Biogas is the product of a biologically mediated process, which is known as anaerobic digestion (AD). AD is one of the established processes producing biofuel. AD produces biogas by decomposing biodegradable matter in an anaerobic environment by consortia of microorganisms (Khanal, 2008; Sawatdeenarunat et al., 2015). The microbial consortium works in collaboration to break down recalcitrant biomass structures (like lignocellulose) into their elementary constituents. Complex and unsterile raw material can be transformed into energy-rich biogas through AD. Biogas is a concoction of CH_4 (50–60%), CO_2 (30–40%), H_2 (1–5%), N_2 (0.5%), CO, H_2S, and water vapors (traces). The nature of the feedstock and the reactor pH mainly determine the CH_4 and CO_2 content in biogas (Muñoz et al., 2015). The quality of biogas depends on the methane content. More the methane content more is the calorific value. The other constituents are considered unimportant. More the other constituents like CO_2, H_2S, N_2, etc. lower will be the

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calorific value. The removal of CO_2, H_2S, and other constituents from biogas is a very crucial point to the technological and economic feasibility of upgrading process of the gas (Lovane et al., 2014). H_2S and NH_3 are toxic and extremely corrosive, damaging the combined heat and power (CHP) unit and metal parts via the emission of SO_2 from combustion. Moreover, the presence of siloxanes in biogas, even in minor concentrations, is also associated with problems. It is very well known that during combustion, silicone oxides generate sticky residues, which deposit in biogas combustion engines and valves causing malfunction (Abatzoglou & Boivin, 2009). Therefore, need of the hour is to have robust technologies to remove these impurities from the biogas and enhance the efficiency of biogas production.

The present chapter provides a comprehensive outline of the development of biogas around the globe and different advanced biogas technologies and concepts. It also discusses issues pertaining to biogas production and current trends in this field. It deals with the role of biotechnology and nanotechnology in the enhancement of biogas production. Finally, it covers various physical, chemical, and biological biogas-upgrading approaches.

11.2 Biogas Developments Worldwide

The steady growth in the number of European biogas plants over the past decade indicates the robust nature of the biogas sector. By the end of 2017, there were 17 783 biogas plants and 540 biomethane plants in operation Europe wide. The total installed electric capacity (IEC) of biogas plants in Europe continued to increase in 2017, growing by 5% to reach a total of 10 532 MW (Deremince & Königsberger, 2017; EBA, 2018). The electricity produced from biogas amounted to a European total of 65 179 GWh. Biomethane production has also increased, reaching 19 352 GWh or 1.94 billion cubic meter (bcm) in 2017. Within the past five years, 3122 new plants have been installed to give an increase of 18%. In the year 2017, an increase of 2% in the number of biogas plants was achieved (Deremince & Königsberger, 2017; EBA, 2018). The feedstocks for these biogas plants mostly include agricultural, sewage, and landfill waste.

Germany has been the driving force for biogas development for many years and is still the foremost country in terms of the number of operational plants. The number of plants is highest for Germany (10 971 plants) and Italy (1 655 plants), followed by France, Switzerland, United Kingdom, and the Czech Republic with more than 500 plants each (Figure 11.1). Traditionally, the bigger countries are also the ones with a stable biogas market.

The number of plants relative to the population of some European countries is shown in Figure 11.2. Germany stays in the lead but is now followed by Switzerland, Czech Republic, Luxembourg, and Austria to make up the top 5, while Italy, France, and the United Kingdom fall behind, making clear that there

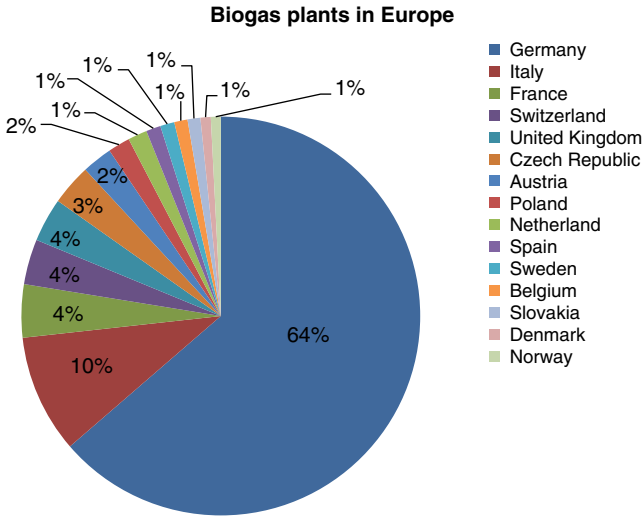


Figure 11.1 Number of biogas plants in European countries. *Source:* Modified from EBA (2018).

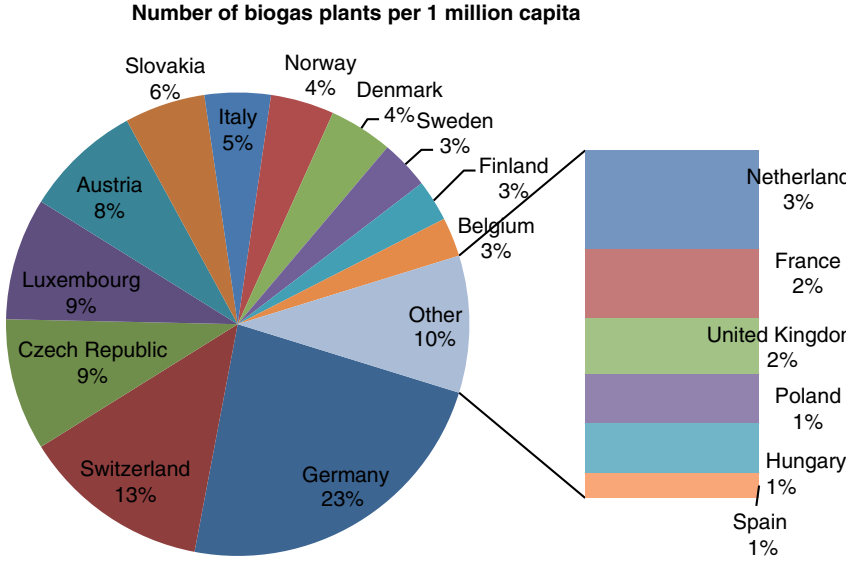
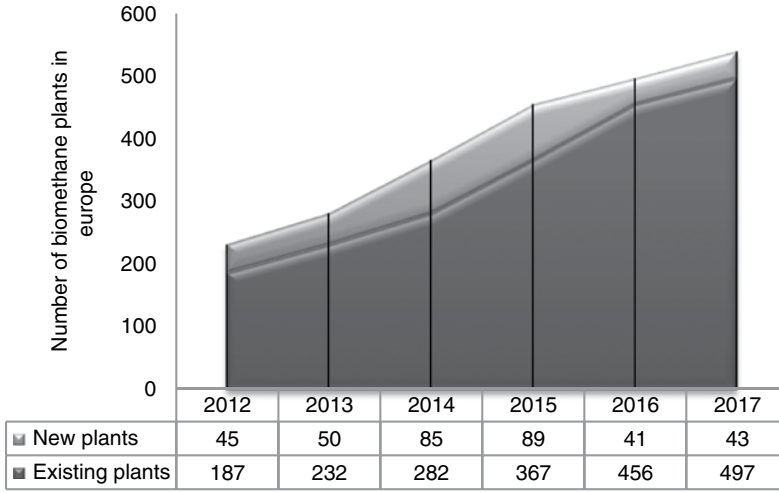


Figure 11.2 Number of biogas plants (total) relative to the population of European countries in 2017. *Source:* Modified from EBA (2018).

is still a very great potential for all European countries to further develop their biogas markets. The European countries, namely, Austria, Switzerland, Germany, Denmark, Spain, Finland, Switzerland, France, Hungary, Iceland, Italy, Luxembourg, Netherland, Norway, Sweden, and the United Kingdom reported biomethane production in 2017. Other countries like Estonia, Ireland, and Belgium have also reported their first biomethane-producing plants in 2018. This demonstrates the fast development of biomethane sector in Europe. The number of the biomethane plants has risen quickly in recent years, from 187 plants in 2012 up to a total of 540 plants in 2017, as indicated in Figure 11.3.

In the United States, there were more than 2100 biogas plants in 2017, of which 250 farm-based digestion plants using livestock manure and 654 biogas recovery plants from landfills (USEPA, 2017a). From a number of 15000 wastewater treatment plants (WWTPs), there were about 1240 WWTPs operating anaerobic digesters producing biogas. Almost all wastewater biogas plants are installed at large-scale facilities, treating from one to several hundred million gallons per day of wastewater. Biogas plants had an installed electricity capacity of 2400 MW in 2015 and 2438 MW in 2016 and generated 1030 GWh electricity (ABC USA, 2017; USEPA, 2017a, 2017b). The energy potential of biogas in the United States was assessed at 18.5 billion m^3 of biogas/year, of which 7.3 billion m^3 from manure, 8.0 billion m^3 from landfill sites, and 3.2 billion m^3 from WWTPs, which could generate about 41.2 TWh of electricity.



	2012	2013	2014	2015	2016	2017
New plants	45	50	85	89	41	43
Existing plants	187	232	282	367	456	497

Figure 11.3 Development of the number of biomethane plants in Europe. *Source:* Modified from EBA (2018).

11.3 Biogas Development in India

In 1970, the first oil crisis made it obvious to the Indian policymakers that the rural and urban poor section of the population would not afford conventional energy like petrol, diesel, kerosene, etc. (Deo et al., 1991; Mittal et al., 2018). The oil products were solely imported from other countries. The energy crisis at the global level and shortage at the local level worsened the national energy security due to rising costs of energy imports. This affected the national budget and laid pressure to meet the rising energy subsidy for domestic fuels, like kerosene, utilized by the poor urban and rural people for cooking and lighting purposes (Mittal et al., 2018). Therefore, it became imperative to the Indian policymakers to devise policies wherein conservation and augmentation of local resources like agricultural waste and animal waste are the priority, so that these can be used in efficient ways to derive energy. Several rural programs like the National Biogas and Manure Management Program (NBMMP), off-grid biogas power generation program, and waste to energy program have been implemented by the government for biogas development in India (Milieu & Shukla, 2007; MNRE, 2015). The National Project on Biogas Development of the Ministry of Non-Conventional Energy Sources started in 1981–1982 for promotion of family-type biogas plants, to provide clean alternate fuel to the rural masses and enriched organic manure for agriculture, was also a part of multi-prolonged strategy endorsed to attenuate the rural energy crisis (Milieu & Shukla, 2007). The implicit objective of this program is to reduce the use of nonrenewable fuels and fuelwood.

NBMMP is a Central Sector Scheme, which provides a subsidy for the setting up of family-type biogas plants mainly for rural and semi-urban/households. Central subsidy is being extended for the setting up of a biogas plant. In India, the total target for setting up family-type biogas plants under the NBMMP for 2012–2013 to 2016–2017 was 562 000 and out of which the total achievement was 415 140 with an estimated quantum of 3 030.5 lakh m^3 biogas produced. The number of biogas plants installed and the number of villages covered under NBMMP for the year 2015–2018 were 166 918 and 43 380, respectively. The cumulative number of biogas plants installed in India from 1981 to 2017 was 4 966 628 with an estimated biogas production of 18 128.1 lakh m^3/year 2016–2017. The selected state-wise number of organic waste-based biogas power plants installed in India from 2015 to 2018 was 96. These were installed in Andhra Pradesh, Delhi, Haryana, Karnataka, Madhya Pradesh, Maharashtra, Punjab, Tamil Nadu, Telangana, Uttar Pradesh, and Uttrakhand. The total estimated biogas production in India has increased 31% from 2011–2012 to 2016–2017 as shown in Figure 11.4 (Indiastat, 2019).

In India, the biogas plants are based on cattle dung, poultry droppings, municipal waste, fruit and vegetable waste along with agricultural residues (Paddy

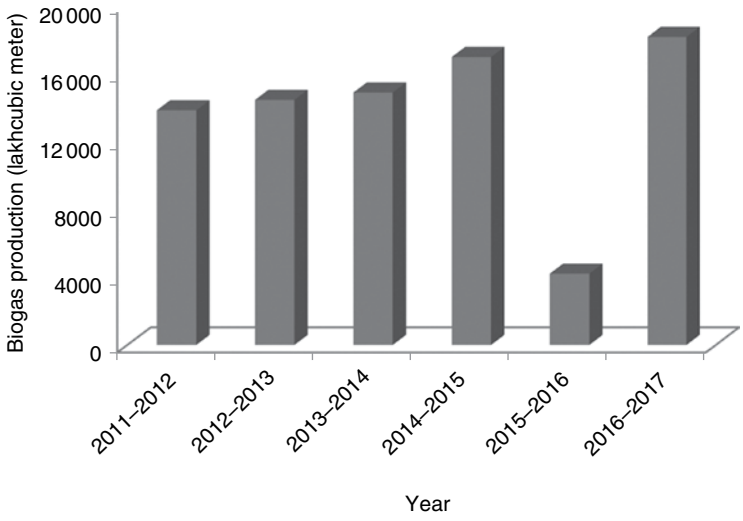


Figure 11.4 Biogas production in India. *Source:* Data from Indiastat (2019).

straw). The household biogas plants based on cattle manure are mainly used for cooking and lighting, whereas medium-sized biogas plants either based on cattle manure or agricultural residues or other wastes like distillery spent wash, poultry droppings, etc. are used for heat and electricity generation and other purposes. Some of the biogas plants in India are high-rate biomethanation and power generation and compressed biogas plant in Ludhiana (1.0 MW power from biogas produced from about 250 tonnes/day of cattle manure), biogas plant in Jabalpur (1.2 MW power project based on cattle manure), biogas plant in Tamil Nadu (bagasse wash water based), biogas-plant-based power generation plant at village Thamna Gujrat, mixed-feed biogas-fertilizer plants (BGFP) at Abohar Punjab, Biogas bottling project (1000 m^3/day capacity) installed at Singla Bio-Energy, Sri Ganganagar (Rajasthan). Paddy-straw-based biogas plant, one of its kind, has been installed by Sampuran Agri Ventures at Fazilka Punjab. The Indian Govt. has sanctioned financial assistance for the installation of plants, which will produce compressed biogas (CBG) of CNG quality. The 11 biogas bottling projects of this type have been commissioned in the India after acquiring required permissions from state and central government agencies. These 11 commissioned biogas bottling projects are in Maharashtra (2), Punjab (3), Karnataka (1), Haryana (4), and Rajasthan (1) (https://mnre.gov.in/. . ../case-study-biogas).

11.4 Recent Issues in Biogas Production

The environmental benefits of biogas technology are often highlighted, as a valid and sustainable alternative to fossil fuels with the final goal of mitigating global warming (Cecchi & Cavinato, 2015). Although in the environmental safety way, biogas production is one of the good alternative to burning fossil fuels, but some constraints are faced during its production. Some of them are low-quality construction of biogas plants, leakage of pipelines, low biogas production, and lack of maintenance, which hinder the effective operation of specialized biogas services (SBS). In Kenya Nzila et al. (2012) employed a multi-criteria perspective for assessing the sustainability of biogas systems focusing on various dimensions like technical, economic, and environmental sustainability. For the technical issues, they referred to operational reliability, which is weighed by non-failure rate; however, the study does not present details on operational reliability. Technical analysis done through fault-tree approach helps in identification and analysis of potential risks in SBS and to improve SBS. Operational reliability was used to analyze operation problems and failures based on data like problems related to relative positioning of plant components that are not appropriate like plant is too far from animal shed, inlet tank is too high for feeding, outlet tank is too remote to be reached, cleaning the clogged inlet pipes during feeding or big cracks on the wall of the inlet tank or outlet chamber (because of poor construction quality or damage done by natural disasters such as floods etc.). Accidentally biogas slurry is discharged into water bodies without any disposal treatment causing environmental pollution. The slurry is only stored as waste without being reused as organic fertilizer. Slurry is applied to farmlands directly without composting or stabilization. The main greenhouse gases (GHG) produced during this process leads to environmental and health concerns. Emission factors of biogas plants operating direct biogas combustion of carbon monoxide (310 g/GJ), sulfur dioxide (25 GJ^{-1}), nitrogen oxides (540 GJ^{-1}), non-methane volatile organic compounds (21.15 GJ^{-1}) and formaldehyde (14 GJ^{-1}) (Paolini et al., 2018). Sustainability criteria set challenging GHG emission targets for biogas plants that are achievable and consistent with expectations of facility operators providing a robust, consistent, and logical methodology is crucial for the confidence of industry, investors, and future development of the sector.

11.5 Current Trends in Biogas Production

Biogas formation is a complex and multistep microbial process where microbes have a synergistic, interdependent relationship with each other. For efficient biogas formation, robust pretreatment methods and efficient anaerobic digesters

are need of the hour. Further to gain a better insight into the digestion process, its development, and optimization, it is necessary to have knowledge of the microbial community.

Development in the pretreatment strategies aims to enhance the product yields from lignocellulosic feedstocks and lower the methane emissions to the atmosphere, thus positively contributing toward environmental protection. The effect of pretreatment techniques on AD has only recently been investigated, and it is still necessary to optimize these techniques in terms of efficiency, cost, and application range. Further research will likely focus on whole-process engineering, in which pretreatment is integrated into the digester, rather than viewing pretreatment as a separate process. Pretreatment must overcome the structural barriers of lignocellulose and its polymers (cellulose and hemicellulose) by subjecting them to microbial breakdown activities, resulting in enhanced biomass degradation and increased biogas yield (Achinas & Euverink, 2016). Ideal biomass pretreatment aims to make the substrate more accessible to microorganisms by completely or partially decomposing the feedstock into fermentable sugars, thus eliminating the lignin resistance and decreasing the crystalline structure of the cellulose. The problem of recalcitrance has not yet been solved; further research is required to investigate new genetic engineering approaches to solve problems related to degradation and thereby succeed in achieving higher efficiency (Abramson et al., 2012).

The dynamics of the different microbial groups involved in AD are complex and interactive. The quantities of the microbial groups are disproportionate and influence the overall process reaction rate. It is reported that among the four microbial groups involved in AD, methanogens have the slowest growth rate and are the most sensitive to changes in process conditions such as temperature, pH, redox, and inhibitors. Hence, methanogenesis is the key pathway for biogas production and is commonly considered to be the rate-limiting step of the whole process (Chen et al., 2008). Recent studies report that one strategy to improve process economics is the optimization of the metabolic pathways in order to genetically modify the metabolic efficiency of the microbes. The investigation of different metabolic pathways has led to energy-rich biofuels (Xu & Koffas, 2010).

Various molecular techniques have been reported for the identification of bacterial and archaeal communities from AD. Combinations of 16S ribosomal RNA gene clone library sequencing and dot blot hybridization, denaturing gradient gel electrophoresis, real-time polymerase chain reaction (PCR), terminal restriction-fragment length polymorphism, and fluorescence in situ hybridization have all been reported for assessing microbial community structures and their shift during AD of different wastes (Bialek et al., 2012; Ike et al., 2010; Shin et al., 2010). Morris (2011) hypothesized that several methanogens are related with the production rate of methane. Using a quantitative PCR, the researchers investigated some gene copies and mRNA transcripts of the methanogen-specific gene mcrA from DNA

and RNA of different anaerobic digesters. Metagenomics is the extraction and sequencing of the bulk DNA from an uncultured, environmental sample (Culligan & Sleator, 2016). Metagenomics allows the metabolic potential of the microbial community to be accessed. The "omics" methods that are the combination of metagenomics, metatranscriptomics, and metaproteomics are very helpful molecular biology tools used to diagnose anaerobic digesters. Other important parameters should also be considered in developing AD process models. Such parameters include cation concentrations, nutrients availability, inhibitors' concentration, degree of acclimation to the feedstock, startup conditions of the digester, feedstocks' solid concentration and their type, hydraulic loading, organic loading, and biogas production per unit volume (Karthikeyan et al., 2016; Ma et al., 2016).

Design of reactors is very important for an efficient AD process and biogas formation. A considerable number of research projects have been developed to evaluate different configurations (e.g. single- or multiple-stage reactors) in order to enhance the efficiency of AD. Recent studies report that the separation of the AD process into two stages, such that hydrolysis/acidogenesis and acetogenesis/methanation carried out in separate reactors (EPA, 2006), can increase the conversion rate of organic material to methane; however, the cost of such a complex system is a significant drawback (Yu et al., 2017). The application of multiple bioreactor systems generally has a specific goal, such as improved process stability and higher efficiency. A multiple-stage bioreactor system permits different conditions (e.g., organic loading rate and temperature) to be applied. At present, few multiple-stage AD units operate to produce biogas fuel on a commercial scale. The complexity and high cost of this multiple-stage technology are barriers to commercial use. An alternative technique has been developed that is based on a high working pressure (up to 100 bar, 1 bar = 100 kPa); with this technique, the production of biogas with more than 95% methane content is feasible. The technique aims to integrate biogas production and in situ increased-pressure purification into a single process in order to produce clean biogas (99% methane) that can be fed directly into the natural gas networks. Lindeboom et al. (2011) reported that pressure of up to 20 bar can increase methane yield and that in situ upgrading is successful with a high-pressure autogenerative method. The biogas produced by this method contains less than 5% CO_2, because more CO_2 dissolves in the water under high pressure.

11.6 Advanced Anaerobic Digestion Methodologies

Biogas technology being an old process is developing day by day. In the last decade, many approaches and modifications in the AD systems have been made to enhance process efficiency. New reactors have been made to overcome the already

existing issues like retention of the slow-growing methanogenic biomass, separation of inhibitory compounds, reducing downstream processing cost, etc. Now molecular biology techniques have made it possible to comprehend the microbiological aspect involved in biomass degradation and made it possible to control and regulate the process more efficiently than the traditional methods. Some of the important technologies are as follows:

11.6.1 Anaerobic Membrane Reactor (AnMBRs)

The latest progress in biogas generation is the incorporation of AD process with membrane separation techniques in membrane bioreactor. It aims to enhance the concentration of biomass extensively in the reactor. This allows the sustenance of high cell concentrations under high hydraulic load. This is possible as hydraulic retention time (HRT) is uncoupled from the solid retention time by sufficient mixing. The capability of effective retention of microorganisms in AnMBRs results in higher productivity and concentration of product, apart from providing pronounced resistance to toxic substances. It also simplifies the biomass or product formation. All this improves the biogas production economy (Ylitervo et al., 2013). This technology has promising capability to generate biogas, high quality effluent, minimize waste, and demands less capital costs and energy requirements. Different types of AnMBRs available for production of biogas are broadly divided into two categories: conventional and modified AnMBRs. The conventional AnMBRs include completely stirred tank reactor (CSTR) AnMBRs, upflow anaerobic sludge blanket (UASB) AnMBRs, expanded granular sludge bed reactor (EGSB) AnMBRs, anaerobic fluidized-bed membrane bioreactor (AFBR) AnMBRs, jet flow anaerobic bioreactor (JFAB) AnMBRs. The modified AnMBRs consists of Anammox AnMBRs, anaerobic dynamic membrane bioreactors (AnDMBRs), anaerobic membrane distillation bioreactors (AnMDBRs), anaerobic osmotic membrane bioreactors (AnOMBRs), anaerobic membrane sponge bioreactors (AnMSBRs), gas-lifting AnMBRs (Gl-AnMBRs), vibrating AnMBRs (V-AnMBRs), anaerobic bio-entrapped membrane bioreactors (AnBEMRs). Although research is being carried out in the direction to develop and make potential AnMBRs, it still demands more research inputs to surmount various challenges like inhibition of product, methane recovery, fouling of membrane, and cost of membrane (Chen et al., 2016).

11.6.2 Dry Anaerobic Digestion Technology (DADT)

AD process can be divided into two categories depending upon the total solid (TS) content: wet and dry AD. The process is wet if TS content is less than 15% and dry if it is more than 15%. Biogas from solid organic wastes can be directly produced

through a technology known as dry digestion. It is considered as a practicable way to treat solid organic wastes like agricultural residues etc. It consists of both batch and continuous processes. The DADT is luring the attention as it does not involve foam problems, sedimentation, surface crust, and does not need size reduction or removal of inert substances. Besides these advantages, it also enhances biogas efficiency cum production rate, reduces the cost, and simplifies the operation. The digested products can be directly utilized as fertilizers or soil conditioners without dewatering (Wang et al., 2011; Wu et al., 2006).

11.6.3 Anaerobic Co-digestion Technology (AcoD)

Anaerobic co-digestion technology (AcoD) is a reliable alternative option to resolve the disadvantages of a single-substrate digestion system related to substrate characteristics and system optimization. Co-digestion involves digestion of more than one feedstock together. It is carried out to maintain the C/N ratio of the mixture close to optimal. The execution of AD is strongly determined by the C/N ratio of the substrate. Generally, the nitrogen content is high in algal biomass, so its C/N ratio is around 6–10 (Yen & Brune, 2007). This is quite lower than the required range of 25–32. The biomass with high protein content may lead to the production of high levels of ammonia and further accretion of the toxic volatile fatty acids. Therefore, such sort of feedstocks having high N content must be supplemented with carbon-rich co-feedstocks to corroborate good conversion to methane.

Co-digestion of substrates was earlier thought of providing synergistic interactions during digestion, but now it is established that the methane production is mainly dependent on total organic rate preferably than on synergistic effect of different substrates (Mata-Alvarez et al., 2014). Co-digestion of pig manure with cultivated algae (Astals et al., 2015), dairy wastewater with cattle manure (Toumi et al., 2015), sewage sludge with glycerol (Jensen et al., 2014) under different operating conditions confirmed that the biogas production is improved comparing with single-substrate feed. The AcoD technology helps to obtain enhanced nutritional balance in the system and reduces the possibility of lipids and ammonia inhibiting the digestion environment. Zhang et al. (2016) conducted experiment using the high-solid AD to enhance the biogas production and achieved the biogas yield of 478 ml/gVS at a $C : N$ ratio of 25. Adding organic materials (e.g. activated carbon, zeolites, polymers) in system designing and integrating the thermodynamic and kinetic modeling may improve the optimization process for biogas production and thus making it more economically feasible. To enhance the biogas production, the addition of clinoptilolite shortens the lag phase of co-digestion, improves the organic carbon matter destruction, and controls the optimal C/N ratio. Zeolite, one of the environmentally friendly inorganic additive material, is used to enhance the performances of AcoD. Because of its conducive

characteristic for microorganisms, adhesive zeolite has widely been used as ion exchangers for the removals of ammonium and absorbs heavy metals, which are toxic for microorganisms in AcoD process (Wang et al., 2015). Song and Zhang (2015) reported the pretreatment of co-substrates using wet-state H_2O_2 can improve effectively the biodegradability and methane yield from wheat straw and cattle manure. AD process and biogas production, including different mathematical models about the process, have been studied up till now, but the stability and optimization problems still require further investigations. ADM1 model is a powerful tool to predict, control, and optimize the AcoD system. Thus, innovative AcoD design may significantly improve biogas production as renewable energy and the environmental quality.

11.7 Role of Biotechnology in Enhancing Biogas Production

Recent interest toward shifting to non-food-based feedstock for biofuel production, in addition to the biomass burning issue, has resulted in exploiting agricultural residues as feedstock. However, the recalcitrant nature of this biomass makes it extremely difficult to hydrolyze into fermentable sugar. Devising cost-effective processes to produce novel enzymes effective against the cellulosic biomass is an example of how energy can be extracted from biological sources. Biotechnology offers a concrete promise for the development of more effective and sustainable processes. The use of biotechnological methods like genetic engineering and other modern technologies (protein engineering, biological pretreatment, and bioaugmentation) have enormous potential for the production of clean and renewable energy from biological sources. The microbial engineering will help to engineer bacteria with enzymes capable of degrading lignocellulosic biomass. It will disrupt the catabolite repressor and overexpress the cellulase gene activator to enhance the enzyme production by several folds. The protein engineering will develop novel enzymes (cellulases, xylanases) with higher specificity toward cellulosic biomass. Many leads have been obtained to understand the role of several unannotated transcription factors involved in the cellulase production via transcriptomic and proteomic studies, which are being valorized to construct a superior biocatalyst.

The biological pretreatment and bioaugmentation for enhancing the performance of AD are quite interesting, recent, and demand more research inputs. Biological pretreatment using enzyme or microorganism to pretreat lignocellulosic material for producing biogas is a promising technology due to its environmental friendliness and cost-effectiveness, although the process is time-consuming compared to physical and chemical pretreatment (Zheng et al., 2014). The

biological pretreatment involves the use of pure culture, mixed culture, and enzymatic pretreatment for enhancing biogas production from lignocellulosic material. The effect of enzymatic pretreatment on enhancing biogas production in most cases is still lower than that of physical or chemical pretreatments, but it can be made more promising through strain mutation, genetic engineering, protoplast fusion, and process optimization might be helpful. Various lignocellulase genes with thermostability, alkalostability, overcoming feedback inhibition, and other economically important traits have been cloned and expressed (Kuhad et al., 2016). Lima et al. (2016) used four *Aspergillus nidulans* recombinant strains to simultaneously produce a multi-enzymatic cocktail of arabinofuranosidase, endo-1,4-xylanase, endo-1,5-arabinanase, and xyloglucan-specific endo-β-1,4-glucanase; the recombinant enzymatic pretreatment was residue-free and seemed to be more efficient than the applied alkaline method. With the developing of biotechnology, the use of enzymatic pretreatment for enhancing biogas production from lignocellulosic materials could be unquestionable economically wise in the future.

It is well established that each stage, i.e. hydrolysis, acidogenesis, acetogenesis, and methanogenesis of AD process, is carried out by different microorganisms; the successive methanogenesis depends on the balance of the four steps; and any rate-limiting step would limit the overall rate of biogas production (Vanwonterghem et al., 2016). Therefore, using bioaugmentation technology, i.e. supplying the desired organisms to increase the activity of microorganisms involved in the rate-limiting stage is an attractive option to increase the biogas yield.

In short, genetic engineering will produce strains having capabilities like wide pH tolerance, toxin resistance, high enzyme titer producing and efficient lignocellulose degraders, etc. Such strains are very much needed. With the development of biotechnology, recombinant strains would be frequently used in the AD system since they have the potential to increase biogas production (Wei, 2016).

11.8 Application of Nanotechnology in Biogas and Methane Production

Nanoparticle additives show positive and negative effects on the biogas production rate. The materials are classified into three categories: (i) metal oxides, (ii) zero-valent metals, and (iii) nano-ash and carbon-based materials. The efficiency of the biogas production process, i.e. the methane production and the degree of degradation, has been increased by the addition of trace elements (Feng et al., 2010). Positive effects by trace elements addition have also been observed in specific methanogenic activity assays with various substrates, including methanol Co and Ni (Zandvoort et al., 2006), acetate, and propionate (Ni, Co, and Fe) (Zitomer et al., 2008). Qiang et al. (2012) mentioned that the growth of

methanogenic bacteria is dependent on Fe, Co, and Ni during their enzyme synthesis. Otero-González et al. (2014) investigated that the addition of CuO nanoparticles (NPs) decreased the methane production by 15% in comparison with the control reactor. Ni NPs produced the highest significant biogas and methane production compared to Co, Fe, and Fe_3O_4 NPs and the control. However, high concentrations of these elements can inhibit the biological degradation process in anaerobic reactors. The methanogenesis activities showed a significant decrease in case of acetate, while it had not strongly been affected in case of hydrogen, proving that CuO NPs inhibit the acetoclastic activities rather than the hydrogen trophic activities (Otero-González et al., 2014). The nanosized CuO showed the most negative effect on the biogas production rate followed by nanosized ZnO. In acetoclastic methanogenesis, NPs CuO was found to completely inhibit the methanogenesis, while NPs FeO decreased the methanogenesis activities to 85% and NPs AgO showed no significant effect on methanogenesis activities (Gonzalez-Estrella et al., 2013). Zero valance iron proved in literature to be a suitable low release electron donor for methanogenesis during the AD process. The addition of trace metals in form of nanoparticles reduced not only the lag phase but also the time to achieve the highest biogas and methane production (peak). Nanoparticles have demonstrated biostimulating effects on the methanogenic activity during the start-up of the AD of cattle slurry process through the HRT.

11.9 Biogas Upgrading Technologies

The biogas upgrading technologies involve the removal of impurities like CO_2, H_2S, N_2, etc. from the biogas. The removal of these impurities is imperative for biogas recovery and purification. There are various approaches to achieve this objective. The main processes involved in this have been summarized in Table 11.1.

11.10 Conclusion

Biogas production is a sustainable process. It creates a renewable source of energy and at the same time utilizes organic wastes. It encounters certain limitations in terms of efficient substrate digestion, robust purification process, and dissemination of technology properly. Various technologies developed for biomass pretreatment, digestion, and biogas purification have proven to be very promising. It still needs colossal research inputs to make the biogas production process more efficient and sustainable. Lastly, the governments need to frame proper policies under which subsidies are to be given to set up biogas plants at family, community, or industrial levels to boost up the biogas market and its dissemination.

Table 11.1 Biogas upgrading technologies.

S. No.	Technologies for biogas purification	Advantages	Disadvantages	References
A	**Old technologies**			
1.	*Pressure swing adsorption (PSA)* This technology separates the different gasses from biogas based on their molecular characteristics and the affinity of the adsorbent material	1) 95–99% CH_4 concentration 2) The humidity of the raw biogas can be removed 3) Less energy demand with low emissions, elimination of nitrogen and oxygen is also possible 4) Relatively fast installation and easy start up	1) High capital investment and operational costs 2) H_2S elimination step is needed, and tail gas from the process needs to be treated 3) Water should be removed before PSA process 4) High CH_4 losses when valves malfunction	Chen et al. (2015), Cavenati et al. (2005), Zhao et al. (2010), Bauer et al. (2013), and Augelletti et al. (2017).
2.	*High pressure water scrubbing* Separation of CO_2 and H_2S from the biogas due to their increased solubility in water compared to CH_4.	1) >97% CH_4 concentration. 2) Removal of both CO_2 and H_2S. 3) No special handling and chemicals are required. 4) Tolerant for impurities. 5) Easy in operation with low CH_4 loses (<2%). 6) Regeneration of water is possible.	1) High investment and operating costs 2) Less efficient 3) Low flexibility toward variation of input gas 4) High pressure, need higher energy to compress the gas and to pump water 5) Clogging due to bacterial growth	Chen et al. (2015), Zhao et al. (2010), Krich et al. (2005), Weiland (2009), Wang et al. (2015), and Ryckebosch et al. (2011).
3.	*Organic physical scrubbing* Absorption of CO_2 and H_2S is accomplished by the use of organic solvent instead of water.	1) Anticorrosion nature of solvents but shares only 6% of the upgraded biogas market. 2) >97% CH_4 concentration 3) Remove organic components such as H_2S, NH_3, HCN, and H_2O	1) Complex operation with high investment and operational costs 2) Expensive for small-scale applications 3) Reduced operation when dilution of glycol with water 4) Solvent regeneration is difficult if H_2S is not removed first	Chen et al. (2015), Krich et al. (2005), Angelidaki et al. (2018), and Bekkering et al. (2010).

(*Continued*)

Table 11.1 (Continued)

S. No.	Technologies for biogas purification	Advantages	Disadvantages	References
4.	*Chemical scrubbing* Chemical absorption involves reversible reaction between absorbed substances and solvent	1) >99% CH_4 concentration with low operational costs 2) Complete H_2S removal is possible and can operate at low pressure 3) High selectivity for CO_2 with low CH_4 loss 4) The process is faster than water scrubbing, and solvent is easier to regenerate	1) High investment cost, and heat is required for regeneration of solvent 2) Problems of contaminant build-up, corrosion, and amine breakdown 3) Waste chemical may require treatment	Chen et al. (2015), Zhao et al. (2010), Krich et al. (2005), Ryckebosch et al. (2011), Andriani et al. (2013), and Bauer et al. (2013).
5.	*Cryogenic separation* Cryogenic separation of biogas is based on the principle that various gases like CO_2 and H_2S liquefy under different pressure and temperature conditions	1) High purity of CH_4 with 98% concentration 2) CO_2 purity is also high and can be used as a dry ice 3) Low energy and cost is required to obtain highly pure liquefied biomethane (LBM) with less than 1% CH_4 loss. 4) Environmentally friendly technique with no chemicals use	1) High investment, maintenance, and operational costs 2) High energy requirements 3) Use of different expensive process equipment	Ryckebosch et al. (2011), Andriani et al. (2013), Bauer et al. (2013), Muñoz et al. (2015), and Grande & Blom (2014).
6.	*Membrane separation* The membrane acts as a permeable barrier that allows specific compounds to pass through differently and control their permeability based on the applied driving forces such as the difference in concentration, pressure, temperature, and electric charges of different species	1) Less operational and capital investment costs and high CH_4 recovery up to >96% 2) Small space requirements and available at low capacities 3) Easy maintenance without hazardous chemicals 4) Low maintenance cost 5) Highly reliable and cheap process	1) For high-purity product, multiple steps of membrane are required 2) Low CH_4 yield in single step 3) Low membrane selectivity 4) Consumes more electricity per unit of gas produced 5) Not suitable for high purity needs	Chen et al. (2015), Zhao et al. (2010), Andriani et al. (2013), Bauer et al. (2013), and Baker & Lokhandwala (2008).

B	**New technologies**			
7.	*Microbial electrochemical method (MEC)* Electrons released by bacteria from the oxidation of organics in anode can combine with protons to generate hydrogen in the cathode chamber, used for biogas upgrading	1) Sustainable and cost-effective way to upgrade biogas 2) Methane could directly be produced by the reduction of CO_2 in the cathode using a biocathode in MEC, and methane was produced at an overall energy efficiency of 80% 3) Microbial electrochemical method is environment friendly way to couple several positive actions together, including consumption of CO_2 to upgrade biogas, chemical oxygen demand (COD) removal in the anode and production of valuable gas and liquid products	4) Needs more energy per unit of removed CO_2 (2.36 vs 0.78 kWh/Nm^3 CO_2 removed)	Lovley & Nevin (2013), Angelidaki et al. (2018), and Zeppilli et al. (2016)
8.	*Chemical absorption using amine solution* Chemical scrubbers use aqueous amine solutions (i.e. mono, di or tri ethanolamine) to bind the CO_2 molecules contained in the biogas	1) H_2S can also be completely absorbed in the amine scrubber 2) Final methane content in the output gas can reach 99% purity	1) Toxicity of the solvents to human and environment 2) The initial cost of the amine solvents and their loss due to evaporation	Angelidaki et al. (2018) and Yoo et al. (2013).
9.	*Hydrogenotrophic CO2 removal*	1) Chemically store renewable energy as CH_4 by H_2 production (via water electrolysis) and the subsequent upgrading of biogas 2) Use the heat from methanation and the O_2 produced during electrolysis used for microaerobic removal of H_2S.	Power-to-gas approach exhibits low operating costs	Rittmann et al. (2013) and Rodero et al. (2018).

(*Continued*)

Table 11.1 (Continued)

S. No.	Technologies for biogas purification	Advantages	Disadvantages	References
10.	*Ex situ upgrading* It relies to the provision of CO_2 from external sources and H_2 in an anaerobic reactor that contains pure or enriched hydrogenotrophic culture, resulting in their subsequent conversion to CH_4.	3) It secures the stability of the conventional biogas process because the upgrading is occurring in a separate unit 4) Biochemical process is simpler since there is no degradation of organic substrate (i.e. initial steps of anaerobic digestion, such as hydrolysis and acidogenesis are not performed 5) It is a biomass independent process	6) Low gas–liquid mass transfer rate	Burkhardt & Busch (2013), Savvas et al. (2017), and Guneratnam et al. (2017).
11.	*In situ upgrading* H_2 is injected inside a biogas reactor in order to be coupled with the endogenous CO_2, which is produced in the anaerobic digester and be converted into CH_4 by the action of autochthonous methanogenic Archaea	1) Using pure N_2 gas for desorption, technology is able to generate CH_4 at a purity of 95% and 87% at laboratory and pilot scale, respectively 2) Process can lead up to approximately 99% methane recovery only in cases that operational parameters (e.g. pH) are fully monitored and controlled	1) CH_4 losses are high according to pilot-scale tests, amounting to 2–8% 2) Concentration of CO_2 in the flue gas is very low, since a large amount of air or N_2 is used for CO_2 desorption 3) Increment of pH level to values above 8.5, leading to inhibition of methanogenesis	Angelidaki et al. (2018) and Agneessens et al. (2017).
12.	*Upgrade through fermentation processes* CO_2 in biogas can be biologically converted to methane with the addition of H_2 to achieve biogas upgrading, the production of valuable liquid products from CO_2 in biogas	1) Mixed culture fermentation of CO_2 and H_2 has potential advantages such as no need for sterilization and possibility of using wastewaters for nutrients supply 2) CO_2 and H_2 could be converted to acetate by mixed culture 3) Methane content of 95% in the biogas was achieved by this technology	4) The technology is very promising compared to those based on H_2, since H_2 has low solubility and generally requires high energy to achieve high gas-liquid mass transfer	Nie et al. (2008), Luo & Angelidaki (2012), Martin et al. (2015), and Angelidaki et al. (2018).

13.	*Chemical hydrogenation process* The reduction of CO_2 with H_2 can be either conducted biologically or chemically	5) Due to high selectivity, complete conversion of CO_2 and H_2 can be practically achieved	1) Sustainability is affected by the presence of trace gasses in the biogas, which degenerate the catalysts leading to increased need for periodical replacement 2) Need for pure gasses and the high energy cost to maintain the operational conditions	Jürgensen et al. (2014), Gübitz et al. (2015), and Xia et al. (2016).
14.	*Photosynthetic CO2* Removal an alternative method to sequester the CO_2 in order to obtain a CH_4-rich gas	1) Removal of H_2S and CO_2 in a single-step process 2) H_2S removal efficiencies of 100% concomitant with CO_2 removals of 80–95% are typically reported during photosynthetic biogas upgrading		Bahr et al. (2013), Posadas et al. (2015), Posadas et al. (2017), and Toledo-Cervantes et al. (2016).
15.	*Hydrate formation*	1) Used to remove CO_2 from contaminated. natural gas (2a) 2) CH_4:CO_2 ratio of 75%:25%, the concentration of CO_2 can be reduced to 16%	3) The capture of CO_2 by hydrate formation consumes a large amount of energy, mainly due to the extremely high pressure required by hydrate formation	Van Denderen et al. (2009) and Tajima et al. (2004).
16.	*Biological chemautotrophic methods* Based on the action of hydrogenotrophic methanogens that can utilize H_2 to convert CO_2 to CH_4	4) Using *Methanobacterium thermoautotrophicum* can increase the concentration of biogas from 60% to 96%, while H_2 and H_2S are not detectable	1) H_2 has the inherent disadvantage of a very low volumetric energy density, which renders difficult its storage 2) H_2 as a fuel for the transportation sector is still under development	Luo et al. (2012), Angelidaki et al. (2018), and Muñoz et al. (2015).
17.	*Biological photoautotrophic methods*	1) H_2S removal is additionally achieved, while >54% of CO_2 is consumed 2) The methane recovery of photoautotrophic technologies can reach up to approximately 97% depending on the reactor type and the selected algal species 3) Production of active biomass that can be used either for the extraction of high value-added products or as feedstock for biogas production oriented to circular economy	4) Closed systems have high photosynthetic performance but needs high investment cost and energy demands	Angelidaki et al. (2018), Jürgensen et al. (2014), Guedes et al. (2011), Mussgnug et al. (2010), Meier et al. (2017), Bahr et al. (2013), and Yan et al. (2016).

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12

Biofertilizers

A Sustainable Approach Towards Enhancing the Agricultural Productivity

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12.1 Introduction

The ever so increasing population has resulted in exerting immense pressure on the agricultural sector to cope with the increasing demands for food supply. This necessitates agricultural development to increase the production to successfully match with the growing population. It is estimated that by the year 2037, the global population will be approximately nine billion (Chamie, 2020). Hence, to increase the annual crop yield, various effective strategies such as (i) expansion of irrigational facilities; (ii) use of pesticides and chemical fertilizers; (iii) development of high yielding seed varieties; (iv) advanced mechanization of agriculture; and (v) changed agricultural practices have been employed (Vasile, Popescu, Ion, & Dobre, 2015). Unfortunately, indiscriminate use of these synthetic chemicals (also known as xenobiotes) as fertilizers and pesticides pose a grave menace to the natural environment by polluting soil, water, and air (Rahman & Zhang, 2018; Savci, 2012; Sujanya & Chandra, 2011). Since most of these xenobiotes are nonbiodegradable in nature and cannot be absorbed by the plants, they get accumulated in the soil resulting in undesirable altercations of the soil characteristics (Savci, 2012). These chemical fertilizers were also reported to be responsible for the eutrophication of the water bodies and greenhouse effect, as well as decline in the soil nutrient content and making the crops more susceptible toward phytogenic pathogens (Aktar, Sengupta, & Chowdhury, 2009; Goenadi, Mustafa, & Santi, 2018; Liu, Sung, Chen, & Lai, 2014; Youssef, & Eissa, 2014).

Owing to the above-mentioned adverse effect of the chemical fertilizers, potential alternatives in terms of organic farming have been in demand for quite a time

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to meet the rising demand of food (Mahanty et al., 2017; Reddy, 2015). The cumulative effect of increasing the agricultural productivity along with decreasing the utilization of chemical fertilizers, thus reducing the risks associated with it, is collectively been termed as "Green Revolution," where the researchers are preparing the eco-friendly alternatives for the chemical fertilizers by exploiting the beneficial nature of the microorganisms (Chittora, Meena, Barupal, & Swapnil, 2020). This approach has also been supported by the recent policy of European Union where introduction of eco-friendly agricultural practices has been drafted to prevent further environmental pollution (European Parliament and the Council of the European Union, 2018). One such method to reduce the excessive utilization of chemicals is application of biofertilizers (Mishra & Dash, 2014).

Biofertilizers, also known as "microbial inoculants," are those substances that contain live or latent microbial strains, which colonize the rhizosphere or the interior of the plants and promote growth by increasing the supply and uptake of the nutrients to the host plant (Arora, Khare, & Maheshwari, 2010; Barman, Paul, Choudhury, Roy, & Sen, 2017; Mahanty et al., 2017; Malusá, Sas-Paszt, & Ciesielska, 2012; Malusá & Vassilev, 2014). These microorganisms have the potential to enhance the plant growth by accelerating the microbial processes that increase the availability of the native nutrients in the plant assimilating form and production of various compounds that have plant-pathogen inhibiting properties (Abd El-Lattief, 2016; Toyota & Watanabe, 2013). Use of biofertilizers tends to improve the activity of the indigenous soil microflora, protects the plants from various stress conditions (both abiotic and biotic), and remediates the complex, chemical pollutants present in the soil such as pesticides or heavy metals (Fragoeiro & Magan, 2008; Pant, Pandey, & Kotoky, 2016; Pelcastre et al., 2013; Raja, 2013; Siddiquee, Aishah, Azad, Shafawati, & Naher, 2013; Singh, Singh, Chandra, Patel, & Rai, 2009). Biofertilizers make up an abstract part of integrated nutrient management and play a key role in improving the productivity and sustainability of the soil in eco-friendly and cost-effective manner (Khosro & Yousef, 2012; Reddy et al., 2020). These are the renewable sources of plant nutrition that harvests the naturally available nutrient mobilization system, thus enhancing the soil fertility and crop yield enormously (Mahanty et al., 2017; Pandey & Singh, 2012; Swapna, Divya, & Brahmaprakash, 2016).

12.2 Types of Biofertilizers

Based on their mode of action, the biofertilizers have broadly been classified into three major groups (Figure 12.1). They are nitrogen-fixing biofertilizers, those microorganisms that fix the atmospheric nitrogen and make them available for the plants; phosphorus biofertilizers, those microorganisms that solubilize the insoluble phosphates and transport the solubilized phosphorus through the

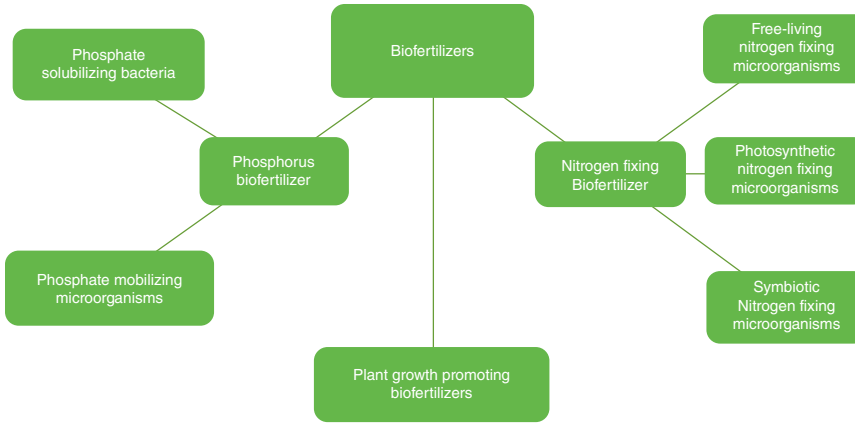

Figure 12.1 Types of biofertilizers.

rhizosphere; and plant-growth-promoting biofertilizers, the ones which produces plant-growth-promoting compounds as by-products of their metabolic pathway.

12.2.1 Nitrogen-Fixing Biofertilizer

Nitrogen is one of the most important macronutrients necessary for the plant growth. It is being used for various important metabolic processes such as formation of nucleic acid base pairs, amino acids, various plant hormones, alkaloids, and transportation within the plant via xylem and phloem (Khosro & Yousef, 2012). However, nitrogen is one of the most limiting nutrients since the plants cannot use molecular nitrogen from the atmosphere and availability of the fixed nitrogen is limited (Itelima, Bang, Sila, Onyimba, & Egbere, 2018). Deficiency of nitrogen is a common stress in the plants that leads to stunted plant growth due to reduced photosynthetic capacity, impaired enzyme synthesis, and chlorophyll content (Shengqi et al., 2012). Thus, nitrogen is being added separately during the crop cultivation in the form of nitrates and ammonium salt. However, the best alternative for these chemical-based fertilizers is the biological nitrogen fixation by a specialized group of microorganisms, which converts the freely available atmospheric nitrogen into nitrates, ammonia, and sometimes urea (Bakulin, Grudtsyna, & Pletneva, 2007; Gothwal, Nigam, Mohan, Sasmal, & Ghosh, 2008). The nitrogen-fixing microorganisms also known as "nitrogen fixers" were divided basically into three groups: free-living bacteria such as *Azotobacters*, blue green algae such as *Azolla*, *Anabaena*, and symbionts such as *Rhizobium* (Kumar, 2016). The biological nitrogen fixation can be represented by the following reaction (Biswas & Gresshoff, 2014):

$$N_2 + 16ATP + 8e^- + 8H^+ \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

12.2.1.1 Free-Living Nitrogen-Fixing Microorganisms

Microorganisms belonging to the family *Azotobacteriaceae* were known to have the nitrogen-fixing ability and were reported to be used as potential biofertilizers in nonleguminous crops such as rice, coconuts, barley, wheat, maize, tobacco, sunflower, oat, beetroot, line, sugarcane, coffee, and tea (Bhat, Ahmad, Ganai, Shams-Ul-Haq, & Khan, 2015; Gupta, Annapurna, & Jaitley, 2016; Wani, Chand, & Ali, 2013). They are free-living, aerobic, heterotrophic bacteria that are mostly found in neutral or alkaline soils (Bag, Bappa Paramanik, & Ashok Choudhury, 2017). They can reportedly be able to fix up to 20 kg of nitrogen per hectare annually (Mahanty et al., 2017). Six members of this family have been reported till date for their ability of nitrogen fixation, out of which *Azotobacter chroococcum* is the most commonly occurring species that is found at an average of 10^4–10^5/g of soil. The other members are *Azorhizophilus paspali, Amblyseius armeniacus, Acanthosis nigricans, Acinetobacter beijerinckii, Azotobacter vinelandii, Aethalochroa insignis*, and *Azomonas macrocytogenes* (Chandra, Arora, Barh, & Sharma, 2018). As biofertilizers, *Azotobacters* not only increase the nitrogen availability of the plants but also produce various plant hormones, growth-promoting factors, and antifungal agents that help in plant growth, improved seed germination, and protection of the plants from various pathogens (Gothandapani, Sekar, & Padaria, 2017). Along with this, these microorganisms stimulate the activity of the soil microflora and are recorded for the increase in the plant height, overall biomass crop yield (Mahato & Kafle, 2018; Sethi & Adhikary, 2012).

12.2.1.2 Photosynthetic Nitrogen-Fixing Microorganisms

This category of nitrogen-fixers mainly consists of blue-green algae also known as cyanobacteria, which are basically diazotrophs found mostly in various water bodies and transform atmospheric nitrogen into various nitrogenous and ammonium compounds (Singh, Kumar, Rai, & Singh, 2016). Cyanobacteria such as *Nostoc linkia, Anabaena variabilis, Aulosira fertilisima, Cylindrospermum* sp., *Calothrix* sp., *Tolypothrix* sp., *Stigonema* sp., and *Scytonema* sp. were considered to be the efficient nitrogen-fixers since they have a specialized thick-walled modified cells called heterocyst where the nitrogen fixation takes place (Chittora et al., 2020). However, non-heterocystous cyanobacteria such as *Aphanothece* sp. and *Chroococcidiopsis* sp., or filamentous cyanobacteria such as *Oscillatoria* sp. and *Trichodesmium* sp., were also been reported for their nitrogen-fixating ability (Berrendero et al., 2016; M. Kumar, Reddy, Phogat, & Korav, 2018). These microorganisms have been reported to be used as biofertilizers in various crops such as barley, chilli, cotton, lettuce, maize, oats, radish, sugarcane, and tomato (Thajuddin & Subramanian, 2005). Studies on utilization of cyanobacteria as biofertilizers have previously reported that along with controlling the nitrogen

availability for the plants, they are also responsible for improving the soil porosity, aeration, and water holding capacity. They are also been reported for the production of phytohormones such as gibberellins, auxins, and so on, vitamins such as vitamin B12 and amino acids, and various adhesive substances that increase the water holding capacity of the soil (Chittora et al., 2020; Paumann, Regelsberger, Obinger, & Peschek, 2005).

The most extensively studied group of nitrogen-fixing microorganisms belongs to the family *Rhizobiaceae* (α-proteobacteria) that includes the microorgranisms belonging to the genera *Rhizobium, Azorhizobium, Allorhizobium, Bradyrhizobium, Mesorhizobium,* and *Sinorhizobium* (Patel & Sinha, 2011). Commonly known as Rhizobia, these microorganisms form a mutualistic association with the plants, especially belonging to the family Leguminosae, where they form special tumor-like structures on their roots called root nodules, which act as the zone of nitrogen fixation. Within these nodules, the microbes differentiate into bacteroids, which convert the atmospheric nitrogen into ammonium (Mącik, Gryta, & Frąc, 2020). Studies revealed that microorganisms belonging to this group is able to fix around 50–200 kg of nitrogen per hectare (Bhat et al., 2015; Gopalakrishnan et al., 2015; Mitra, 2019). The beneficial effect of these microorganisms with commercially important leguminous plants such as pea, chickpea, soybean, alfalfa, beans, and clovers has been well documented (Verma, Yadav, Tiwari, Lavakush, & Singh, 2010). Studies report that not only these microorganisms increase the nitrogen content of the plants in root and shoot matter but they also increase the plant dry matter, seed yield, protein content along with the production of compounds such as indole acetic acid and 1-aminocyclopropane-1-carboxylate deaminase, and other plant-growth-promoting factors (Aremu, Alori, Kutu, & Babalola, 2017; Bramhachari, Nagaraju, & Kariali, 2018; Wani, Khan, & Zaidi, 2008; Wdowiak-Wróbel et al., 2017).

12.2.2 Phosphorus Biofertilizer

Phosphorus is a major growth-limiting macronutrient, which is indispensable just like nitrogen (Anand, Kumari, & Mallick, 2016). It constitutes about 0.2–0.8% of the plant dry weight and is an essential element in plant growth and development. It is a major structural component of various enzymes, co-enzymes, nucleic acids, nucleotides, phospholipids, and phosphoproteins (Sharma, Sayyed, Trivedi, & Gobi, 2013). Metabolically, phosphorus plays a major part in several physiological and biochemical processes in the plants such as photosynthesis, development of roots, flowers, seeds and other plant reproductive systems, carbon metabolism, energy transfer, and so on (A. A. Khan, Jilani, Akhtar, Saqlan, & Rasheed, 2009; Amarjeet Kumar, Kumar, & Patel, 2018; Satyaprakash, Nikitha, Reddi, Sadhana, & Satya Vani, 2017). Along with that it also aids to nitrogen

fixation, plant protection, transformation of sugar to starch, and most importantly the crop maturity and quality. A substantial amount of the phosphorus absorbed gets accumulated in grains as phytase, and hence it deficiency affects the crop yield adversely (Anand et al., 2016; Charana Walpola, 2012).

Phosphorus is usually found in its insoluble form in the soil, which cannot be utilized by the plants. Hence, to increase the phosphorus availability for the plants, it is added as chemical fertilizers during the cultivation. However, around 70–90% of the phosphorus applied either gets adsorbed to the soil or gets immobilized by the cations present in the soil such as Ca^{2+}, Al^{3+}, or Fe^{3+} to form complexes such as calcium phosphate, aluminum phosphate, or ferric phosphate, which are insoluble forms of the phosphorus and hence unusable by the plants (Charana Walpola, 2012; Amarjeet Kumar et al., 2018; Satyaprakash et al., 2017). Thus, the application of the phosphate fertilizers needs to be carried out frequently. However, these immobilized phosphates, if converted to the soluble, plant usable form, will act as a huge reservoir for the phosphates, which will be adequate enough to sustain the worldwide phosphate requirement for about 100 years (Kalayu, 2019). Hence, conversion of these immobilized insoluble phosphates and its mobilization using low-priced, eco-friendly method is highly necessary. To achieve this, microorganisms having the ability to solubilize and mobilize phosphates from insoluble to soluble form can be used in the agricultural soil. These types of microorganisms are known as "phosphate biofertilizers." Based on their application, they have been categorized as phosphate-solubilizing and phosphate-mobilizing microorganisms, respectively.

12.2.2.1 Phosphate-solubilizing Bacteria (PSB)

These are a particular group of microorganisms who have the ability to convert the insoluble phosphate present in the soil into soluble form. The role of the soil microorganisms in determining the phosphate availability of the plants has been studied since 1950s (Krasilnikov, 1961; Kudashev, 1950). Bacteria belonging to this group belong to the genera *Azotobacter, Bacillus, Bradyrhizobium, Burkholderia, Ralstonia, Enterobacter, Rhodococcus, Serratia, Salmonella, Rhizobium, Erwinia, Paenibacillus, Sinomonas, Kushneria,* and *Thiobacillus* the prominent being *Agrobacterium* spp., *Bacillus circulans*, and *Pseudomonas* spp. (Alori, Glick, & Babalola, 2017). Even fungi have also been reported for their phosphorus solubilization ability. Fungi featuring this ability belongs to *Achrothcium, Aspergillus, Cephalosporium, Chaetomium, Cladosporium, Fusarium, Paecilomyces, Penicillium, Rhizopus, Rhizoctonia, Saccharomyces, Schizosaccharomyces, Trichoderma*, and *Yarrowia* (Elias, Woyessa, & Muleta, 2016; Pal, Singh, Farooqui, & Rakshit, 2015). Studies have revealed that bacteria that tend to be more effective phosphate-solubilizing microorganisms than fungi

constitute about 50% of the total soil microbial population, while the phosphate-solubilizing fungi constitute of about only 0.1% (Anand et al., 2016). Beneficial effect of the PSMs on the plant growth has been well documented on various crops such as variety of beans, millets, sugarcane, barley, chickpeas, wheat, lettuce, maize, and so on (Shrivastava, Srivastava, & D'Souza, 2018).

There are various approaches such as lowering soil pH, chelation, and mineralization, which describe mechanism of phosphate solubilization by the PSBs. The primary mechanism of phosphate solubilization by the microorganisms is by lowering the pH of the soil by production of organic acids such as acetic acid, 2-ketogluconic acid, adipic acid, citric acid, malic acid, lactic acid, succinic acid, propionic acid, fumaric acid, malonic acid, oxalic acid, glutaric acid, glyoxalic acid, tartaric acid, and butyric acid, which are the result of microbial metabolism, fermentation, or oxidative respiration (Ahmed & Shahab, 2012; Charana Walpola, 2012; Amarjeet Kumar et al., 2018; Selvi, Paul, Vijaya, & Saraswathi, 2017; Yousefi, Khavazi, Moezi, Rejali, & Nadian, 2011). With increase in the pH of the soil, the divalent and trivalent forms of phosphate such as francolite and fluorapatite develop, which are insoluble in soil (Satyaprakash et al., 2017). It was observed that a strong correlation exists between the microbial production of organic acids and solubilization index, and PSBs were known to create acidity by production of carbon dioxide (Alam, Khalil, Ayub, & Rashid, 2002). The phosphate solubilization efficiency of the microorganism depends upon the nature, strength, amount, and type of organic acid produced by the microbial strain, which differs from one microbial strain to another. For example, studies establish that di- and tricarboxylic acids were found to be better solubilizers than aromatic acids just like the aliphatic acids that are found to be more effective than the phenolics and fumaric acids (Mahdi, Talat, Dar, Hamid, & Ahmad, 2012). Of all the acids mentioned previously, gluconic acid and 2-ketogluconic acids were the most reported organic acids that are majorly found in the PSBs (Charana Walpola, 2012; Rodríguez & Fraga, 1999; Satyaprakash et al., 2017). These acids dissolve the insoluble phosphates and then chelate the cations resulting in soluble phosphates thus, acts as strong chelators. Similarly, inorganic acids such as carbonic acids, nitric, and sulfidric acids have been reported for the conversion of insoluble phosphates to soluble form by the microbial strains (M. S. Khan, Zaidi, & Wani, 2009; Zaidi, Khan, Ahemad, Oves, & Wani, 2009). Another vital process that involves the enhancement of the concentration of phosphorus in the soil is solubilization of organic phosphate compounds such as clay particles, plants, or animal remains, which mostly consists of nucleic acids, phosphonates, sugar phosphates, phytic acids, etc. (A. A. Khan et al., 2009). The phosphate-solubilizing microorganisms produces phosphatases like compounds such as phytases, phosphonates, and C-P lyases that hydrolyze the organic phosphates and

organo-phosphonates resulting in inorganic phosphorus that can be assimilated by the plants directly (Shrivastava et al., 2018). The microorganisms commonly reported for the production of these phosphatase-like substances belong to the genera *Aspergillus, Penicillium, Trichoderma, Bacillus, Streptomyces, Enterobacter, Arthrobacter, Azotobacter*, etc. (Aseri, Jain, & Tarafdar, 2009; Neira-Vielma et al., 2018; Selvi et al., 2017; Tarafdar, Bareja, & Panwar, 2003).

12.2.2.2 Phosphate-mobilizing Microorganisms

The group of microorganisms having the ability to increase the phosphorus availability for the plants by moving it within the soil rather than solubilizing the insoluble phosphates were identified as phosphate mobilizers (M. Kumar et al., 2018). *Mycorrhizae*, the root-symbionts are considered as the most significant microorganism belonging to this group. These types of symbiotic relationships were predominantly found in commercially important crops such as rice, wheat, potato, maize, and so on (Barman et al., 2017). Mycorrhizal fungi or more precisely endomycorrhizal fungi penetrate their hyphae into the cortical cells of the root and form the structures known as arbuscules (Frąc, Hannula, Bełka, & Jędryczka, 2018). The arbuscular-forming mycorrhizal fungi produce long external hyphae that increase the close interaction of the root with the soil phosphates and thus increases the phosphate uptake by the plants as compared to the non-mycorrhizal plant systems. Presence of these fungi in the root nodules have also reported to enhance the stress tolerance ability of the plants and protects them against various plant pathogens (Bi, Zhang, & Zou, 2018). Fungi belonging to the genera *Acaulospora, Scutellospora, Glomus, Entrophospora, Gigaspora,* and *Sclerocystis* were basically reported for their arbuscular forming and phosphate-mobilization ability (Sadhana, 2014). The advantageous effect of these microorganisms on the enhanced yield has been documented on various plants such as asparagus, cucumber, beans, muskmelon, onion, pepper, tomato, and watermelon (Chen et al., 2017).

12.2.3 Plant-Growth-promoting Biofertilizers

A group of microorganisms, especially the rhizobacteria that enhances the plant growth and yield by producing a variety of compounds such as growth hormones, chelating agents, siderophores, HCN (hydrogen cyanide), antibiotics, anti-fungal agents, and lignocellulosic enzymes were identified as plant-growth-promoting rhizobacteria or PGPR (Khosro & Yousef, 2012; Lugtenberg & Kamilova, 2009; Majeed, Abbasi, Hameed, Imran, & Rahim, 2015). They colonize in the root rhizospheres, modify the root architecture by increasing its branching and number, and provide important nutrients to the plants via

phosphate and potassium solubilization and nitrogen fixation (Ashok Kumar et al., 2015). The microorganisms belonging to the genera *Achromobacter, Actinoplanes, Agrobacterium, Alcaligenes, Amorphosporangium, Arthrobacter, Azotobacter, Bradyrhizobium*, *Bacillus*, *Cellulomonas*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Micromonospora, Pseudomonas*, *Rhizobium*, *Streptomyces, Streptosporangium, Thermobifida*, and *Xanthomonas* were reported of having the ability of enhancing the plant growth and yield by producing various growth-promoting compounds and substances that controls the plant pathogens, especially antifungal agents, such as siderophores, hydrolytic enzymes, and antibiotics (Chowdhury, Hartmann, Gao, & Borriss, 2015; Franco-Correa et al., 2010; Gouda et al., 2018; Vejan, Abdullah, Khadiran, Ismail, & Nasrulhaq Boyce, 2016). Siderophores are the low-molecular-weight compounds produced by the microorganisms for chelating Fe^{3+} from the soil. The siderophore-producing microorganisms enhances the plant growth and yield by increasing the supply of Fe^{3+} ion chelated from the soil and creating a Fe^{3+}-deficit micro-environment within the soil, thus creating inhibiting microhabitat for other competitive and antagonistic microorganisms (Bamboriya, Bamboriya, & Shanti, 2018). It has been observed that PGPRs such as *Burkholderia cepacia* exhibit dual functionality of both biofertilizers and biopesticides depending upon the growth conditions and requirement of the plants. They were reported to produce mycotoxins that act against the plant pathogen *Fusarium* sp.; it also produces the growth-boosting siderophores that encourage the plant growth in the deficiency of micronutrient iron (Bhattacharyya & Jha, 2012).

The working mechanism of PGPRs may be broadly divided in to two distinct categories such as direct and indirect mechanisms (Glick, 2014). While the supplement of compounds such as phytohormones, siderophores, nitrogen fixation, solubilization of macro- and micronutrients by these microorganisms comes under the direct mechanisms, production of antibiotics, lytic enzymes, and other compounds to boost the plant immunity and biocontrol of the phytopathogens is considered the indirect mode of action of the PGPRs (Beneduzi, Ambrosini, & Passaglia, 2012; Bhattacharyya & Jha, 2012; Patel, Thakkar, & Subramanian, 2015). Similarly, based on their location, PGPRs can be divided into two groups, i.e. extracellular and intracellular PGPRs. Extracellular PGPRs or ePGPRs exist either in the rhizosphere or within the spaces between the cells of root cortex while the intracellular PGPRs or iPGPRs were found within the nodules, the specialized structures on the roots (Ahemad & Kibret, 2014). Like all other plant–microbe interactions, the ability to colonize is most important. The plant roots produce signals recognized by the soil microbes, thus initiating the crosstalk. In response, the microbes initiate the process of colony formation in the rhizosphere guided by their response to the chemotactic signal. Post-attraction, the bacterial

cell gets attached to the root surface and start proliferation to form a dense aggregate of cell colonies known as biofilm or macro-colonies and then interacts with the plants through the root surface (Berg, 2009; Nihorimbere, Ongena, Smargiassi, & Thonart, 2011).

12.3 Effect on Bioremediation of Environmental Pollutants

Rapid industrialization, extensive usage of agrochemicals, and perpetual anthropogenic activities have led to release of several pollutants such as organic contaminants, toxic wastes, and heavy metals (Shinwari et al., 2015). These pollutants have an adverse effect on the environment and human health and hence been the primary concern of the researchers for decades. One of the major categories of the inorganic pollutants are heavy metals, which are water-soluble in nature and accumulates easily within the biosphere for long period without degeneration (Akhtar, Chali, & Azam, 2013). The most widely reported heavy metals are arsenic (As), cadmium (Cd), chromium (Cr), copper (Cu), lead (Pb), mercury (Hg), nickel (Ni), and zinc (Zn) of various valence states (Mahanty et al., 2017). When these metal ions are found in excess in the environments, the root cells absorb them rapidly and translocate them to the other parts of the plant. Even though plants utilize various metals as micronutrients, disproportional accumulation has detrimental effect such as reduced growth, disturbed metabolism, or death of the plant (Mehes-Smith, Nkongolo, & Cholew, 2013). Many studies have reported the negative impact of these pollutants on soil fertility and metabolic activity of the soil microbial community (Lenart & Wolny-Koładka, 2013). Similarly, another very important cause of soil pollution is the extreme usage of agrochemicals, which are a significant element of the modern agriculture. Pesticides are broadly categorized into different groups such as herbicides, fungicides, insecticides, rodenticides, nematicides, and so on based on their targets (Mohanty & Jena, 2019). These compounds are extremely persistent by nature and do exist for a prolonged period in the environment, thus possessing a potential threat to the ecosystem (Puri & Kumar, 2012). Remediation of these pollutants is an extremely challenging business since they are highly persistent in nature and have higher half-lives. Heavy metals are nonbiodegradable, and it has been observed that the reduced forms tend to be less harmful compared to their oxidized states and hence heavy metals can be detoxified only by changing their oxidation states (Wuana & Okieimen, 2014). Several physiochemical methods have been applied for the removal of these pollutants from the ecosystem, but each of them tends to have certain limitations such as secondary intermediate pollution or involves higher cost with less efficiency. Nonetheless, bioremediation technology has proved to be the best remediation strategy due to its higher proven efficiency, cost-effectiveness,

eco-friendliness, and stability compared to the physiochemical technologies (Lim, Shukor, & Wasoh, 2014; Nawaz & Ahsan, 2014).

Role of microorganisms for the bioremediation of the pollutants has been explored to the fullest. A wide range of microbial strains that are found to produce plant-growth-promoting substances have also been examined to have the ability to remediate the metal toxicity, as well as other organic pollutants from the environment (Dixit et al., 2015; Saravanan et al., 2020). PGPR strains belonging to the genus *Achromobacter, Bacillus, Brevibacillus, Bradyrhizobium, Mesorhizobium, Rhizobium, Sinorhizobium, Kluyvera, Pseudomonas, Ralstonia, Variovox, Ochrobactrum, Psycrobacter, Xanthomonas* have been reported to play a vital role in the remediation of heavy metals from the environment (Shinwari et al., 2015). These microorganisms are known to produce the enzyme 1-aminocyclopropane-1-carboxylate deaminase also known as ACC deaminase that inhibits the plant-growth-suppressing, stress-inducing hormone ethylene that has been produced due to the presence of heavy metal accumulation within the plant (Hossain, Piyatida, da Silva, & Fujita, 2012; Singh, Shelke, Kumar, & Jha, 2015). Another important mechanism to reduce the adverse effect of the heavy metal accumulation is the production of siderophores by the microorganisms that forms stable metal complexes, thus minimizing the stress of metal toxicity (Radzki et al., 2013; Saha et al., 2016).

Similarly, soil bacterial strains such as *Actinomycetes*, *Azospirillum, Serratia, Klebsiella, Bacillus, Pseudomonas, Gordonia, Azotobacter, Enterobacter, Paenibacillus*, and so on known for their plant-growth-promoting ability has been reported for the effective bioremediation of a variety of pesticides from both soil and contaminated waterbodies (Mahanty et al., 2017). These microorganisms were known to produce various enzymes in response to the chemical contaminant present in its microenvironment (Herwijnen et al., 2003; Zhang, Yuan, Xiong, Wang, & Jiang, 2020). These enzymes catalyze the processes such as oxidation, reduction, or hydrolysis, which degrade the pollutants into a more water-soluble, nontoxic end products (Verma, Jaiswal, & Sagar, 2014). These enzymes are encoded by the genes residing in the anti-catabolic plasmid present in these microorganisms (Laemmli, Leveau, Zehnder, & Van Der Meer, 2000). For example, it was found that the *ese* gene that encodes enzymes such as Ese monooxygenase and Esd monooxygenase were found in several endosulfan-degrading microorganisms (Kataoka & Takagi, 2013). Similarly, the *lin* gene detected in several hexachlorocyclohexane-degrading PGPRs was reported to encode enzymes such as hydrolase, dehydrogenase, and dehalogenase, which play a vital role in the degradation of these compounds (Cérémonie, Boubakri, Mavingui, Simonet, & Vogel, 2006; Pan, Siegrist, & Crimi, 2012). Thus, based on the available literature, it can be concluded that utilization of PGPR is a promising alternative for the bioremediation of various contaminants from the environment in a sustainable approach.

12.4 Bioformulations and Its Types

Biofertilizers are basically viable microorganisms intended for the enhancement of the soil fertility and productivity. One of the most important steps during the preparation of the biofertilizer is its formulation, a multistage process that unifies single or a combination of the selected microbial strain with an appropriate carrier compound along with some cell-protecting additives that increase the shelf life of the biofertilizer (Bargaz, Lyamlouli, Chtouki, Zeroual, & Dhiba, 2018; Herrmann & Lesueur, 2013). It determines the quality, stability, and activity of the biofertilizer to a greater rate (Arora et al., 2010; Bashan, De-Bashan, Prabhu, & Hernandez, 2014). To achieve the desired results, the developed formulations must fulfil the following properties (Catroux, Hartmann, & Revellin, 2001; Herrmann & Lesueur, 2013; Mahanty et al., 2017; Malusá et al., 2012):

1) It should be eco-friendly in nature and should neither change the soil characteristics nor have any antagonistic effect on the soil natural microflora on addition.
2) It should have a long shelf life and better stability even under the extreme conditions of production, storage, transportation and application.
3) The carrier material used should be cost-effective and adequately available.
4) The formulation should be able to adjust its pH according to the surrounding and must be capable for the addition of the nutrients and controlled release of microbes to the rhizosphere.

Based on the type and characteristics of the carrier material used for the preparation of the biofertilizers, bioformulations are broadly categorized into two types such as solid carrier based and liquid bioformulations (Figure 12.2) (Brahmaprakash et al., 2017). In case of a solid bioformulation, the intended microbial strain is attached with a carrier molecule that transfers the microorganism to the rhizosphere (Patil, Jadhav, Ghorpade, & Sharma, 2013). Carriers are the mode of conveyance of the live microorganisms to the subjected soil as biofertilizers. They play a very important role in defining the activity and the efficiency of the biofertilizers as they determine the delivery of the right amount of viable microbial cells to the site of interest (Bashan et al., 2014). The carrier not only acts as a microhabitat for the subjected microbial strain but also protects them from the antagonistic behavior of the indigenous soil microflora and provides nutritional support to promote their growth (Arora et al., 2010; Balasubramanian & Karthickumar, 2017). Choice of carrier for the formulation of biofertilizers depends on a number of factors such as cost-effectiveness, the requirement of the subjected microorganisms and targeted crop, method of application, and so on (Abd El-Fattah, Eweda, Zayed, & Hassanein, 2013; Stamenković, Beškoski, Karabegović, Lazić, & Nikolić, 2018). Properties of a good

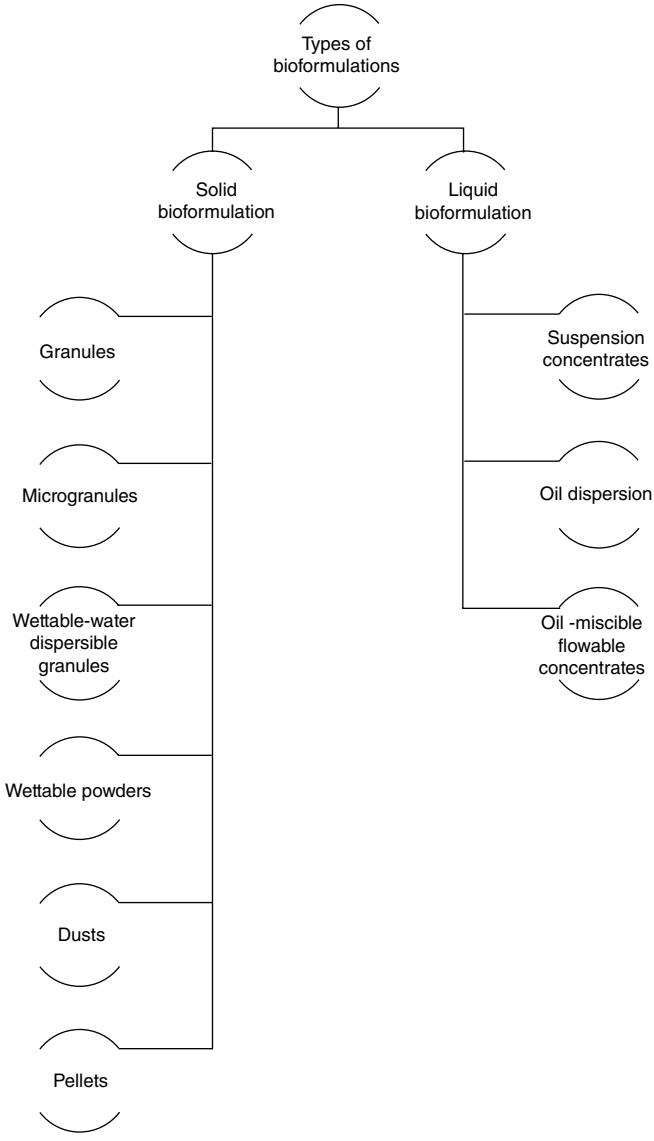

Figure 12.2 Types of bioformulations.

carrier material are that it should be nontoxic and inert in nature, have an excellent moisture binding ability, cost-effective, adequate availability, efficient buffering capacity, etc. (Malusá et al., 2012; Siddiq et al., 2018; Tabassam, Sultan, Akhtar, Hassan, & Ali, 2015; Zayed, 2016). It must also have high organic matter content

and must be capable of easy exchange of gases (Abd El-Fattah et al., 2013). Peat is one of the most widely used material as carrier, while others include coal, charcoal, polymers, vegetable oils, plant waste materials mud, etc. (Sahu & Brahmaprakash, 2016). Solid bioformulations can be obtained in six different forms such as granules, microgranules, wettable water-dispersible granules, pellets, dusts, and wettable powders. Granules and microgranules have basically the same composition: selected microbial strain, a carrier, and cell-protectant additives. However, they differ from each other only by the size of the particles of the final product. While the granules have a size between 100 and 1000 μ, the microgranules is of a size of 60–100 μ. These types of bioformulations are predominantly used for the biotreatment of soil since they have an advantage of ease of application, storage, and are safer to be used (Mishra & Arora, 2016). Another form of carrier-based bioformulations is wettable powders that are one of the oldest forms of preparation, which includes around 50–80% of technical powders, with fillers, dispersants, and surfactants (Brar, Verma, Tyagi, & Valéro, 2006). The wettable/water-dispersible granules are freely flowing, non-dusting granules, which also have the similar composition as that of wettable powders. Even though both the forms are promptly miscible with water, the wettable powders are more commercially appealing alternatives as they have high concentration of dispersing and wetting agents and are considered to be safe (Hazra, Karmakar, Poi, Bhattacharya, & Mondal, 2017). The carrier-based solid bioformulations have their own set of advantages and disadvantages. The advantages include cost-effectiveness, ease of production, and low maintenance, while the disadvantages include high risk of contamination, low count of viable cells, reduced shelf life, and higher sensitivity toward the temperature variations (Mishra & Arora, 2016). Hence, liquid-based bioformulations have been worked out as an alternative to the solid biofertilizers.

Liquid bioformulations are the aqueous-based biofertilizers where the microbial broth culture (around 10–40%) is resuspended in carrier liquids such as mineral oils or organic oils or oil–water suspensions (around 35–65%) along with surfactants, dispersants, and suspender mediums (Bharti et al., 2017; Yadav & Chandra, 2014). Compared to the solid-based biofertilizers, these are more preferable due to its higher shelf life, ease of processing and application, and higher viable cell counts (Surendra Gopal & Baby, 2016). These were found to be more efficient in terms of activity and cost-effectiveness compared to their solid counterparts (Schulz & Thelen, 2008). However, in practicality, the absence of microhabitat provided by the solid carriers to the subjected microorganisms in the solid-based bioformulations leads to lose of viability on application, requirement of specific physiological conditions such as low temperature containers for longer storage periods and increased sensitivity toward the indigenous soil microflora and varying environmental conditions (John, Tyagi, Brar, Surampalli,

& Prévost, 2011). However, these limitations can be overcome by the addition of various excipients such as glycerol or sucrose, which not only protects the microorganisms from the toxic environments but also increases their survivability postinoculation (Kaur, Gangwar, & Pandove, 2018; Tittabutr, Payakapong, Teaumroong, Singleton, & Boonkerd, 2007). There are various types of liquid formulations such as suspension concentrates, oil dispersion, oil-miscible flowable concentrates, and so on (Mishra & Arora, 2016). As the name suggests, suspension concentrates are the type of bioformulation where the liquid microbial broth also known as active ingredients along with the necessary additives are dispersed in a small quantity of water and needs to be diluted to the prescribed concentration before application. They are more effective than the wettable powders (Tadros, 2013). The oil-miscible flowable concentrates are very much similar to the suspension concentrates in terms of its composition. However, they need to be diluted using an organic liquid before their application to the soil (Faria & Wraight, 2007; Singh & Merchant, 2013). Similarly, the oil dispersion is the bioformulation where the active ingredients were suspended in mineral oils or any organic oils, which act as a binder for the microbial strain to the target resulting in a higher efficiency (Mishra & Arora, 2016).

12.5 Preparation of Biofertilizers

Preparation of biofertilizers varies depending upon the microbial strain selected for the study. However, the overall process can be generalized into the following steps (Chittora et al., 2020): For the large-scale production of biofertilizer, first the inoculum is prepared by inoculating the pure culture of the selected strain in liquid medium and incubating them in an orbital shaker incubator under the optimum growth conditions. Depending on the growth rate of the microorganism, the incubation period should be set in such a manner that the harvested microorganisms must be in their exponential growth phase resulting in a concentration of 10^5–10^6 viable cells/ml. This is termed as the mother culture or seed culture. The mother culture is gradually scaled up in higher volumes, in larger containers under optimum conditions until the viable cell count reaches to 10^9–10^{10} cells/ml with a thicker consistency. Now, this broth culture will be used for the large-scale production of the subjected microbial strains using large-scale bioreactors or fermenters operated at the optimum growth and nutritional conditions. Post-downstream processing and harvesting the microbial biomass, the inoculant is mixed with the prescribed carriers and additives to increase its shelf life. The pH of the formulation is maintained around 6.0–7.5 and the storage temperature of 25–30 °C. The viable cell count for the prepared biofertilizers must be checked and maintained as per the international standards. In case of liquid

biofertilizers, only two third of the volume of the container needs to be filled, and the rest one third volume must be left void for aeration.

12.6 Various Modes of Biofertilizer Application

There are various ways in which the biofertilizers can be applied with each mode having its own set of advantages and disadvantages (Chen, 2006). They can either be applied directly to the soil or may be applied on the seeds and seedlings before planting them in the soil. Various factors such as crop types, inoculant properties, types of formulations, soil physiological conditions need to be considered prior to the adoption of the mode of application of the biofertilizer (Mahmood, Turgay, Farooq, & Hayat, 2016). Owing to its ease of use, simplicity, and minimal requirement, seed treatment is one of the most preferred practice for the biofertilizer applications (Asif et al., 2018). In case of seed inoculation, the bioformulations may either be sprinkled on the seed or be mixed with the seeds using a seed hopper or mixed with a small amount of water to prepare a slurry, and the seed is mixed with them and left overnight before plantation (Malusá & Ciesielska, 2012). In some cases, the formulation is mixed with certain nontoxic adhesives such as sucrose solutions, carboxymethyl cellulose, vegetable oils, or gum arabic and then coated on the seeds before inoculation (Bashan et al., 2014; Mącik et al., 2020; Malusá & Ciesielska, 2012). During the process of dusting, the inoculants were mixed directly with the dry seeds, thus resulting in a weak adherence between the two and being considered to be the most ineffective method of all. In order to protect the microorganisms from harsh environmental conditions or chemicals present in the soil prior to the application of the biofertilizers, the seeds incubated with the bioformulation slurry is coated with inert materials such as charcoal, clay, calcium carbonate, lime, talc, and so on (Malusá & Ciesielska, 2012). In order to introduce a large population of the microorganisms to the soil directly, the soil inoculation technique is being encouraged. In case of solid biofertilizers, the seeds were placed within the bed of bioformulation, while in case of liquid biofertilizers, the seeds were placed within the furrows prepared for the purpose and the bioformulations is sprayed over them. Advantages pertaining to this technique involve minimization of loss of inoculants, increased contact time between the seeds and the biofertilizers, protection of the subjected microorganisms from the negative impact of the harmful agrochemicals. However, the technique is less economic in nature since it involves larger storage area, more specialized techniques, and advanced machineries thus predominantly used in developed countries (Bashan et al., 2014; Deaker, Roughley, & Kennedy, 2004).

12.7 Challenges to Commercialization of Biofertilizers

Despite of its advantages, large-scale production and utilization of biofertilizers are still not considered in most of the countries for their agricultural practices. This may be due to several constraints associated with the commercialization of the biofertilizers, which can be perceived at various levels starting from production unit till its application by the farmers. One of the major constraints in the commercial production of biofertilizer is the lack of availability of a suitable, specific microbial strain, which can survive both in solid and liquid bioformulations. In general, the microbial metabolic activity varies according to its surrounding environment. The selected strain not only needs to have a good adaptability but should also be able to display the required activity even at the adverse physical conditions and must compete with the indigenous microorganisms for nutrition and nodulation of hosts (Panda, 2013). Requirement of biofertilizer differs from region to region and from one crop to another. Hence, even for a single crop cultivated in two different regions, the microorganism and the carriers to be used in the biofertilizer may differ according to the need of the soil, in which the crops are being cultivated. Hence, commercial production of a biofertilizer, which could be utilized efficiently for a wide spectrum of crops or for a diverse region, is a Brobdingnagian task. As discussed previously, a suitable carrier material is highly essential for an effective bioformulation. However, studies suggest that production units in search of alternate, cost-effective carrier molecules resort to unsterilized carriers, thus resulting in contamination and a low count of viable microbial strains per gram of the biofertilizers (Barman et al., 2017; Borkar, 2015; Mazid & Khan, 2014). Moreover, the lack of knowledge about the storage conditions of these biofertilizers that are actually live microbial cells ultimately leads to their death, and thus the farmer is unable to explore the actual potential of the biofertilizer (Barman et al., 2017). Another important factor that may cause the low level of acceptance and willingness among the farmers to opt for this alternate technology is the slow response compared to its chemical counterparts (Panda, 2013).

12.8 Future Perspective

Effective substitution of biofertilizer in place of chemical fertilizer as an integral component of modern agricultural practice is the emerging area of interest among the researchers. Even a few countries have implemented various strains of beneficial bacterial strains for enhancing the soil fertility and productivity (Weekley, Gabbard, & Nowak, 2012). Hence to make this practice more widespread,

the following issues needed to be addressed (Gamalero, Berta, Massa, Glick, & Lingua, 2010; Mahanty et al., 2017):

1) The first and foremost challenge in the widespread application of biofertilizer is to find an effective microbial strain, which can be used for a large variety of crops and be able to cater several necessities of the plant simultaneously.
2) Commercialization of biofertilizers will require novel approach in each stage of formulation, production, storage, transportation, and application.
3) Studies pertaining to the persistence of the microbial strains used as biofertilizers under various environmental stress conditions need a special attention as the physiological conditions of the soil in the agricultural field differ from that in the laboratory. Hence, suitable microbial strains that can resist such harsh conditions should be subjected for the novel formulation.
4) Application of genetically modified microorganisms as biofertilizers can be another more likely area of study on which the researchers can focus on. However, they need to abide to the rules involving to the use of these microorganisms in the first place and be able to convince both regulatory agencies, as well as the public that these modified microbial strains possesses no harm to the environment or human health.
5) Lastly, there is a dire need to educate the people, especially the farmers regarding the advantages of biofertilizers and the long-term benefits associated with it. They need to be enlightened regarding the adverse effect of the chemical fertilizers. It is usually the misconception around the use of microorganisms that hold back the public from using the biofertilizers in first place. It is highly necessary to clear this misapprehension that microorganisms only lead to disease and must be eradicated by any means possible.

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13

Biofertilizers from Food and Agricultural By-Products and Wastes

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13.1 Introduction

Fertilizers boost soil fertility and improve crop yield, wherein these can be broadly categorized as organically and inorganically generated fertilizers (Souza et al., 2016). However, the nutrients that come from beneficial microorganisms present in the soil can be reduced with the excessive use of fertilizers. Hence, to preserve soil fertility, their proper use is necessary, where organic fertilizers can be another efficient way to provide soil nutrients. Organic material in the soil can increase soil fertility and minimize the release of nitrogen, thereby regulating the degradation of soil and the supply of other nutrients (Paul & Nair, 2008; Paul et al., 2009).

Recently, several countries have been employing various traditional and advanced technologies in an attempt to recycle 15–50% of their produced waste (Diaz et al., 1993). The conventionally prepared soil conditioners have been used for fertilizing soil and plant, since ancient times, which has been achieved with the help of the carbon and nutrient-enriched soil compositions using weeds, stalks, stems and fallen leaves, pruning, and dead branches; vermicompost, and farm waste such as cornstalks and sugarcane bagasse, drops, and grapes; and vegetables (Diaz et al., 2007). Initiatives and research in this direction have contributed in the reduction of environmental pollution caused by these wastes and have also contributed in soil reconstitution, which can be further accelerated through biofertilizers that are compositions created by the amalgamation of advanced techniques and microorganisms.

This chapter focused on biofertilizers, different waste sources being employed in the preparation of biofertilizers, corresponding microorganisms contributing toward preparation of effective biofertilizers and different technologies currently

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being employed for the production of these biofertilizers. This chapter has also briefly discussed their targeted effects on the soil composition and plant activity.

13.2 Biofertilizer

Biofertilizers are classified as preparations that contain alive or dormant cells of microorganisms that contribute to plant uptake through corresponding soil or seed-specific interactions of plant nutrients (Laditi et al., 2012). Biofertilizers are environment-friendly engraving goods that reduce the deteriorative effects of any corresponding natural sources and help in purification of soil over certain aspects of residual artificial fertilizers (FAO, 2013). Natural or organic items including sawdust, rice bran and husk, and waste-tattered paper are also often used as components of these biofertilizers, which also often have corresponding nutrients and microorganisms and subsequently serve as their carriers.

The role of plant nutrients for the maximum production of crops is well-established, and it has been observed that 16 plant nutrients are needed in the necessary quantities for the crop to achieve the targeted yield, which must be made available. In numerous studies, nitrogen (N), phosphorus (P), and potassium (K) have been reported as crucial elements for the enhancement of natural plant resistance toward cold, drought, diseases, and pests (Debosz et al., 2002). Furthermore, macronutrients and micronutrients have been defined as the essential plant nutrients, including iron (Fe), zinc (Zn), copper (Cu), manganese (Mn), or chlorine (Cl), along with the basic plant nutrients as the different forms of N, P, calcium (Ca), Mn, sulfur (S), and other essential plant nutrients. The ability to provide insufficient/sufficient amounts (soil requirements for the total supply of nutrients to plant) of plant nutrients should be determined (FAO, 2013). This is also necessary for the efficient formulation of biofertilizer and for the provision of sufficient nutrients to increase soil and plant fertility.

Application of biofertilizers is an essential component of integrated nutrient management, as they complement chemical fertilizers for sustainable farming by making them cost-effective through utilizing renewable sources of plant nutrients. For biofertilizers production, many microorganisms are used, which efficiently contribute through interaction with respective crops (Menardo et al., 2010). Their existence and role can be grouped in different ways, which have been summarized in Figure 13.1.

Europe produces more than 120 million Mg of organic waste annually. Organic waste is treated, and a small amount is burned without the supply of vital nutrients being retrieved. Global production of urban solid waste is projected to increase by about 1.3 billion Mg/yr over the next few years. The main components of urban waste are agricultural and corresponding derived wastes, including food

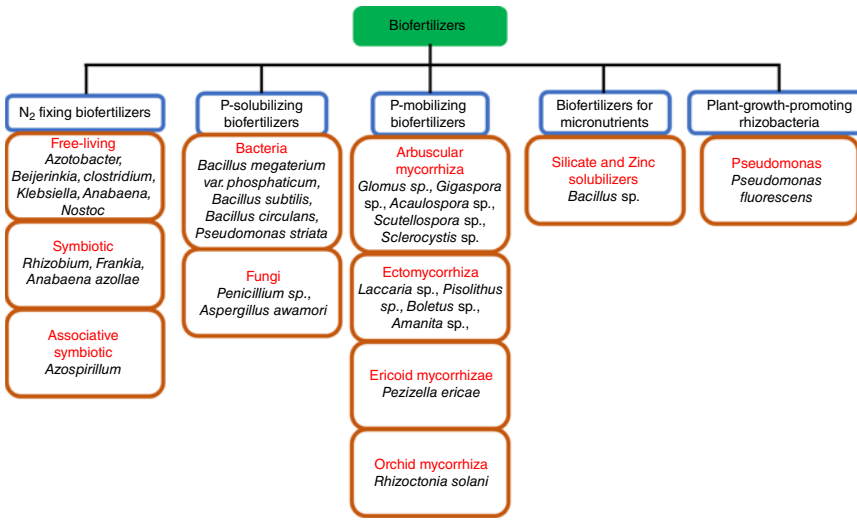

Figure 13.1 Types of biofertilizers. *Source:* Based on Alvi (2019). © John Wiley & Sons.

waste, green waste, or garden waste, which can be treated according to the targeted quality and moisture levels, according to the different scenarios and local situations. Hence, the management of organic waste identified the element compositions of each waste will be used to produce biofertilizer as summarized in Table 13.1. These residues contain several important biological substances, including phenol, vitamins, and carotenoids. A large number of bio-wastes, including three mainly available resources, i.e. agricultural waste, food waste, and sludge, can be used as input for raw materials.

In the following subsections, different biofertilizers with various specific functions/objectives have been briefly discussed with emphasis on different categories of microorganisms efficient for the explicit and/or symbiotic purposes.

13.2.1 N_2-fixing Biofertilizer

Biosynthesis of biological constituent blocks such as amino acids, and subsequently proteins or other constituents such as chlorophyll is essential to plants. The N_2 atmosphere is relatively inert, and N_2 can be converted to ammonia that can be further employed in the soil for plant uses and for the utilization by unique land microorganisms as part of biofertilizers (Abdel Ghany et al., 2013). Two major groups of microorganisms are employed for the development of N_2-fixing biofertilizers, including (i) free-living microorganisms, and (ii) symbiotic microorganisms (Carnahan et al., 1960; Rodrigues et al., 2008; Franche et al., 2009).

13.2.1.1 Free-living N_2-fixing Biofertilizer

Many free-living nitrogen-fixing microbes, such as living in the soil, fix large amounts of nitrogen without any contact with other species. Examples of this type of nitrogen-fixing bacteria include *Azotobacteria* and *Cyanobacteria*. Free-living bacteria function as anaerobic or microaerophilic when fixing nitrogen. Owing to the lack of sufficient carbon and energy sources for these species, their contribution to global nitrogen-fixation rates is usually assumed to be minor.

13.2.1.1.1 Azotobacteria *Azotobacter* is a non-vivid genus of bacteria, which fixes N_2, and is a member of the *Azotobacteria* family. The strains of *Azotobacter* are reportedly used for the production of organic acid, alcohol, and salt, and have been efficiently cultivated in the N-free medium. They are environmentally sensitive, wherein they demonstrate significant sensitivity to high salinity, high pH acidity, and temperatures above 35 °C. These bacteria are mostly found in acidic and/or alkaline soil and plant rhizoids of various commercial crops (food and others), including sugar cane, rice, maize, wheat, cotton, and mustard. *Azotobacteria* produce cyst under various conditions and the corresponding characteristics make them extremely valuable biofertilizers for commercial

Table 13.1 Elemental compositions of possible waste biomass fertilizers.

Waste biomass	Fertilizer type	N(mg/kg)	K(mg/kg)	P(mg/kg)	Cu(mg/kg)	Mn(mg/kg)	Zn(mg/kg)
Chicken manure incineration ash	Ash	NA	120 000	84 500	270	NA	1 600
Sewage sludge ash	Ash	NA	19 100	88 800	2510	NA	6 460
Banana peduncle + sewage sludge	Biochar	NA	NA	5 600	21.5	294.7	186.2
Escherichia coli	Biochar	NA	37 39	84 700	131.6	NA	62
Sugarcane waste straw	Biochar	4 000	16 000	7 930	NA	NA	NA
Anaerobically digested biowaste	Compost	26 700	NA	6 970	97	NA	179
Sewage sludge	Compost	27 000	NA	8 850	162	NA	309
Chicken manure with a mixture of plant biomass	Compost	19 650	28 500	12 090	NA	489	471
Fish waste	Compost	11 400	3 070	2 610	19.3	NA	159.9
Manure from beef cattle	Compost	23 200	NA	10600	NA	NA	NA
Slaughterhouse waste	Digestate	7 900	1 600	900	69.7	201	474
Distiller's waste from ethanol production	Digestate	5 900	2 800	700	69.4	266	465
Pharmaceutical organic waste	Digestate	94 200	6 700	5 600	NA	NA	NA
Pig slurry	Slurry	5 300	2 500	1 400	218	426	801
Dairy manure	Algal turf scrubber biomass	45 100	9 100	7 300	84	250	270

N – nitrogen, K – potassium, P – phosphorus, Cu – copper, Mn – manganese, Zn – zinc, NA – not available. *Source:* This table has been adapted from the open access article "Chojnacka et al. (2020)." Licensed under CC BY-NC-ND 4.0. Hence, readers can refer to the original work for further details.

purposes, which include (i) survival under harsh conditions; (ii) the cells being surrounded by highly slime secretions, which add to the water; (iii) high metabolic rates and a full range of N_2-fixation enzymes that make it possible for N_2 to be fixed in the presence of oxygen (O_2); (iv) ability to use source of ~20–40 mg N/g of carbon (C), equivalent to fixing 20–40 kg N_2/ha (Patil et al., 2013); and (v) the capability of production of plant growth-promoting phytohormones like gibberellic acid (GA) and indole acetic acid (IAA) (Kukreja et al., 2004).

13.2.1.1.2 Cyanobacteria *Cyanobacteria* are a gram-negative phylum of the bacteria, which are non-motile, obtain their energy through photosynthesis, which fixes N_2 and are also known as blue-green algae. They are free-living or have a symbiotic relationship with plants or fungi. Highly efficient N_2 fixing cyanobacteria are used to prepare biofertilizers with the added benefit of propagating in different environments, minimization of greenhouse gas emissions, while increasing the quality of soil during short-generation periods (Singh et al., 2016).

13.2.1.2 Symbiotic N_2-Fixing Biofertilizer

13.2.1.2.1 Rhizobium Biofertilizers

The symbiotic genus of N_2-fixing rhizobium has been widely studied. In a specific group/species of plant, rhizobial species are effective, and meanwhile for the other plant groups/species, they may be less productive or ineffective. *R. leguminosarum bv. phaseoli, Phaseolus vulgaris, R. leguminosarum bv. viciae, R. leguminosarum bv. trifolii, R. etli, R. tropici, Teramnus labialis*, and *R. indigoferae* are few such examples of N_2-fixing rhizobium species, along with their distinct hosts (van Rhijn and Vanderleyden, 1995; Wei, 2002). *Rhizobium*-containing biofertilizers have been efficiently employed to fix 40–250 kg N/ha each year, which has subsequently improved agricultural returns by around 20% (Pindi and Satyanarayana, 2012).

13.2.1.2.2 Azolla Biofertilizers *Azolla* is a wild, water-based fern, which fixes N_2 along with *Cyanobacteria*. The interest in the application of *Azolla* as an effective N_2-fixing system has increased across all parts of the world, particularly in areas with extensive paddy cultivation (Kobiler et al., 1981). The free-living cyanobacteria in the paddy ecosystems have been estimated to be around 15–30 kg N/ha/year, compared to 312–600 kg N/ha per year for *Azolla-Anabaena* (Prasanna et al., 2012). For the residues of paddy crops to be used as a component of biofertilizer, *Azolla* can act as an effective additive for the rapid pulverization of soil and delivery of N to paddy efficiently. As a biofertilizer, *Azolla* is used for rice cultivation in many countries, including the Philippines, China, Thailand, and Vietnam. An increase in rice yield by 0.5–2.0 t/ha has been reported attributed to the use of the *Azolla* biofertilizer during a study by Singh et al. (2014).

13.2.1.2.3 Azospirillum Biofertilizers *Azospirillum* is a gram-negative, mobile, and non-spore-formative category of bacterium belonging to the genus *Rhodospiriliae*. These bacteria have a symbiotic relationship to cereal hosts, in which bacterial cells remain linked with host plants without forming any apparent structure, such as a nodule. The strains of *Azospirillum*, in particular C3 and C4 (maize, sugar cane, oils, cotton, sorghum, pearl millet, etc.), are generally associated with many species and have been identified as organic sources of nutrients for these crops. Biofertilizers of *Azospirillum* have been applied in fields for the treatment of crops, root dipping, or soil use (Wani et al., 2016). The application of these *Azospirillum* has been associated with various positive results, such as (i) assisting plant root in the consumption of minerals and water and thus improving crop yield; (ii) extreme damage reduction; (iii) reduction of parasitic weed germination; (iv) application as a cereal inoculator for phytostimulants (this has been observed extensively); (v) hormonal balance modulation of the plant; and (vi) significant increase in the production of paddy and subsequently rice, equivalent to 15–20 kg N/ha (Rodrigues et al., 2008).

13.2.2 Phosphate-solubilizing Biofertilizers

Phosphorus is the second most essential plant growth nutrient after N for various important physiological processes, including photosynthesis, C metabolism, membrane formation, seed growth, root elongation, and spread (Kumar et al., 2016). Phosphorus is provided to the plant as soil phosphate (P). Furthermore, phosphate-solubilizing soil microorganisms (PSMs) can handle the insoluble form of P in the field and make it available to plants (Roychowdhury et al., 2015). PSM include bacteria and fungi, which play an important role in the supply of P for plants. They excrete organic acids that solubilize P along with the reduction of soil pH, wherein soil enzymes including phosphatases and phytases are formed to make it easy for the plants to absorb P (Vessey, 2003; Ponmurugan & Gopi, 2006).

13.2.3 Phosphate-mobilizing Biofertilizer

P is commonly present in soils; however, low mobility of P is a major factor affecting plant growth, wherein phosphate-mobilizing microorganisms can play a significant role in overcoming this issue. In the cases of many soil fungi, their hyphal structures have been found to mobilize the immobile form of P; hence, these can be efficiently used as phosphate-mobilizing biofertilizers. Mycorrhiza has been extensively used for this purpose (Javaid, 2009), where two major mycorrhizae forms exist (i) ectomycorrhiza, which extracellularly colonizes host plant roots, and (ii) endomycorrhiza, which penetrates the root cells and colonizes intracellular root plants (Moore et al., 2011). They are both used as biofertilizers for

P-mobilization. Fungi functions as extended roots after colonization and thereby increases plant coverage and absorption of soil water and nutrients.

13.2.4 Plant-Growth- promoting Biofertilizers

These consist of a special group of rhizosphere microorganisms. The rhizosphere is the tiny area around the root of the plant, which is affected directly by plant root secretions and soil microorganism growth (Brimhall et al., 1992; Richter & Markewitz, 1995). Some of the organisms colonizing the rhizosphere include bacteria, fungi, algae, and protozoa. Among these, bacteria are most commonly found (95%) in the rhizosphere, which play an important role in increasing plant growth by using different mechanisms, including (i) increased abiotic stress tolerances, (ii) regulation of phytohormone secretions and plant growth, and (iii) siderophores and volatile organic compounds development.

Hence, it can be observed that microorganisms including different bacteria and fungi are important components of efficient biofertilizers, and recent developments along with further investigation in the field of enhancement of these microorganisms through genetic manipulation can further improve the effectiveness of the corresponding biofertilizers.

13.3 Agricultural Waste

Agricultural waste includes organic substances derived from agriculture. The community consists of forest waste, cereal biofuels, plants, and animal manure. Lignocellulose in agricultural and forestry biomass waste is the source of cellulose, hemicellulose, and lignin. Fermentation and thermochemical transformations have been the most common method of exploitation of this resource, to date (Cao et al., 2017). The energy plants in Europe (Sweden and Finland) produce large quantities of wood ash as by-products. The type and form of incinerator and process parameters for ash incineration are different depending on the feedstock.

Wood ash are alkaline sources and possess valuable fertilization properties, which can be used as neutralizing agent for acidic soil. Apart from nitrogen, woody ash contains other nutrients (mainly significant concentrations of phosphorus) that are extremely needed for plant growth. Application of wood ash to fertilize peatland forests has been observed as a promising and efficient method for the management of waste obtained from the combustion of wood (Ellaiah et al., 2002; Glick et al., 2007). Plant ash co-granulation can provide a fertilizer with all the nutrients needed by the plant, including nitrogen.

Fossil fuels and biomass co-incineration can avoid heavy metal application through fertilization with fossil-fuel-based ashes only, attributed to the presence

of numerous undesired substances in excessive content. Agricultural waste sources can also be used for the derivation of biodegradable polymers, such as polylactic acid (PLA). Different PLA biocomposites were derived from root celery fibers in a study by Spiridon et al. (2018), and there are numerous other similar studies available, during which biocomposites have been developed useful for different food packaging and agricultural applications that could be further composted and could add to the nutrient level of the soil (Kale et al., 2007). Grain straw and maize stalks are also valuable combustion sources with significantly lower water content (Menardo et al., 2011).

Poultry litter is a waste obtained from poultry farms, containing feathers and traces of animal feed. The amount of this waste is increasing significantly with increase in demand and supply of chicken meat; hence, this needs to be handled as soon as possible. The valuable micro-elements present in poultry litters can be efficiently employed in the agriculture sector, after preprocessing. Besides processing the poultry litter for fertilizers, thermal processing method has also been a more widely considered technique for the further conversion and application of poultry litter (Yusiharni et al., 2007; Irshad et al., 2009).

Likewise, pig manure and livestock manure are the sources of several useful nutritional constituents, which can be recovered through certain microbiological processes. Anaerobic co-digestion of the sweet potato and cattle manure has been demonstrated as a major method for the increase in biogas and biofertilizer production relative to mono-digestion of only sweet potatoes. The structure, hydration, aeration, and diversification of the microorganism consortia were observed to be influenced by the mixture of manure and plant materials, which was between 1:1 and 1:3 ratios.

The disposal of different locally produced manures is a major issue in several parts of the world. The most productive chemical fertilizers in Europe are imported, while some specific designated local processing plants are used to handle the disposal of manure, which is also hazardous waste product. In other parts of the world, specific structures such as fermentation plants and/or biogas plants along with various techniques are also being exploited to utilize this resource (Boechat et al., 2013; Chiumenti et al., 2018). Currently, several technologies are accessible to produce manure fertilizers (inorganic or organic, liquid, or solid). They are formed by solid/liquid separation, followed by anaerobic digestions or by incineration, drying, composting, liming, pelletizing, P-precipitation, followed by aerobic precipitation and ammonia stripping. Several membrane processes are currently being applied to extract ammonium fertilizer from manure to increase the efficiency of the applied organically derived manure, including nanofiltration, reverse osmosis, and membrane distillation (Zarebska et al., 2015). There are, however, still problems associated with the processing, storage, handling, and minimization of loss of nutrients for more refined and marketable goods.

13.3.1 Agro-industrial Wastes

Two different types of agro-industrial waste, namely, agricultural residues and industrial residues have been shown in Figure 13.2. Further subdivision of agro-industrial wastes as agricultural residues and process residues is also possible. Agricultural residues are residues found on the land following the growth and harvesting phase. These residues in the field include leaves, stalks, seed pods, and sticks, and process residues are found after processing into a useful substitute resource for the further processing plant (Table 13.2), which include molasses, husks, bagasse, nuts, sheets, and stalks. These residues are used as livestock feed for soil fertilization employed in crop production and various other processes.

Large amounts of other residues, including stubble, peel, and roots, etc., are also found in the fields, which are mostly under-utilized. Furthermore, controlled use of the remains/residues left in the fields will improve irrigation and erosion management skills. Wheat and barley are the most common crops in the Middle East and are extensively cultivated in many parts of the world. In addition, numerous other grains, pulses, beans, vegetable, and fruit plants are also grown around the world. The characteristics of corresponding crop residues and subsequent features generally differ from other solid fuels like charcoal, wood, and char briquette (Nayak & Patangray, 2015; Maraveas, 2020), which also contributes toward the difference in the developed biofertilizers.

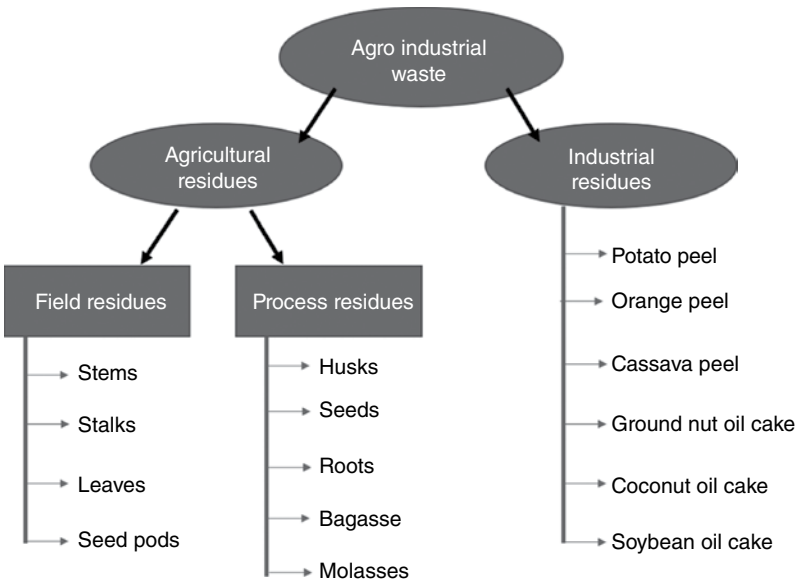

Figure 13.2 Types of agro-industrial wastes. *Source:* Sadh et al. (2018). © Springer Nature.

Table 13.2 Agro-industrial wastes and their compositions.

	Chemical composition (% w/w)					
Agro-industrial wastes	**Cellulose**	**Hemicellulose**	**Lignin**	**Ash (%)**	**Total solids (%)**	**Moisture (%)**
Sugarcane bagasse	30.2	56.7	13.4	1.9	91.66	4.8
Rice straw	39.2	23.5	36.1	12.4	98.62	6.58
Corn stalks	61.2	19.3	6.9	10.8	97.78	6.40
Sawdust	45.1	28.1	24.2	1.2	98.54	1.12
Sugar beet waste	26.3	18.5	2.5	4.8	87.5	12.4
Barley straw	33.8	21.9	13.8	11.0	—	—
Cotton stalks	58.	14.4	21.5	9.98	—	7.45
Oat straw	39.4	27.1	17.5	8.0	—	—
Soya stalks	34.5	24.8	19.8	10.39	—	11.84
Sunflower stalks	42.1	29.7	13.4	11.17	—	—
Wheat straw	32.9	24.0	8.9	6.7	95.6	7.0

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13.4 Food Waste

It is estimated that 3.7 × 109 Mg of agricultural residues and 1.3 × 109 Mg of food residues are produced annually worldwide. On an average, food waste production is 95–115 kg/yr per person in North America and Europe. Worldwide, approximately 1/3 of the edibles produced globally are wasted, which amounts to 1.3 billion Mg/yr (Laditi et al., 2012). Food waste consists of household and restaurant residues, processing waste streams, and crop residues. The waste contains protein, carbohydrates, fat, and lignin, as well as high humidity levels. According to the recent observations, 20% of the 50 biggest global food businesses have introduced food loss and waste reduction and have potentially addressed the issue. Also, about 40–60 % of the material used for food production is used in the manufacturing of animal feed products. Incineration, pyrolysis, aerobic digestion, anaerobic fermentation, or compost may use this large amount of produced waste to convert into heat, power, or fertilizers.

The food losses are primarily due to cultural practices and corresponding applications, wherein food supply chain contributes approximately 30%. Several organizations (e.g. UN-Sustainable Development Goal) are proposing and encouraging strategies for a 50% reduction in food waste to be implemented by policy makers by 2030. Food waste is a valuable outlet for compounds that are otherwise incinerated or disposed by urban fuel waste management.

There are several studies that are reporting the effect of various vegetable and fruit wastes on the growth of other crops. During a study by Tuttobene et al. (2009), the effects of orange waste on durum wheat growth as an organic fertilizer were assessed during a field research, where the levels of nutrients were close to those of mineral fertilizer that had been well selected. It was concluded during the study that application of dried orange as a source of organic fertilizer could partially solve the corresponding environmental problems of the region for dealing with the high production of orange peel waste, while simultaneously providing an alternate organic fertilizer.

Aerobic digestion, aerobic composting, and hydrolysis can also lead to the production of the biofertilizers from food waste, where various farm wastes (i.e. wheat straw, vegetable waste, corn waste, and so on) can be directly returned to the soil. Appropriate management of factors (e.g. composition, temperature, pH), different techniques (anaerobic digestion, and pyrolysis), and reuse of processing by-products will enhance crop yield, introduce useful nutrients for agriculture, and boost the physio-chemical structure and productivity of soil Stylianou et al. (2018), along with reduction of some of the phytotoxic effects of spent coffee ground, attributed to the presence of excess caffeine and propionic acid.

The usage of an organic fertilizer and applicability of the resulting combination of agricultural and mineral waste vary with the composition of the mixture and

the plant requirements. Fertilizer mixtures can vary in their action, where a certain fertilizer mixture can be found to improve root growth in certain plants and/or adversely affect the root growth in others. To increase the efficiency and effectiveness of the biofertilizers, dynamic aerobic high-temperature fermentation of residue food can also be the potential way of fast organic fertilizer manufacture (Jiang et al., 2015). The novel process developed by Jiang et al. (2015) included continuous friction and collision along with relatively high temperature application, which accelerated the progress of composting, wherein the process was completed in 96 hours, which took 24 days to observe similar results of composting under traditional static composting situation. Furthermore, digestate rich in nutrients can be processed to eliminate excess water and nutrients, using concentration through reverse osmosis and aerobic digestion during agricultural applications.

In a different study, potassium-rich banana waste in combination with large-scale phosphorus-rich sewage sludge was used to produce biochar (thermal processes) through slow pyrolysis treatment. These sources were observed to be ideally suitable for the production of biochar. However, presence of arsenic in some samples can be a major drawback, attributed to which this biochar was not used as fertilizer for food plants. Hence, in case of the application of biochar for soil and water remediation, these heavy and hazardous metals are preferably removed before initiating the cultivation process. In case of meat-processing industry by-products, skin and fat are partly recovered to a lower degree protein, while primarily using blood, residual unwanted meat, and bone as part of feed (Jayathilakan et al., 2012). Bones constitute an important and concentrated phosphorus supply (Saeid et al., 2014), which has been highly encouraged to be further exploited. However, the replacement of fertilizers derived from phosphate rock with bones could lead to new technical problems, such as the increased sulfuric acid consumption required in wet-process phosphoric acid for hydroxyapatite solubility. For the bones to be utilized as a feed, acid digestion is required to solubilize the organic bone matrix. Furthermore, pyrolysis of slaughter waste can lead to production of phosphate fertilizers, which can be further combined with other biomass such as meat, wood, and maize. Fish meal and fish waste, especially when composted with a bulking agent, can be a very useful biofertilizer rich in nitrogen, phosphorus, and calcium.

Keratin waste, such as feathers, can be directly digested with sulfuric acid and converted to a mixture of hydrolyzed amino acids and used as fertilizer for plant application. Yarn, which is another keratin waste, when converted to or added as a part of the fertilizer, injects essential nutrients in the field and absorbs moisture, even after little or non-extensive treatment. Through hydrolysis and super-heated water treatment, wool can be converted into fertilizer without any further pretreatment. Additionally, ashes from power stations, particularly those using

biomass as feedstock, may form a renewable source of potassium. Ash obtained from the burning of animal waste also contains high concentrations of phosphorus and calcium and can be used as agricultural fertilizer, wherein a mixture of the animal waste hydrolysates and ash can act as a full collection of plant nutrients. This waste can be further transformed using a biotechnological method and used as a further enriched nutrient source (Chojnacka et al., 2020).

For the manufacture of substrates for the efficient production of various industrial compounds, such as lactic acids, succinic acids, fatty acids, and other dietary additives (Pleissner et al., 2016), several other further advanced technologies are also being developed. Some of those concepts or the process technologies focus on preliminary hydrolysis of food-waste-based organic residues for converting them into respective monomers, including glucose and amino acids, which could be converted to uniquely tailored value-added organic residues for the further development of specific chemicals. This is the *theory of biological economy*, which involves integrated processes for the processing, development, and production of different products (chemicals, sorbents, polyolates, polyurethane sprays, carotenoids, phenolic antioxidants) and bioenergy sources (biodiesel, bio-oil, biogas, etc.), which revolves around the process of recovering integrated waste obtained from different food and agriculture sectors (Pleissner et al., 2016). It is of great importance to use food waste as a tool for bio-based products and resources in waste biorefineries.

13.5 Biofertilizer Production Using Fermentation Technology

The two major forms of fermentation used in the manufacture of these abovementioned biofertilizers are solid-state fermentation (SSF) and submerged fermentation (SmF). For each type of biofertilizers, some of the primary parameters considered include the selection of an appropriate strain of microorganism, development on a particular nutrient medium, scaling, and formulations on a solid or liquid basis. The performance of fermentation processes also depends on parameters such as temperature, pH, aseptic environment, and time of incubation. The production of biofertilizers has been reported to be comprised principally of three key steps: (i) strain growth, (ii) preparation of inoculant, and (iii) upscaling of the biomass (Sethi & Adhikary, 2012). Other important considerations include the identification of the carrier needed for formulation, packaging method and/or material, storage parameters, and transportation methods (Albareda et al., 2008; Atieno et al., 2012; Chojnacka et al., 2020). Preferred characteristics of appropriate carrier material used for the formulation of biofertilizers include high sustainability, low salt solubility, higher water-retention capability, cost-effectiveness, non-toxicity, and biodegradability (Gomare et al., 2013).

13.5.1 Solid-State Fermentation (SSF)

Any biotechnological process in which organisms evolve from the solid substrates or non-soluble materials, while free water is or is not present is referred to as fermentation from a solid state. SSF is a procedure that occurs with solid substrates or similar support, in the absence of free water (Bellon-Maurel et al., 2003; Mienda et al., 2011). Cereal grains, legumes, wheat bran, lignocellulose (sawdust) products, and a large range of other plants and animals have been commonly used for production of valuable and value-added compounds or commodities using the SSF. The above-mentioned different sources could be polymeric, insoluble, or sparingly water-soluble, but most of them are economical, simple to obtain, and a source of concentrated microbial nutrients. Different organic acids, enzymes, biofertilizers, bio-pesticides, bioethanol, aroma compounds, feed of animals, pigments, vitamins, antibiotics, and several other bio-products have been widely documented by SSF.

Though food processing by fermentation is one of the established processes, recent research has further boosted the development of SSF as an applicable method. There are extensive benefits of developing various bioprocesses and products with the use of SSF. Recent literature studies show that low water or lack of water during the SSF process offers a number of advantages, including rapid recovery of the product, low full production cost, smaller sizes of fermenters, less downstream processing, and lower energy consumption for stirring or sterilization (Pandey, 2003). Hence, SSF is an acceptable method for producing efficient and cost-effective biofertilizers that could increase crop yields, wherein the efficient use of biofertilizers is expressed in terms of the growth rate of crops, which in turn reflects an improvement in plant weight and consequently the crop yield.

Various variables, including microbial strains, physiochemical properties of solid support, motion of water through the matrix, temperature, aeration, and fermenter size, should be considered during the development phase of the SSF before any fermentation process is started. Specific environmental factors, including water activity, moisture content, temperature, pH levels, and amount of oxygen, were considered during the development phase in SSF (Perez-Guerra et al., 2003). Humidity content is also a key factor in SSF processes as it correspondingly affects the production and biosynthesis of various metabolites (Krishna & Chandrasekaran, 1996; Ellaiah et al., 2002). In SSF, higher humidity levels result in decreased solubility of substrate nutrients, higher swelling levels, and high water tension (Perez-Guerra et al., 2003). On the other hand, higher levels of humidity also reduced enzyme yield by reducing the porosity (interparticle spaces) of the solid matrix and interfering with the transferring oxygen attributed to a steric hindrance to the growth of the production strains.

Microorganisms used in SSF can occur and obtained from individual pure crop plants, crop plants of different species grown together, or can be isolated from mixed indigenous microorganism consortia. In some SSF processes, such as for the production of tempeh, selective growth is required in microorganisms, such as molds that use the extracellular enzymes for fermentation at low moisture content. Table 13.3 shows the various microorganisms used in SSF systems, such as fungi, yeasts, and bacteria. For the maximum output of value-added goods, molds are also used for SSF because they naturally grow on solid media including wood pieces, seeds, stems, and roots. However, SSF has also been carried out with

Table 13.3 Recent studies in the area of solid-state fermentation using various agro-industrial wastes and microorganisms.

Microorganisms	Solid supports	References
Bacteria		
Amycolatopsis Mediterranean MTCC 14	Groundnut oil cake, coconut oil cake	Vastrad and Neelagund (2011a, b)
Xanthomonas tapestries MTCC 2286	Potato peel	Vidhyalakshmi et al. (2012)
Pseudomonas spp. BUP6	Groundnut oil cake, coconut oil cake, sesame oil cake, and cotton seed cake	Faisal et al. (2014)
Bacillus licheniformis MTCC 1483	Wheat straw, sugarcane bagasse, maize straw, and paddy straw	Kaur et al. (2015)
Fungi		
Aspergillus niger	Rice bran, wheat bran, black gram bran, groundnut oil cake and coconut oil cake	Suganthi et al. (2011)
Aspergillus niger	Rice bran, wheat bran, black gram bran, and soybean	Kumar and Duhan (2011)
Streptomyces spp.	Household kitchen wastes	Ezejiofor et al. (2012)
Aspergillus oryzae	Soybean meal (waste)	Thakur et al. (2015)
Rhizopus arrhizus and *Mucor subtillissimus*	Corncob cassava peel, soybeans, wheat bran, and citrus pulp	Nascimento et al. (2015)
Aspergillus niger	Rice bran, wheat bran, black gram bran, groundnut oil cake, and coconut oil cake	Mahalakshmi and Jayalakshmi (2016)
Aspergillus terreus	Palm oil cake	Rahman et al. (2016)

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bacteria and yeasts that require a relatively higher moisture content but produce lower yield.

SSF is a multistage process consisting of the following steps:

1) Substrate collection.
2) Mechanical, chemical, or biochemical pretreatment for obtaining enhanced qualities of the substrates and to minimize the particle size, which is generally achieved through the pulverization of straw and the shredding of vegetable by-products, so that the efficiency is attained in the physical aspects of the manufacturing process. However, pretreatment costs have been recommended to be balanced with the potential value of the product.
3) Hydrolyzation of the predominantly polymeric substrates, such as polysaccharides and proteins.
4) Method of fermentation for the use of products obtained through hydrolysis.
5) Downstream refining to purify and measure end products.

Sustainable green bioconversion processing of organic waste into useful goods, which could substitute nonrenewable materials and transform chemicals through safer industrial practices, underlines SSF's potential. The SSF's major benefit is its application of fairly simple method for utilizing large and inexpensive biomaterials with extremely low bioconversion pretreatment, along with generation of lower quantities of wastewater, and application of similar microenvironments capable of simulating microorganism growth (Singhania et al., 2009). In addition, employment of SSF has led to a new bioconversion model for conversion and utilization of organic solid waste by producing organically active metabolites on a laboratory and industrial scale. The SSF also simulates natural microbial processes including composting and sedimentary processes (Thomas et al., 2013).

13.5.2 Submerged Fermentation (SmF)

SmF has been used for the manufacturing of most commercial industrial goods and is carried out through inoculation of microbial culture in a liquid state for the manufacture of the desired product (Subramaniyam & Vimala, 2012). Immersed fermentation is a form of manufacturing of biomolecules in which liquids such as alcohol, oil, or nutrient broth are immersed in enzymes and other reactive compounds. SmF uses fluid substrates including molasses and broths free of flux. In the SmF, the microorganism is grown and dispersed as a suspension in a liquid medium, in which different nutrients are either dissolved in many commercial media or suspended as particulate solids.

SmF is also a mechanism involving microorganisms' production in a liquid bubble containing nutrients, which helps in the processing of industrial enzymes, antibiotics, and other products. The method involves extracting the chosen

microbial pool and placing in a small, closed bottle with a deep, nutrient-rich broth suitable for a particular microorganism, such as fungi. The method often requires a high quantity of oxygen. The formation of enzymes occurs as microorganisms interact with the broth's nutrients, which they break down for further utilization. The bioactive compounds are isolated from the fermentation broth (in case of extracellular secretion) or from the centrifuged tablet consisting of the concentrated biomass.

There are two common methods of SmF: batch-fed and continuous fermentation. Sterilized growth nutrients are incorporated into the growth media during the batch-fed fermentation process. In bio-industries, it is most common, as it happens during the biomass growth in fermenters. The growth rate in the cultivation is sustained by the addition of nutrients, thereby minimizing the possibility of overflow.

For continuous fermentation, an open system is created. Slowly and continuously sterilized liquid nutrients are added into the bioreactor at the rate like the rate of transfer/collection of the final nutrient product solution. The fermentation broth is therefore produced steadily. There are also optional anaerobic species involved in certain fermentation processes targeting production of end-product (such as ethanol), which includes *Saccharomyces cerevisiae* that can grow efficiently under aerobic condition along with generating cell biomass and turn toward anaerobic route during the ethanol fermentation phase. However, enzymes such as amylases and proteases produced by the SmF method typically follow the aerobic fermentation path.

With the ease of automation, SmF processes can be further easily extended, which would not encounter the heat transfer limitations (which are SSF's key disadvantage). However, low efficiency, high production costs, and medium complexity are the main disadvantages associated with SmF processes (Babbar & Oberoi, 2014). In recent years, several studies have shown that SSF has a significant impact on performance, resulting in higher yields and better product characteristics compared to SmF, along with low production volumetrics, relatively lower product concentrations, higher effluent generation, and application of complex fermentation equipment.

Table 13.4 presents some of the enzymes generated by SSF and SmF using different microbial strains. From the table, it can be observed that the titers of the enzyme were significantly higher in the case of SSF, except some cases with xylanase production, where SmF led to higher production. Good fermentation conditions encompass maintenance of optimum levels of several variables including temperature, pH, oxygen, and carbondioxide levels. Some common substrates used in the SmFs are soluble sugars, molasses, water, fruit and vegetable juices, and wastewater.

For the extraction of subcontracting metabolites as a liquid, SmF is mainly used. In the conventional processing of microbial enzymes, submerged liquid

Table 13.4 Enzyme production using food waste via solid-state fermentation (SSF) and submerged fermentation (SmF).

		Substrate		Productivity	
Enzyme	**Microorganism**	**SSF**	**SmF**	**SSF (U/g)**	**SmF(U/ml)**
Amylase	*Bacillus* spp.	Oil cake, wheat bran	Starch broth	50 000	400
Xylanase	*Bacillus* spp.	Corn cob, wheat bran	Corn cob and yeast extract	6.18	16.13
Cellulase	*Bacillus* spp.	Banana waste	Carboxymethylcellulose, cellulose	9.6	1.2
Cellulase	*Trichoderma viridae* ATCC 13631	Wheat bran	Mandel's liquid medium	60.5	28
Lipase	*Aspergillus niger* NCIM 1207	Wheat bran, olive oil	Synthetic oil-based medium	630	18
Pectinase	*A. Niger*	Wheat bran, coffee pulp	Pectin-based production medium	2.28	0.48
Pectinase	*A. Niger*	Orange peels	Orange peels	1,224	2.29
Pectinase	*Penicillium citrinum*	Orange waste	Orange waste	34.5	0.067
Pectinase	*Bacillus* spp.	Orange peel waste	Orange bagasse	200	0.30
Xylanase	*Trichoderma koeningi*	Pineapple peel powder, corn cobs	Maize straw	2.868	4.62
Laccase	*Pieurotus* spp.	Orange waste	Mandarin peel	12.2	2.37
Xylanase	*Aspergillus awamori*	Tomato waste	Oat straw	100	820
Xylanase	*A. Niger*	Apple pomace	Palm leaf	5,662	1,906
Xylanase	*Trichoderma* spp.	Watermelon rinds	Melon peels	70	26.5
Xylanase	*Trichoderma* spp.	Sunflower sludge	Pineapple peel	8.75	73.09

Source: Sharma et al. (2016). Hence, readers can refer to the original work for further details.

fermentations are used. Lesser time periods, lower cost, and higher returns are the benefits of SmF technology. Control of the fermentation parameters is easier in the case of liquid culture, which enables large reductions in fermentation time. Besides, it is easier to purify products in the case of SmF. The use of submerged crops also benefits from the processing of many secondary metabolites, which simultaneously decreases the cost of production by minimizing labor in SSF methods.

13.5.3 Production of N_2-fixing Biofertilizer

N_2-fixing biofertilizers are called microorganism-containing biofertilizers that can fix N_2 to N-containing chemical compounds in the soil, which plants can absorb. The most powerful and commonly used N_2 fixers are *Azospirillum*, *Azotobacter*, and *Rhizobium*.

13.5.3.1 Production of *Rhizobium* Biofertilizer

Species selection is the main phase in the production of biofertilizers with *Rhizobium*. During the fermentation procedure, the chosen strain should be specific to the host, and the selected conditions should be conducive for the growth of the microbial strain. It has been stated that a specific species of *Rhizobium* is required to produce effective nodules for each crop. The most frequently used medium for the species *Rhizobium* is mannitol yeast extract (YEM). YEM *Rhizobium* medium contains (g/l): mannitol, 10.0; yeast extract, 1.0; K_2HPO_4, 0.5; $MgSO_4.2H_2O$, 0.2; NaCl, 0.1; $CaCO_3$, 1.0; and pH, 6.8 ± 0.2 (Allen & Allen, 1950; Subba Rao, 1977). However, the use of YEM can be costly for large-scale processing. Thus, commercial producers prefer cost-effective biomass for production that is readily accessible (Ben Rebah et al., 2002). Many researchers have used agricultural waste and industrial waste as medium ingredients for the production of products derived from *Rhizobium*, including molasses, steep-corn liquor, deproteinized leaf extracts (Chanda et al., 1987; Jain et al., 2000; Estrella et al., 2004; Ben Rebah et al., 2007; Singh et al., 2011). The development is carried out in two stages: pilot and mass production using fermenters of different sizes (Bissonnette et al. 1986). Finally, the culture obtained is used in the formulation or in the liquid formulation of the carrier of biofertilizers.

13.5.3.2 Production of *Azotobacter* Biofertilizer

The N-free medium of Ashby is commonly used in the cultivation of *Azotobacter*, which contains sucrose (g/l), 20.0; K_2HPO_4, 0.2; $MgSO_4.2H_2O$, 0.2; NaCl, 0.2; K_2SO_4, 0.1; $CaCO_3$, 5.0; pH, 7.4 ± 0.2. Sucrose is used as the source of C in this medium and N_2 as an ambient source of N. K_2HPO_4 supplies the system with a buffer, while other ingredients of the medium provide different ions essential for *Azotobacter* development. Medium composition and growth conditions can vary, as the selected strain can belong to different genera.

13.5.3.3 Production of *Azospirillum* Biofertilizer

Azospirillum is a commonly used microorganism, which grows in OAB media, which is made up of solution A and solution B. Details of Solution A (g/l): malic acid, 5; NaOH, 3; $MgSO_4.7H_2O$, 0.2; NaCl, 0.1; NaCl, 1.01; Leas through, 0.1; $FeCl_3$, 0.02; (mg/l): $NaMoO_4.2H_2O$, 2; $MnSO_4$, 2.1, and H_3BO_3, 2.8; $Cu(NO_3)_2.3H_2O$, 0.04; $ZnSO_4.7H_2O$, 0.24. K_2HPO_4, 6; KH_2PO_4, 4; 100 ml of distilled water. Details of Solution B (g/l): solution pH 6.8 is blended after autoclaving and cooling (Okon et al., 1977; Bashan et al., 1993). In this case, the fermenter must also be constructed in a manner that helps it to maintain the microaerophilic condition for the growth of *Azospirillum* during fermentation with this medium.

13.5.4 Production of Phosphate-solubilizing Biofertilizer

The selection of appropriate strain is performed based on their P-solubilizing potential and area of application for the P-solubilizing biofertilizer. The constitution and the corresponding characteristics of soil in different fields vary, including physical and chemical structure, the organic matter, and the P substance (Kim et al., 1998). The next step is to choose the required medium of production. The medium should be conducive for the growth and increment of the number of the cells of the selected strain. Pikovskaya medium (Pikovskaya, 1948; Roychowdhury et al., 2015) is a simple medium and has been used commonly for propagating organisms appropriate for the preparation of PSBs, including *Pseudomonas* and *Bacillus*. Mother culture is produced by inoculation into the sterile medium of the most strictly maintained bacterial culture, followed by inducement by P-solubilizing strain and medium production targeted to reach 109 CFU/ml population under stress conditions. Besides, the mother culture is transferred for scale-up in a pilot fermentation system and then in a larger bulk fermenting unit. A fermenter's specifications are chosen depending on the final biofertilizer volume required for usage. During a study by Pindi and Satyanarayana (2012), fermentation process was performed with continuous rest and ventilation for approximately seven days to reach the concentration of 109 CFU/ml for the population of selected strain cells. Every day, the pureness and growth of the selected strain should be checked for quality control. The broth is then harvested, processed at cooler temperatures, and then mixed under aseptic conditions with the right carrier content.

13.5.5 Production of Phosphate-mobilizing Biofertilizer

Many scientists have demonstrated the ability of the P-mobilization through biofertilizer using *Arbuscular mycorrhiza* (AM) fungi (Rillig et al. 2015; Berruti et al. 2016). However, due to its mandatory symbiotic existence, the use of AM biofertilizers is limited. AM cannot be produced in a large-scale laboratory using synthetic media as observed in the cases of other microorganisms propagated to

be used as biofertilizers. The variety of plant genotypes and soil composition also affect the potential application of AM as a P-mobilizing biofertilizer. Consequently, culture in pot typically produces AM to keep contamination under regulation (Klironomos & Hart, 2002). A concentrate collection of propagules of the same form, typically contained in natural soil inoculum, is present in the inoculum prepared by this process (Berruti et al., 2016). AM-based biofertilizer application has reportedly increased host plants' content of nutrients, growth, and corresponding yield. The success of the application or failure of the inoculum (native or alien) is therefore dependent on its growth (greenhouse or open field) and method of application (single or consortium. (Secilia & Bagyaraj, 1987; Herrmann & Lesueur, 2013). The commercial production and application of this biofertilizer is not highly recommended, but it is feasible.

13.6 Biofertilizer for Organic Farming

The demand for nutritious and convenient, balanced foods along with sustainable long-term sustainability and environmental contamination issues related to the indiscriminate use of agrochemicals have made organic farming a major priority globally. While the use of chemical inputs in agriculture is inevitable to meet increasing global food demand, there are vital opportunities for the introduction of organic production to the domestic export market in the case of selected crops and niche areas. Furthermore, remediation measures for the depletion of soil quality has led to the development and application of modern machinery and fertilizers. Bio-culture includes preparing live cells or latent cells that are successfully fastened with a microbial strain for nitrogen fixation and/or solubilization of phosphates or cellulolytic microorganisms used for the treatment, acceleration, or strengthening of available microorganisms.

By fixing atmospheric nitrogen in combination with and without plant roots, biofertilizers have a big part to play in enhancing soil fertility, solubilizing insoluble soil phosphates, and producing components necessary for maintaining soil quality conducive for plant growth. They are encouraged to harvest the bionutrients through mobilization method (Venkatashwarlu, 2008) that can be naturally used. Several authors have also explored the role and significance of biofertilizer in sustainable crop production (Biswas et al. 1985; Wani & Lee 1995; Katyal et al. 1994). In Asia, however, development in biofertilizer technology has always remained less satisfactory attributed to various local restrictions.

The basic P adsorption by functional groups can influence the load balance and cause particle dispersion. The most known advantage of mycorrhizae has been reported as enhancement of the P nutrition of plants. The mechanism usually involves the mycorrhizal feature involving exploration of the ground with mycorrhizal fungi (hyphae) more physically than with roots. The manufacture of

glomalin is a theoretical mechanism for P absorption by mycorrhizal fungi. Glomalin contains very large quantities of iron (Lovelock et al., 2004). Moreover, leaf wilting in mycorrhizal plants does not occur after soil drying until the water level in soils decrease significantly. The drought-tolerance caused by Mycorrhiza can be related to AM-related conditions, including improved leaf and turgor water pressure and preservation of the general function and transpiration, increased hydraulic conductivity, and the root length.

A part of the soil structure is the ground aggregation. The skeletal structure of the soil is modified further by Mycorrhizal fungi, which (i) contributes to the soil growth through external hyphae; (ii) establishes conditions propitious for the creation of micro-aggregates by external hyphae; (iii) encourages interweaving of micro-aggregates to form macro-aggregates by external hyphae; and (iv) promotes the direct use of soil from the plant's carbon capital (Miller & Jastrow, 1990, 2000). This direct access affects soil aggregate formation, as soil carbon is essential for the formation of organic materials required for soil particles.

13.7 Conclusion

Fermentation is a biofertilizer processing technology. Biofertilizers are living microorganisms that have solid or liquid formulations, which contribute importantly to maintain and improve sustainable soil fertility and crop yield. Biofertilizers are used to promote N_2-fixation, phosphate solubilization, phosphate movement, and other plant-growth-promoting microorganisms with various characteristics. The efficient utilization of biofertilizers depends on their fermentation technology in this area. Different parameters such as a plant-specific host strain, the production of a professional fermentation bioprocess; a standardized, cost-effective, and easily accessible growing media; the growth rate, capacity, efficiency, and survival of the selected strain, and the properties of the supporting materials used are taken into consideration. The pool of available information from agriculture sector has increased knowledge and understanding of the above criteria, which has led to great progress in fermentation technology. There have been improved performance, reliability, cost-effectiveness, ease of use, and biofertilizers multifunctionality. It has increased both soil fertility and agricultural crop productivity. Further groundbreaking research is needed to develop enhanced biological fertilizers, which can fully substitute and sustainably lead to healthy growth of many different crops.

Conflict of Interest

Authors declare no conflict of interest.

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