Om V. Singh · Anuj K. Chandel *Editors*

Sustainable Biotechnology-Enzymatic Resources of Renewable Energy

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Foreword

Even with greater efforts from developed and developing nations, it seems unlikely to resolve the issues involving with atmospheric CO_2 levels. The effects of this issue are affecting the climate, the adoption of global conservation measure, and the stabilization of fossil fuel prices. It is still a certainty that global oil and gas supplies will be largely depleted in a matter of decades. However, nature provides abundant *renewable* resources that can be used to replace fossil fuels, if not completely but at least to some extent. However, major issues remain at the forefront such as cost, technology readiness levels, and compatibility with existing distribution networks.

In current scenario, the cellulosic fuel remains unsuccessful to reduce societal independence from fossil fuel. There is a need to continue to bridge the technology gap and focus on the critical aspects of lignocellulosic biomolecules conversion. In addition, the value-added products of industrial significance are among top priority during bioconversion to liquid fuels. Therefore, the respective molecular mechanisms regulating the bioconversion of liquid fuel may remain to be discovered so that biofuel could become a reality at a reasonable cost.

Commercialisation of biofuels and biochemicals is a great challenge, however not impossible to met with the demands of sustainable energy. This book provides the key aspects of molecular mechanism of liquid fuel and value-added products of industrial significance. Uniquely, the editors focused on technological updates on biomass processing, system biology, microbial fermentation, catalysis, regeneration, and monitoring of renewable energy and recovery process. This book also offers facts of techno-economic analysis, climate change, and geopolitical interpretation of bioenergy aspects.

It is my pleasure to present this book to the scientific community as a unique source of information of sustainable biotechnology. The editors of this book are among the champions of bioenergy research. I thank all of the contributors for sharing their research and ideas with the scientific community using this unique platform of bioenergy research.

Lorena, São Paulo, Brazil

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Contents

1 Introduction 1
Om V. Singh and Anuj K. Chandel

2 Role of Systematic Biology in Biorefining of Lignocellulosic Residues for Biofuels and Chemicals Production 5
Vishal Sharma, Bilqeesa Bhat, Mahak Gupta, Surbhi Vaid, Shikha Sharma, Parushi Nargotra, Satbir Singh and Bijender Kumar Bajaj

3 Biotechnological Advances in Lignocellulosic Ethanol Production 57
Latika Bhatia, Anuj K. Chandel, Akhilesh K. Singh and Om V. Singh

4 Sustainable Production of Biofuels from Weedy Biomass and Other Unconventional Lignocellulose Wastes 83
Anurup Adak, Surender Singh, A. K. Lavanya, Anamika Sharma and Lata Nain

5 Technological Aspects of Lignocellulose Conversion into Biofuels: Key Challenges and Practical Solutions 117
Catia Giovanna Lopresto, Alessandra Verardi, Cecilia Nicoletti, Debolina Mukherjee, Vincenza Calabro, Sudip Chakraborty and Stefano Curcio

6 Beyond Ethanol: Contribution of Various Bioproducts to Enhance the Viability of Biorefineries 155
Ruly Terán Hilares, Muhammad Ajaz Ahmed, Marcos Moacir de Souza Junior, Paulo R. F. Marcelino, Silvio S. da Silva and Júlio César dos Santos

7 Techno-economic Assessment of Bioethanol Production from Major Lignocellulosic Residues Under Different Process Configurations 177
Pornkamol Unrean

8 Lignocellulolytic Enzymes from Thermophiles 205
Vikas Sharma and D. Vasanth

9 Application of Enzymes in Sustainable Liquid Transportation Fuels Production 219
Nivedita Sharma and Poonam Sharma

10 The Realm of Lipases in Biodiesel Production 247
Daniela V. Cortez, Cristiano Reis, Victor H. Perez and Heizir F. De Castro

11 Nanotechnology-Based Developments in Biofuel Production: Current Trends and Applications 289
Avinash P. Ingle, Priti Paralikar, Silvio Silverio da Silva and Mahendra Rai

12 Ester-Based Biofuels from Wastes 307
Konstantina Boura, Panagiotis Kandylis, Argyro Bekatorou, Agapi Dima, Maria Kanellaki and Athanasios A. Koutinas

13 Sustainable Production of Biogas from Renewable Sources: Global Overview, Scale Up Opportunities and Potential Market Trends 325
Lilia E. Montañez-Hernández, Inty Omar Hernández-De Lira, Gregorio Rafael-Galindo, María de Lourdes Froto Madariaga and Nagamani Balagurusamy

14 Microbially Originated Polyhydroxyalkanoate (PHA) Biopolymers: An Insight into the Molecular Mechanism and Biogenesis of PHA Granules 355
Akhilesh Kumar Singh, Laxuman Sharma, Janmejai Kumar Srivastava, Nirupama Mallick and Mohammad Israil Ansari

15 Biopolymer Synthesis and Biodegradation 399
Sudhakar Muniyasamy, Özgür Seydibeyoğlu, Boopalan Thulasinathan and A. Arun

16 The Application of Microbial Consortia in a Biorefinery Context: Understanding the Importance of Artificial Lichens 423
Cristiano E. Rodrigues Reis, Aravindan Rajendran, Messias B. Silva, Bo Hu and Heizir F. de Castro

17 Green Microalgae as Substrate for Producing Biofuels and Chlorophyll in Biorefineries 439
Bruna C. M. Gonçalves and Messias B. Silva

18 Potential Applications of Enzymes in Sericulture 463
Yeruva Thirupathaiah, Anuj K. Chandel and V. Sivaprasad

Promotional Text 473

Author Index 475

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His contributions span the biomass science, biotechnology, and policy domains, and include sustainable development of biofuels and renewable chemicals under biorefinery concept. He is a frequently invited presenter on technical and strategic aspects of biomass energy and biochemicals, in prominent forums and international conferences. e-mail: anuj.kumar.chandel@gmail.com; anuj10@usp.br

Chapter 1
Introduction

Om V. Singh and Anuj K. Chandel

The ever-increasing appetite of energy relies upon the use of unsustainable conventional resources. Even though, the nature offers abundant *renewable* resources to replace unsustainable sources, the technology readiness levels and compatibility with existing distribution networks remains a challenging issue. Multiple renewable energy resources such as solar, tidal, hydrothermal, ocean thermal, and wind energy have ben explored as alternative resources, however each comes with its limitations. Lignocellulosic biomass is one of the most immediate source of energy that can serve as potential alternative of fossil fuel (Singh and Harvey 2008; Chandel and Singh 2011; Chandel and Silveira 2017). After successfully introducing Edition one of "Sustainable Biotechnology: Sources of Renewable Energy" in 2010, here we continued to extend our efforts towards bridging the technology gap and focusing on other critical aspects of lignocellulosic biomolecules. We also considered the respective mechanisms regulating the bioconversion of liquid fuels into energy and value-added products of industrial significances.

The lignocellulosic biomass (LB) is an inexpensive feedstock. Due to the nature of availability, it is an easily accessible as agricultural and forest residue, municipal wastes etc., and has potential to act as a valuable substitute for fossil fuels. Structurally, LB contains significant amounts of polysaccharides that can be subjected to microbial fermentation into energy and other value-added products of industrial significance. Considering the enormous potential of LB, in Chap. 2, *Sharma* et al. provided strategies for bio-refining and valorization of biomass to value-added prod-

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ucts. The chapter also emphasizes on recent advancements in the fields of pretreatment, metabolic engineering, enzyme production and fermentation that would help in developing a suitable technology which could replace the deteriorating effects of fossil fuels. Further, *Bhatia* et al. in Chap. 3, give the matter a more detailed consideration by elaborating on biotechnological advancements in cellulosic ethanol production from lignocellulosic biomass. In continuation, *Adak* et al. in Chap. 4 provides a detail description of weedy biomass and other unconventional lignocellulosic wastes for sustainable production of biofuel.

Earth's most plentiful and renewable energy resources typically include sunlight, wind, geothermal heat, water (rivers, tides and waves), and biomass. All of these are suitable for the generation of electricity but biomass is the current main renewable feedstock for the production of "liquid" fuels—typically ethanol, and biodiesel and possibly to include butanol, hydrogen and methane. However, the efficient and cost-effective production of bioethanol from various lignocellulosic biomasses is depending on the development of a suitable pretreatment system and technological aspects of bio-engineered feedstock. *Lopresto* et al. in Chap. 5 interpreted the existing pretreatment methods of bioengineered feedstock that can be effectively utilized via biocatalytic hydrolysis. In addition to technological aspects of lignocellulosic conversion into biofuels, *Hilares* et al. in Chap. 6 discussed how high cost of 2G ethanol process can be supplemented by producing biopolymers, biopharmaceutical, nutrients, pigments, surfactants, and other biochemical using varying fractions of lignocellulosic biomass. The concept was named as "Bio-refinery" that can contribute with the economic viability of current state of bio manufacturing of specialty chemicals. *Unrean P* continued the discussion in Chap. 7, provided techno-economic analysis to compare different upstream process configuration for lignocellulose-to-ethanol process and to determine the cost effectiveness process option suitable for commercialization based on minimal selling price of ethanol produced.

Novel enzyme mediated bioconversion of biomass can address the multiple challenges of effective hydrolysis of lignocellulosics. Discovering new and sustainable resources, which can help refuel industrial biotechnology. The adverse environmental conditions which normal earth microbiota do not tolerate, offer potential sites to explore specific sets of microorganisms designated as 'Extremophiles'. The discovery of these microorganisms has enabled the biotechnology industry to innovate unconventional bioproducts i.e. 'Extremolytes' (Schiraldi and DeRosa 2002; Singh 2012; Beeler and Singh 2016). In Chap. 8, *Sharma and Vasanth* provided an overview of thermophilic habitats. The applications of extremophiles and their products, extremozymes, with their possible implications with lignocellulolytic activity are also discussed broadly.

Studies show that different types of biomasses are being used for production of sustainable fuel with the help of biofuel enzymes. According to the BCC Research report, Global Markets and Technologies for Biofuel Enzymes (EGY009B), the global market for biofuel enzymes have higher projection in the future ahead. *Sharma and Sharma* in Chap. 9 discussed multiple applications of enzymes in sustainable liquid transportation fuels production. In continuation, several enzymes including lipases are known for the hydrolytic activity on carboxylic fatty ester bonds. There is broad

industrial interest in lipases due to their applications in a wide array of value-added products of commercial significance such as detergents, cleaning agents, pharmaceuticals, food industries, and for biodiesel production. In Chap. 10, *Cortez* et al. discussed the realm of lipases in biodiesel production.

Technological implementations have always been amazed multidisciplinary areas of science. Nanotechnology represents one of the most fascinating techno-scientific revolutions ever undertaken in various sectors including biofuel and bioenergy. Varying nanomaterials have been explored to play important role in energy fields due to their unique structure, relatively high specific area and comparatively good efficiency of lighting and heating (Ansari and Husain 2012; Singh 2015; Rai and da Silva 2017). In Chap. 11, *Ingle* et al. discussed recent trends and applications of nanotechnology in biofuel production.

The agro-industrial waste is defined as the organic and non-organic residues generated by the activity of the production and processing or raw materials from agricultural, livestock, and dairy industries. Utilization of these raw products could potentially reduce the overall cost of biofuel production (Balan 2014). In Chap. 12, *Boura* et al. presents different aspects of the production of ester-based biofuel from agro-industrial wastes emphasizing on the production of 2nd generation biofuel. The anaerobic digestion is the most prominent bioenergy technology and has been profitable alternative providing a sustainable solution to treat organic wastes and reduce the greenhouse gases emission. *Montanez-Hernandez* et al. in Chap. 13 provided sustainable production of biogas as potential biofuel from renewable sources.

Among value-added products of commercial significance, Polyhydroxyalkanoates (PHAs) have received substantial attention as an alternate of conventional non-biodegradable plastic. Microorganisms especially bacteria and cyanobacteria have the ability to synthesize PHAs granules intracellularly as carbon and energy storage compounds. In Chap. 14, *Singh* et al. provides microbial molecular mechanism for biogenesis of PHA as green plastic molecule. Further, *Muniyasamy* et al. in Chap. 15 presents synthesis of biopolymer and aspects of their biodegradability. This chapter also discusses the management of conventional plastic materials to secure the environment.

The technological advances have enabled an effective use of natural sources to obtain clean energy, thus reducing emission of gaseous pollutants into the environment. The use of microalgae as raw material to obtain biofuel has been proved promising. Thus in Chap. 16, *Reis* et al. discussed the applications of microbial consortia in a bio-refinery context and provided insight understanding of the importance of artificial lichens. In continuation, *Goncalves and Silva* in Chap. 17 proposed green microalgae as substrate for producing biofuel and chlorophyll as value-added product of commercial significance in the bio-refineries.

Apart from biofuel, the sericulture is another important sustainable agro based industry that plays pivotal role in the rural and urban economy. Similar to biofuel, the sericulture utilizes lignocellulosic biomass as a potential resource of raw material for profitability. In Chap. 18, *Thirupathaiah Y* provided a unique contribution to this book explaining the future and perspectives of potential applications of enzymes used in the sericulture sector of environmental sustainability.

This book "Sustainable Biotechnology: Enzymatic Resources of Renewable Energy" is a collection of articles elucidating several broad-ranging areas of progress and challenges in the utilization of sustainable resources of renewable energy, especially in biofuels. After the release of "Sustainable Biotechnology-Resources of Renewable Energy" in 2010, this book comes just at a time when industrialists are accelerating their efforts in the exploration of alternative energy and other value-added products of commercial significance to establish long-term sustainability in bio-refineries. Apart from liquid fuel this book also provides in-sights of value-added products, which may help in revitalizing the biotechnology industry at a broader scale.

We hope readers will find these articles interesting and informative for their research pursuits. It has been our pleasure to put together this book with Springer press. We would like to thank all of the contributing authors for sharing their quality research and ideas with the scientific community through this book.

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Chapter 2
Role of Systematic Biology in Biorefining of Lignocellulosic Residues for Biofuels and Chemicals Production

Vishal Sharma, Bilqeesa Bhat, Mahak Gupta, Surbhi Vaid, Shikha Sharma, Parushi Nargotra, Satbir Singh and Bijender Kumar Bajaj

Abstract World has witnessed most unprecedented economic/industrial growth during past few decades. But this resulted in massive depletion in the fossil fuel reserves, and grave environmental concerns like green house gas emissions, climate change etc. Keeping in view the serious consideration there is a paradigm shift towards the exploration of renewable energy resources, and development of processes/products that are green, clean and ecobenign. Lignocellulosic biomass, being an inexpensive and abundant energy source could be exploited for the production of bioenergy and other oleochemicals. But due to recalcitrant nature of lignocellulosic biomass, which is attributed to presence of lignin and hemicelluloses making the substrate inaccessible to hydrolytic enzymes. Therefore, the major challenge in biomass to biofuel/bioactives is conversion delignification of lignocellulosic biomass. With the application of appropriate pretreatment technique, the complex biomass can be partially loosened and made accessible for hydrolysis. Environment friendly and cost effective biological pretreatment method using microorganisms offers advantages in getting the desired results in energy efficient manner. Appropriate combination of hydrolytic enzymes is required for complete degradation of cellulose and hemicelluloses into simpler sugars which served as raw material for further transformation. Successful saccharification of lignocellulosic biomass results in release of fermentable sugars which could act as starting material for production of bioenergy (Bioethanol, biobutanol, biohydrogen, biogas etc.) and other value-added products (Bioplastic, animal feed, composites, enzymes, xylooligosaccharides etc.). With the advancement in technology (green biotechnology), the conversion costs of lignocellulosic biomass could be lowered and product yields could be enhanced making the production processes more economical and alleviating the deleterious effects of harsh chemicals and fossil fuels on environment.

Keywords Biofuel · Lignocellulosic biomass · Pretreatment
Xylooligosaccharides · Polyhydroxybutyrate · Biohydrogen · Biobutanol
Saccharification

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2.1 Introduction

The current global energy appetite relies upon the use of fossil fuels which accomplish the needs of industrial and automobile sector. The massive use of fossil fuels as a major source of energy has not only left shortage of fuels but has also created alarming environmental concerns viz. green house gas emission, global warming etc., economic and social concerns (Nargotra et al. 2016). This has motivated the researchers to work intensively for finding out renewable sources of energy. Numerous renewable energy resources like wind energy, hydrothermal energy, oceaothermal energy, tidal energy, solar energy etc. have been explored as alternate sources, but suffer with their individual limitations. Among various renewable sources of energy, abundantly present lignocellulosic biomass serves as the most potential alternative to overcome the energy crisis (Vaid and Bajaj 2017).

Lignocellulosic biomass (LB) is an inexpensive feedstock and available on the earth's crust in plenty. It is easily accessible as agricultural and forest residues, municipal wastes etc. (Saini et al. 2015) and may act as a valuable substitute for fossil fuels (oil, natural gas and coal), which are finite and mostly non renewable (Mohr and Raman 2013). LB generally composed of 40–50% cellulose, 20–30% hemicellulose and 10–25% lignin (Saini et al. 2015). About 50–80% of polysaccharides available in LB can be subjected to microbial fermentation for production of several useful products. Cellulose is a highly stable polymer which is majorly composed of (1, 4)-D-glucopyranose units attached by β-1,4 linkages (Sindhu et al. 2016). The LB contains cellulose molecules which are held together by intermolecular hydrogen bonds in native state, but they have a strong tendency to form intra-molecular and intermolecular hydrogen bonds and this tendency increases the rigidity of cellulose making it crystalline, insoluble and highly resistant to most organic solvents. The major bottleneck in bioconversion of LB into biofuels is the recalcitrant nature of LB (Sharma and Bajaj 2014).

Pretreatment of the LB is imperative for efficient conversion of LB into useful based products. The process of pretreatment is essential for the modification of the plant cells so as to reduce the recalcitrance of the cell wall. An ideal pretreatment process should not only be cost effective, energy efficient and having a high performance rate but should also lead to the yield of high fermentable sugars and least inhibitor formation (Vaid and Bajaj 2017). Pretreatment removes the physical and chemical barriers that make native biomass amorphous and accessible to enzymatic hydrolysis (Sun et al. 2016). Several pretreatment approaches (physical, chemical and biological) have been developed for generating suitable raw material for saccharification.

Pretreatment plays an important role in increasing the permeability of the LB but efficiency of biomass to biofuel conversion can only be valued with complete utilization of reducing sugars. Saccharification of biomass after an appropriate pretreatment determines the viability of process (Khare et al. 2015). Saccharification is an important step but still remain as one of the bottleneck in LB-biofuel conversion strategy. Saccharification can be done by various carbohydrases like cellulases, xylanases and

other hydrolases but cellulases and xylanases are primarily the predominant ones for hydrolysis of biomass (Sartori et al. 2015). Cellulases act on cellulose part of LB which comprises of three predominant activities viz. exo-1,4-glucanase, endo-1,4-glucanase and cellobiase. Xylanases like endo-1-4,-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, as well as acetylxylan esterases are required for the degradation of hemicellulosic component of biomass (Sadhu and Maiti 2013). Both enzymes help in determining the efficiency of saccharification when applied in synergism. Saccharifying enzymes may be used that are available commercially or produced *in-house* from microorganisms like bacteria/fungi. Commercial enzymes like Celluclast (cellulase), Novozyme 188 (xylanse) etc. have been very effective (Khare et al. 2015) but fewer studies showed that *in-house* produced enzyme complex gave better saccharification results (Sartori et al. 2015). Effective saccharification in turn may lead to enhanced LB-biofuel product generation.

Systematic biology has developed strongly during the last decades, partly due to improved molecular techniques and more advent of computer sciences. Systematic biology has enabled understanding of how life has developed and all the flora and fauna are on earth are related to one another in an evolutionary manner (Systematic Biology-Editorial Board 2014). The classification of the flora and fauna is carried out by studying morphological, embryology, physiological, molecular, behavioral, ecological and geographic characters (Hecht and Steere 1970). Plants have been classified and evolved as one as sole contributor to enable the sustenance of all other life forms. Plant based biomaterial have a wide range of application particularly lignocellulosic biomass (LB). Plant LB has emerged as a very novel application for biorefinery products. But plant cell walls have evolved to be recalcitrant to degradation as walls contribute extensively to the strength and structural integrity of the entire plant (Vaid et al. 2017). There is an immense structural diversity within the walls of different plant species and cell types within a single plant as well. Depending on diverse nature of LB used in biorefinery, various steps involved in pretreatment (such as chemical/enzymatic reactions) and subsequent fermentation of different sugar components to viable biofuel production needs to be studied and enhanced in terms of various process parameters and product yield (Foster et al. 2010).

The lignocellulosic biomass can be classified into woody biomass (hardwood and softwoods) and the agricultural crops. Woody biomass is structurally stronger and denser, and has higher lignin content than agricultural biomass. As a result, woody biomass is more recalcitrant to microbial and enzymatic actions than non-woody biomass. On the other side the agricultural crops contain less lignin content which make them easily accessible to the microbial enzymes for the production of biofuels like ethanol (Zhu and Pan 2010). Lignin is a complex heteropolymer of three *p*-hydroxycinnamoyl alcohol monomers i.e. *p*-coumaryl, coniferyl and sinapyl alcohols that are called as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) units, respectively, when incorporated into the polymeric form. Their contribution to lignin composition varies significantly among cell types, taxa and between tissues in the same plant. Among woody biomass, the angiosperms (hardwood) contain less amount of lignin which majorly contain G and S units with a little traces of H units whereas lycophytes, ferns and gymnosperms (softwoods) contain G units with

insignificant amounts of H units in their lignin (Cesarino et al. 2012). Hence, both agricultural crops and the woody biomass present as diverse plant forms that may be efficiently utilized in the modern concept of biorefineries.

Utilization of plant based renewable feed stock as a source for fuels and other commodity chemicals is the need of the hour and the concept of developing alternatives to the volatile petroleum market has been widely embraced by governments and scientific community alike. The potential for exploitation of LB is enormous as it comprises the non-edible portion of the plant and therefore, does not compete with food supplies. Moreover LB is extremely eco-friendly as it is renewable and possesses properties like biocompatibility and biodegradability (Almeida et al. 2012). Even though biofuels produced from LB presents a promising alternative, but the major bottleneck encountered in this approach is the high production costs making the overall process unprofitable. Therefore in order to overcome this challenge biorefining of LB for producing renewable oil as well as valuable co-products is being undertaken (Sun et al. 2016). Biorefineries focus on the economical production of value-added chemicals at high selectivities and yields. On one hand cellulosic ethanol, is considered as a strong substitution of petroleum-based polymers, where as hemicellulose could be converted to xylitole, levelinic acid, xylooligosaccharides among other. Lignin, the other principle component, could be marketed as a chemical (Kim et al. 2016).

Considering the enormous potential of LB, the present chapter deals with the strategies for biorefining and valorization of biomass to value added products. Recent advancements in the fields of pretreatment, metabolic engineering, enzyme production and fermentation would help in developing a suitable technology which could replace the deteriorating effects of fossil fuels.

2.2 Concept of Biorefineries

Lignocellulosic biomass (LB) from agriculture and forestry, which includes agro-industrial residues, forest-industrial residues, energy crops, municipal solid waste, and other materials, has emerged as an excellent feedstock for biorefineries that complement oil refineries as sources of fuels and other value added chemicals (Arevalo-Gallegos et al. 2017). Biorefinery in general terms imply the conversion of biomass into a number of useful products ranging from bulk products (bioenergy) up to specialty chemicals. It is a collection of processes that utilizes renewable grain, lignocellulosic or high moisture content biomass to produce a final useful product or a variety of products, in such a way that no waste is left behind. This concept is analogous to today's petroleum refinery, which produces multiple fuels and chemicals from petroleum (Moncada et al. 2016). A biorefinery is a network of facilities that integrates biomass conversion processes and equipment to produce biofuels, energy and chemicals from biomass. Many authors define the biorefinery as the analogy to current oil refineries, which produce multiple fuels and chemicals from petroleum (Morais and Bogel-Lukasik 2013).

The oil refineries and biorefineries are different from each other in two ways. The first is the raw material (biomass) that is used in biorefineries has not undergone the biodegradation of crude oil over millions of years. Biomass is organic matter derived from living organisms (Moncada et al. 2016). The second is the complexity after application of different existing and emerging technologies in order to obtain bioproducts integrally and simultaneously. In addition to this a biorefinery involves assessing and using a wide range of technologies to separate biomass into its principal constituents (carbohydrates, protein, triglycerides etc.), which can subsequently be transformed into value-added products. Lignocellulose is one of the most abundant bioresource in the world that is considered as a best cheap source of carbohydrates and has been applied as a potential substrate for the production of high value products including biofuels such as bioethanol, biodiesel and biogas. Apart from that it is easily available and highly abundant due to the fact that 75% of its composition is contributed by polysaccharide makes it a purely suitable raw material for the biofuel production (Saini et al. 2015).

2.3 Biofuel as Renewable Energy Source

The shortage of the fossil fuels and the increasing pollution rates has shifted the focus to the bioenergy. Biomass energy is a promising source of renewable energy, but the feedstock used for producing it should come from non-food crops or agricultural waste (second generation feedstock), to avoid competition with food sources and arable land. A number of biomass energy resources are found all over the world that mainly includes wood product industry wastes, municipal solid waste, agriculture residues and the energy crops (Saini et al. 2015). These biomass resources have an excellent potential to be used as the future substrate for biofuel production.

First generation (1G) biofuels are produced primarily from foods crops such as grains, sugar cane and vegetable oils. But the limitation in using the 1G ethanol is that it creates food versus fuel conflict (Mohr and Raman 2013). The second generation (2G) biofuels are generally obtained from non-edible LB, including residues of crops or forestry production (corn cobs, rice husks, sugarcane bagasse, forest thinning, sawdust, etc.), and whole plant biomass (energy crops such as switchgrass, poplar and other grasses). Biofuels obtained from vegetable oils produced from sources that do not directly compete with crops for high quality land (jatropha and microalgae) can also be labeled as second generation biofuels. The emergence of 2G biofuels is widely seen as a sustainable response to the increasing controversy surrounding the 1G biofuels as 2G biofuels possesses excellent quality features, and can be better controlled and maintained in time. LBs such as wood, grass, agricultural and forestry residues such as straw, firewood, sawdust, rice husks, coconut shell, groundnut shell, pine needles, bamboo, sugarcane baggase, cotton and chilli stalks etc. are the potential substrates for bioethanol and biogas production (British petroleum 2013). At present the production of the second generation ethanol is still in infancy and is produced only in few demo plants around the world that are not yet commercially feasible. At the

moment, Borregaard company located in Norway declares to be the largest producer of second generation ethanol with an annual production of 20,000 m^3 (Lennartsson et al. 2014).

It has been observed that the first-generation biofuels have increasingly been adopted, and these are projected to steadily increase in production. However, growing interest is being paid to second generation and third generation biofuels, which are not food competitors, and which might have better environmental performance, particularly in terms of lower green house gas emissions. Microalgae are currently being promoted as an ideal third generation biofuel feedstock because of their rapid growth rate, greenhouse gas fixation ability and high production capacity of lipids (Alam et al. 2015). The major advantage of using them is that they don't compete with food or feed crops, can be grown on non-arable land and saline water and a very short harvesting cycle (Harun et al. 2010).

2.4 Lignocellulosic Biomass for Bioethanol Production

Lignocellulosic biomass refers to crops, crops residues or forestry biomass. These include wood, grass, agricultural and forestry residues such as straw, firewood, sawdust, rice husks, coconut shell, groundnut shell, pine needles, bamboo, sugarcane baggase, cotton and chilli stalks, etc. that are considered as potential substrates for bioethanol production (Vaid and Bajaj 2017). LB mainly consists of 40–50% cellulose, 25–30% hemicellulose, 15–20% lignin, and traces of pectin, nitrogen compounds, and inorganic ingredients (Table 2.1, Mori et al. 2015). Cellulose and hemicelluloses together constitute approximately 70% of the entire biomass and are tightly linked to the lignin component through covalent and hydrogen bonds that make the structure highly recalcitrant to any treatment.

Cellulose represents the main constituent of LB, and is a polysaccharide that consists of a linear chain of D-glucose linked by β-(1, 4)-glycosidic bonds to each other. In a plant cell wall, cellulose exists in crystalline (organized) structure as well as in amorphous structure can be easily digested by enzymes (Kulasinski et al. 2014). Hemicelluloses located in secondary cell walls, are heterogeneous branched biopolymers containing pentoses (β-D-xylose, α-L-arabinose), hexoses (β-D-mannose, β-D-glucose, α-D galactose) and/or uronic acids (α-D-glucuronic, α-D-4-O-methylgalacturonic and α-D-galacturonic acids). Hemicelluloses are linked to cellulose by hydrogen bonds and to lignin by covalent bonds. Lignin is a complex hydrophobic, cross-linked aromatic polymer that is susceptible to microbial attack. It is a polyphenolic aromatic compound synthesized from phenylpropanoid precursors. Generally, the plant cell wall microstructure is regarded to be a matrix of lignin and polysaccharides intimately associating with each other that make the cell wall of the plant cell rigid and stable. Thus in order to make the components of lignocellulose (cellulose and hemicellulose) accessible to the microbial enzymes, a suitable pretreatment strategy is required (Behera et al. 2014; Chaula et al. 2014).

Table 2.1 Lignocellulose composition of different biomass

Lignocellulosic biomass	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Barley straw	31–45	27–38	14–19	Saini et al. (2015)
	33.3	20.4–28	17.1	Tye et al. (2016)
Corn stover	42.6	21.3	8.2	Sarkar et al. (2012)
	44	25.1	9	Danish et al. (2015)
	35–42.6	17–35	7–21	Tye et al. (2016)
Banana peels	18.2	10.5	14.8	Wadhwa and Bakshi (2013)
Rice straw	32–47	18–28	5.5–24	Tye et al. (2016)
	32	8.4	30.3	Danish et al. (2015)
	28–36	23–28	12–14	Saini et al. (2015)
Sorghum straw	32.4	27	7	Tye et al. (2016)
	32	24	13	Saini et al. (2015)
Wheat straw	35–45	20–30	12–15	Sarkar et al. (2012)
	37–42	27–32	13–15	Saini et al. (2015)
	33–45	20–32	8–20	Tye et al. (2016)
	45.1	9.2	37.4	Danish et al. (2015)
Sugarcane bagasse	65	–	18.4	Sarkar et al. (2012)
	32–48	19–24	23–32	Saini et al. (2015)
	43.9	21.5	17	Danish et al. (2015)
	45.4	28.7	23.4	Tye et al. (2016)
Oat straw	37.6	23.3	12.9	Tye et al. (2016)
Rye grass	34	20.6	24.4	Zheng et al. (2008)
Tomato pomace	12	12	39	Wadhwa and Bakshi (2013)
Algae (green)	20–40	20–50	–	Saini et al. (2015)
Grasses	25–40	25–50	10–30	Li et al. (2010)

2.5 Pretreatment of the Lignocellulosic Biomass

Lignocellulosics are abundantly available in nature, relatively distributed worldwide and also act as an alternate source of energy. Bioconversion of LB to liquid and gases is one of the prospective approaches for sustainable biofuels, biochemical and biomaterials as combined in a concept called biorefinery. The traditional microorganisms used to produce the valuable products such as ethanol cannot directly ferment the complex LB. Thus, a suitable pretreatment strategy is necessary and essential to hydrolyze the lignocellulosics into fermentable sugars. Due to highly recalcitrant nature LB needs pretreatment (chemical, physical and biological) prior to enzymatic hydrolysis (Sharma and Bajaj 2014).

Pretreatment removes the physical and chemical barriers that make native biomass recalcitrant and makes cellulose amenable to enzymatic hydrolysis, which is a key step in biochemical processing of lignocellulose based on the sugar platform concept. This effect is achieved by increasing the accessible cellulose surface area through solubilization of hemicelluloses and/or lignin, which are coating the cellulose of the native biomass (Jönsson and Martin 2016). Several factors have been reported to play instrumental role for developing optimal cost and energy-effective pretreatment process (Maurya et al. 2015). The aim of the effective pretreatment of lignocellulosic biomass should be focused on to increase the accessible surface area and decrystallize cellulose, along with partial depolymerization of cellulose and hemicellulose, to solubilize hemicelluloses and lignin. It should also maximize the enzymatic digestibility and minimize the loss of sugars, of the pretreated material, to minimize capital and operating costs and finally must also preserve the pentose (hemicellulose) fractions that limit the formation of toxic components which inhibit growth of fermentative microorganism.

A number of pretreatment techniques are available that increase the accessibility the fermentable sugars to the hydrolyzing enzymes.

2.5.1 Physical Pretreatment

The main purpose of physical pretreatment such as milling, grinding, chipping, freezing, radiation is to increase surface area and reduce particle size of lignocellulosic materials. Moreover, it leads to decrease degree of polymerization and decrystallization of feedstock. Combination of physical and other pretreatment method is usually used.

2.5.2 Chemical Pretreatment

Chemical pretreatment for LB involves different chemicals such as acids, alkalis, and oxidizing agents e.g. peroxide and ozone, dilute acid pretreatment using H_2SO_4/HCl are the most widely used methods. Pretreatment could have different effects on structural components of lignocellulose, based on the type of chemical used. Alkaline pretreatment, ozonolysis, peroxide and wet oxidation pretreatments are more effective in lignin removal whereas dilute acid pretreatment is more efficient in hemicellulose solubilization.

Acid pretreatment is one of the most popular methods to attain high sugar yields from LB (Lee et al. 2015). The main objective of acid pretreatment is to increase the accessibility of the enzymes to the cellulosic fractions by solubilizing the hemicellulosic fraction of the biomass. Alkaline pretreatment as compared to other chemical pretreatments, can be conducted at lower temperature and pressure causing less sugar degradation than acid pretreatment but the reaction times take several hours or days, or even weeks for softwood. Alkaline pretreatment of lignocellulosic materials causes swelling leading to an increase in internal surface area, decrease in the degree of polymerization and crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure making cellulose and hemicellulose available for the enzymatic degradation.

Ionic liquids have also been recognized as one of the potent pretreatment methods for the effective dissolution of cellulose and hemicellulose sugars. Ionic liquids (ILs) are thermally stable organic salts with potential application as 'green solvents' and ILs exhibit excellent physical characteristics including the ability to dissolve polar and non-polar organic, inorganic and polymeric compounds. Use of ILs is emerging as an efficient strategy for pretreating recalcitrant LB. Xu et al. (2015) isolated a novel ionic liquid-tolerant microorganism, *Fusarium oxysporum* BN secreting ionic liquid stable cellulase and reported that *F. oxysporum* BN directly converted IL pretreated rice straw to bioethanol yielding 0.125 g ethanol g^{-1} of rice straw.

2.5.3 Biological Pretreatment

A variety of bacteria and fungi can hydrolyze cellulose and hemicellulose into corresponding mono-sugars like glucose, arabinose, xylose, etc. (Table 2.2). Fungi such as brown, white, and soft-rot fungi are widely used for selective degradation of lignin and hemicellulose among which white-rot fungi seems to be the most effective ones. Biological pretreatments unlike physical and chemical pretreatment methods do not involve high temperature and pressure and does not require acids, alkali or any reactive species. This pretreatment is environmental friendly because of its low energy use and mild environmental conditions.

Table 2.2 Culture conditions and performances of various cellulolytic, hemicellulolytic and/or ligninolytic microorganisms

Microorganism	Activity	pH	Temperature (°C)	Treatment time (days)	Degradation (%)	References
Clostridium thermocellum	C, H	6.1–7.8	60	4–5	85–100	Rabemanolontsoa et al. (2015)
Clostridium cellulolyticum	C, H	7.2	32–34	3–6	20–75	Desvaux et al. (2001) as reviewed by Dionisi et al. (2015)
Ruminococcus albus	C, H	6.7–7.1	37	0.5–2	30–70	Pavlostathis et al. (1988) as reviewed by Dionisi et al. (2015)
Ruminococcus flavefaciens	C, H	6.5–6.8	39	2–7	54–87	Shi and Weimer (1992) as reviewed by Dionisi et al. (2015)
Actinotalea fermentans	C, H	6.5	30–55	28	60	Bagnara et al. (1987)
Trichoderma viride	C, H	5	30	0.5–1.2	50–75	Peitersen (1977) as reviewed by Dionisi et al. (2015)
Trichoderma reesei	C, H	4.8	28	7	100	Velkovska et al. (1997) as reviewed by Dionisi et al. (2015)
Pseudomonas spp.	L	5.3–7.8	30	7–60	20–52	Sørensen (1962) as reviewed by Dionisi et al. (2015)
Xanthomonas spp.	L	–	30	7–30	39–48	Odier et al. (1981) as reviewed by Dionisi et al. (2015)
Acinetobacter spp.	L	–	30	30	47–57	Odier et al. (1981) as reviewed by Dionisi et al. (2015)
Streptomyces cyaneus	C, H, L	–	28–37	21–28	29–52	Berrocal et al. (2000) as reviewed by Dionisi et al. (2015)
Phanerochaete Chrysosporium	L	–	39	14–30	28–60	Shi et al. (2008) as reviewed by Dionisi et al. (2015)
Echinodontium taxodii 2538	L	–	25	28	24	Zhang et al. (2007) as reviewed by Dionisi et al. (2015)
Pleurotus ostreatus	L	–	25–30	30–60	40–41	Kerem et al. (1992) as reviewed by Dionisi et al. (2015)

2.6 Enzymatic Saccharification of Pretreated Lignocellulosic Biomass

Lignocellulose can be hydrolytically broken down into simple sugars either enzymatically by cellulolytic enzymes or chemically by sulfuric or other acids (Zhang et al. 2012). However, enzymatic hydrolysis is becoming a suitable way because it requires less energy and mild environment conditions, while fewer fermentation inhibiting products are generated (Brummer et al. 2014). Enzymatic hydrolysis includes the processing steps that convert the carbohydrate polymers into monomeric sugars. The various potential factors that contribute to the resistance of biomass to enzymatic hydrolysis include cellulose crystallinity, accessible surface area and protection by lignin and cellulose sheathing by hemicelluloses. Enzymatic hydrolysis is carried out with cellulases at mild conditions of pH and temperature, 4.5 and 50 °C, respectively. Some proteins like swollenin play an important role in non-hydrolytically loosening the cellulosic fibril network and do not act on β-1,4 glycosidic bonds in cellulose. Swollenin increases the accessibility of cellulases to cellulose chains by dispersion of cellulose aggregations and thereby exposing individual cellulose chains to the enzyme (Santos et al. 2017). Enzyme related factors which affects hydrolysis includes enzyme concentration, enzyme adsorption, end-product inhibition, thermal inactivation and unproductive binding to lignin.

2.7 Ethanol Fermentation

Ethanol fermentation is a biological process in which sugars are converted by microorganisms to produce ethanol and CO_2. An important factor which prevents industrial utilization of lignocelluloses for bioethanol production is the lack of microorganisms able to efficiently ferment both pentoses and hexoses released during pretreatment and hydrolysis (Kang et al. 2014).

In recent years, the concept of consolidate bioprocessing (CBP) has emerged as an efficient method for the saccharification and fermentation of the sugars to produce ethanol and other organic acids. It involves depolymerization of the lignocellulosic matrix with simultaneous production of enzymes and ethanol in one single step. In CBP, the ethanol and all the required enzymes are produced by a single microorganism strain in a single bioreactor. CBP reduces the ethanol production cost by eliminating the operating costs and capital investment associated with purchasing or producing the enzymes. In CBP, mono or co-cultures of microorganisms can be used that directly ferment cellulose to ethanol. Khuong et al. (2014) optimized the alkaline pretreatment (NaOH) of sugarcane bagasse for consolidated bioprocessing fermentation by the cellulose fermenting fungus *Phlebia* sp. MG-60 and got an ethanol yield of 4.5 from 20 g l^{-1} of sugarcane bagasse. Brethauer and Studer (2014) successfully achieved 67% ethanol yield from pretreated wheat straw (dilute acid) using three naturally occurring strains: *Trichoderma reesei*, *Saccharomyces cerevisiae* and

Scheffersomyces stipites. Okamoto et al. (2014) isolated and characterized a strain of the white rot basidiomycete *Trametes versicolor* that was capable of efficiently fermenting xylose and found that strain, designated KT9427, was capable of assimilating and converting xylose to ethanol under anaerobic conditions with a yield of 0.44 g ethanol per 1 g of sugar consumed. So as a whole CBP serves as the most economically viable and less time consuming process that converts LB directly into ethanol in a single step appropriately.

2.8 Biohydrogen as Biofuel

Biohydrogen is considered as the fuel of future as its combustion generates huge energy and results only in the production of water as an end product making it a clean fuel (Singh et al. 2015a). Hydrogen possesses the gravimetric energy density at 141 MJ Kg^{-1} i.e. the highest in comparison to other biofuels. The second generation of biofuels mainly utilizes lignocellulosic materials for the production of liquid (ethanol, butanol) or gaseous (biohydrogen or biogas) fuels (Cheng et al. 2011; Datar et al. 2007). The third generation feedstock in the form of microalgae has received more attention in the biofuel production. Biofuel production from microalgae has gained positive ground because of their high carbohydrate and lipid content.

2.9 Microalgae for Biohydrogen Production

Algae are considered as the potential feedstocks for the production of third generation biofuels as biomass can be converted directly into energy. Microalgae are the photosynthetic organisms and are known as the primary producers in any ecosystem. Microalgae include dinoflagellates, green algae (chlorophyceae), golden algae (chryosophyceae) and diatoms (bacillariophyceae) (Jambo et al. 2016). They have relatively simple requirements for growth when compared to other sources of LB. Some algae may contain a huge amount of cellulose and hemicellulose content in their cell walls with accumulated starch as the main carbohydrate source (Domozych et al. 2012). In addition to this they also contain least amount of lignin which otherwise limits the accessibility of cellulose and hemicellulose to cellulolytic enzymes (Park et al. 2011). Both starch and most cell wall polysaccharides can be converted into fermentable sugars for subsequent biofuel production via microbial fermentation (Wang et al. 2011). The cultivation of microalgae shall not only reduce the need of arable land but may also channelize the waste water, saline and brackish waters for their growth and could be harvested nearly on daily basis (John et al. 2011).

Biohydrogen can also be directly produced by microalgae by photofermentation process. It is an anaerobic process that uses the hydrogenase enzyme for the oxidation of the ferredoxin. But all the hydrogenases produced by microalgae are not efficient enough and compete with many metabolic processes. So looking into the

insights of the hydrogenase action on the ferredoxin and their engineering may lead to generation of efficient hydrogenases in order to form biohydrogen in large amount (Yacoby et al. 2011). For an ideal production of biohydrogen co-culture of micro and macro algae can employed as some micro algae (*Arthrospiraplatensis*) may have a low C/N ratio that is not feasible for hydrogen production. Xia et al. (2016) co-cultured *Laminaria digitata* (macroalgae) and *A. platensis* (microalgae) pre-treated with 2.5% dilute H_2SO_4 at 135 °C for 15 min, with a total yield of carbohydrate monomers of 0.268 g g^{-1} volatile solids (VS) and an optimal specific hydrogen yield of 85.0 ml g^{-1} VS at an algal C/N ratio of 26.2 and an algal concentration of 20 g VS l^{-1}. Ding et al. (2016a) co-fermented *Laminaria digitata* (macroalgae) and *Chlorella pyrenoidosa* and *Nannochloropsis oceanic* (microalgae) that facilitated hydrolysis and acidogenesis, resulting in hydrogen yields of 94.5–97.0 ml per gVS which was 15.5–18.5% higher than mono-fermentation using *L. digitata*. Although hydrogen production from algae still seems years away from commercial viability, continued progress in this area shows its ultimate potential.

Pretreatment of lignocellulosic biomass is must for biohydrogen production. The selection of an effective and suitable pretreatment method is a prerequisite for the biohydrogen production from lignocellulosic biomass. Biological pretreatment method show some unique advantages such as low energy requirement, least inhibitors production and operability at room temperature. This pretreatment involves the microorganisms like white rot fungi that secretes the lignin degrading enzymes and hence increases the accessibility of cellulose to the hydrolyzing enzymes (Ren et al. 2009). After pretreatment, the lignin and hemicellulose are dissolved in the prehydrolysate. The free hemicellulose is then subjected to further hydrolysis that releases pentoses and hexoses like xylan, xylose, mannose, arabinose, galactose and glucose. The detailed pretreatment methods employed for various LB substrates for the production of biohydrogen are enlisted in Table 2.3.

Pretreatment of LB is followed by enzymatic saccharification of the complex sugars. Lignocellulose can be hydrolytically broken down into simple sugars either enzymatically by cellulolytic enzymes or chemically by sulfuric or other acids (Zhang et al. 2012). However, enzymatic hydrolysis is becoming a suitable way because it requires less energy and mild environment conditions, while fewer fermentation inhibitor products are generated (Brummer et al. 2014). Enzymatic hydrolysis is one of the most common and effective methods employed to generate fermentable sugars due to the high yields of sugars that can be obtained, with subsequent fermentation of these sugars to produce biohydrogen. The effectiveness of hydrolysis in the polysaccharides present in the lignocellulose substrates, therefore, is determined by an appropriate pretreatment, good selection of enzymatic complexes and cellulose accessibility (Meng and Ragauskas 2014). It depends on optimized conditions for maximum efficiency like hydrolysis temperature, time, pH, enzyme loading, and substrate concentration (Milagres et al. 2011).

Various microorganisms may be employed for biohydrogen production. Biological processes are carried out largely at ambient temperatures and pressures, and hence, are less energy intensive than chemical or electrochemical ones. A number of microorganisms have been found to produce hydrogen from the fermentable sug-

Table 2.3 Different pretreatment strategies and lignocellulosic biomass (LB) composition for the hydrogen production

Lignocellulose biomass	Cellulose (wt%)	Hemicellulose (wt%)	Lignin (wt%)	Pretreatment	Hydrogen production index	References
Corn stover	37.6	21.5	19.1	NaOH + Enzymatic	12.9 mmol l h^{-1}	Ren et al. (2010)
	N.R	N.R	19.1	H_2SO_4	3305 ml H_2 l^{-1}	Cao et al. (2009)
	36.5	31.3	11.9	H_2SO_4 + Microwave	182.2 ml	Liu and Cheng (2010)
Corn cob	38.9	42.2	10.9	HCl	107.9 ml H_2 g^{-1} TVS	Pan et al. (2010)
Corn stalk	33.64	24.4	8.65	Bio pretreatment	176 ml H_2 g^{-1} TS	Fan et al. (2008)
	38.92	20.87	21.52	HCl	149.69 ml H_2 g^{-1} TVS	Zhang et al. (2007)
Miscanthus	38.2	24.3	25	NaOH + Enzymatic	82.2 mmol H_2	De Vrije et al. (2001)
Rice straw	41.4	19.6	22.8	NH_4OH + H_2SO_4	2.7 mmol H_2 g^{-1} straw	Nguyen et al. (2010)
	33.1	26.7	35.9	NaOH	0.76 mol H_2 mol^{-1} xylose	Lo et al. (2010)
Soybean straw	39.6	14.6	23.4	HCl	60.2 ml H_2 g^{-1} dry straw	Han et al. (2012)
Sugarcane bagasse	33.63	23.88	4.31	H_2SO_4	1.73 mol H_2 mol^{-1} sugar	Pattra et al. (2008)
Sweet sorghum bagasse	38.50%	21.4	17.6	NaOH	2.6 mol H_2 mol^{-1} C6 sugar	Panagiotopoulos et al. (2010)
Wheat bran	8.27	33.7	N.R	HCl + Microwave	128.2 ml H_2 g^{-1} TVS	Pan et al. (2010)
Wheat straw	22.5	21.5	N.R	HCl	68.1 ml H_2 g^{-1} TVS	Fan et al. (2006)
	N.R	N.R	N.R	H_2SO_4	168.4 ml H_2 g^{-1} VS	Nasirian et al. (2011)

ars. Biological processes use the enzyme hydrogenase or nitrogenase as hydrogen producing protein. This enzyme regulates the hydrogen metabolism of uncountable prokaryotes and some eukaryotic organisms including green algae. The function of nitrogenase as well as hydrogenase is linked with the utilization of the products of photosynthetic reactions that generate reductants from water. Recent research studies on dark fermentative organisms have been intensively developed and new bacterial species have been isolated for this purpose. For effective production of biohydrogen, it is necessary to identify suitable fermentative microorganisms that can ferment pentose and hexose sugars from lignocellulosic biomass. The pentoses mainly consist of xylose as the main fermenting sugar.

A number of studies of hydrogen production from xylose have been reported using functional microorganisms and mixed cultures. Abdeshahian et al. (2014) successfully produced the fermentative hydrogen by *Clostridium* sp. YM1 with the cumulative hydrogen volume of 1294 ml l^{-1} with a hydrogen yield of 0.82 mol H_2 mol^{-1} xylose consumed. As the lignocellulosic biomass is a mixture of pentose and hexoses, studies have reported the use of both C_5 (pentose) and C_6 (hexose) fermenting microorganisms simultaneously. Ren et al. (2008) reported a hydrogen yield of up to 2.37 mol H_2 mol^{-1} substrate from a thermophilic strain of *T. thermosaccharolyticum* W16 that simultaneously fermented the mixture of glucose and xylose.

Anaerobic bacteria like those of *Clostridium* sp. have been found to ferment sugars due to its high production rate and the ability to use a wide range of carbohydrates including wastewater. *Clostridium* sp. is a typical acid and hydrogen producer which ferments carbohydrate to acetate, butyrate, hydrogen, carbon dioxide and organic solvent. Chong et al. (2009) isolated *C. butyricum* from palm oil mill effluent sludge with optimum hydrogen production at pH 5.5 with POME as substrate and a potential hydrogen yield of 3.2 l H_2 l^{-1} palm oil mill effluent. Some studies have also been reported where the crystalline cellulose was directly converted into hydrogen. Wang et al. (2008) reported that *Clostridium acetobutylicum* X9 generated the maximum hydrogen production and cellulose hydrolysis rate of 6.4 mmol H_2 h^{-1} g^{-1} dry cell and 68.3%, respectively, using microcrystalline cellulose as the substrate.

Dark fermentation may be employed for production of biohydrogen. Biohydrogen can be produced through thermochemical and biological technologies but biological techniques are preferred over thermochemical processes because of their ecological benefits and lower energy requirements (Bundhoo and Mohee 2016). Biohydrogen can be produced via biological processes from technologies such as dark fermentation (DF), photo fermentation, direct and indirect biophotolysis and water-gas shift reactions. However, the DF process is considered to be an efficient method that can have commercial value and importance in the future (Hallenbeck et al. 2012). DF is a process of degradation of organic substrates by anaerobic bacteria in absence of light and oxygen to produce biohydrogen. A series of biochemical reactions are involved in the breakdown and conversion of complex sugars into biohydrogen (Karthic and Shiny 2012). The complex polysaccharides are initially hydrolyzed into simple sugars by biological methods or different pretreatments. The simple sugars then undergo a chain of biochemical reactions in pathways like glycolysis, pyruvate formate lysate pathway, pyruvate ferridoxinoxidoreductase pathway, etc. In addition metal ions also

play an important role in the dark fermentation process as they assist in bacterial metabolism, cell growth, enzyme and co-enzyme activation and functioning and biohydrogen production (Sinha and Pandey 2011). Trchounian et al. (2017) pointed out the importance of Ni^{2+}, Fe^{2+}, Fe^{3+} and Mo^{6+} and some of their combinations for *E. coli* bacterial growth and H_2 production and their experiments found a 2.7 fold increase of hydrogen production by using different combinations of these metal ions.

2.10 Biobutanol as Biofuel

Amongst the production of biofuels acetone–butanol–ethanol (ABE) fermentation ranks the second largest bioprocess. The major product of ABE fermentation is biobutanol. Biobutanol is used as a solvent in the formation of various valued products like hormones, drugs, antibiotics, cosmetics, hydraulic fluids, vitamins etc. (Ding et al. 2016b). Recently, biobutanol is gaining interest as a direct replacement of gasoline. Besides being renewable it has similar properties to gasoline (Gottumukkala et al. 2013). It is an important alternative to bioethanol due to its superior chemical and physical features like lower viscosity, higher energy density, lower affinity to water, better blending capacities, lower hygroscopicity and less corrosive for certain motor parts (Liu et al. 2015a). It can be produced by two ways viz. petrochemically and through fermentation (Gottumukkala et al. 2013; Su et al. 2015).

Lignocellulosic biomass may be exploited for biobutanol production. Biobutanol production through fermentation of sugars using fermenting organism like *Clostridium* species is an industrially active process (Ding et al. 2016b). *Clostridia* strains produce acetone, butanol and ethanol (ABE) at a mole ratio of 3:6:1 during the biobutanol fermentation process (Plaza et al. 2017). However, high substrate cost, low product yield, and high recovery cost hinders its large-scale productivity. Recently, there have been resurging interests in producing biobutanol using inexpensive substrates like low-cost lignocellulosic biomass, developing microbial hyper producing strains, and optimized fermentation conditions (Ding et al. 2016b; Xue et al. 2016). But the process still suffers from low titer and productivity due to recalcitrant LB. The pretreatment of LB increases the feasibility of the biobutanol fermentation process (Xue et al. 2016). Various pretreatment methods have been executed for the biobutanol production from various lignocellulosic feedstocks like sugarcane bagasse, cassava, rice straw etc. as shown in Table 2.4.

A fermentation-pervaporation (PV) coupled process was investigated for the production of ABE from cassava. Glucose consumption rate and ABE productivity increased by 15 and 21%, respectively, in batch fermentation–PV coupled process as compared to batch fermentation without PV. In a continuous fermentation–PV coupled process, the substrate consumption rate, solvent productivity and yield increased by 58, 81 and 15% after 304 h respectively. Thus, the fermentation–PV coupled process helps in decreasing the cost in ABE production (Li et al. 2014). Batch fermentation of sugar hydrolysate (41 g l^{-1} total sugars) obtained after microwave-alkali pretreatment of sugarcane bagasse in assistance with gamma-valerolactone yielded

Table 2.4 Lignocellulosic biomass for biobutanol production

Lignocellulosic biomass	Pretreatment method	Biobutanol production	References
Cornstalk	Alkali-catalyzed organosolv	9.9 g l^{-1}	Tang et al. (2017)
Corn stover	Ionic liquid, 1-butyl-3-methylimidazolium chloride [Bmim][Cl]	7.4 g l^{-1}	Ding et al. (2016b)
Rice straw	Sulphuric acid	3.43 g l^{-1}	Gottumukkala et al. (2013)
Apple pomace	Autohydrolysis, acids, alkalis, organic solvents and surfactants	9.11 g l^{-1}	Hijosa-Valsero et al. (2017)
Sugarcane bagasse	Gamma-valerolactone	9.3 g l^{-1}	Kong et al. (2016)
Birch kraft black liquor	Acid-hydrolysis followed by CO_2 acidification	7.3 g l^{-1}	Kudahettige-Nilsson et al. (2015)
Cassava	Fermentation–pervaporation (PV) coupled process	122.4 g l^{-1}	Li et al. (2014)
Eastern redcedar	Sulfuric acid, sodium bisulfate	13 g l^{-1}	Liu et al. (2015a)
Switchgrass	Hydrothermolysis	11 g l^{-1}	Liu et al. (2015b)
Brewer's spent grain	Sulfuric acid	75 g kg^{-1}	Plaza et al. (2017)
Rice straw	Sodium hydroxide	0.16 g g^{-1}	Rahnama et al. (2014)
Sugarcane bagasse	Liquid hot water, microwave	6.4 g l^{-1}	Su et al. (2015)
Corn stover	Sodium hydroxide	11.2 g l^{-1}	Xue et al. (2016)
Jerusalem artichoke stalk	Sodium hydroxide or/and hydrogen peroxide	11.8 g l^{-1}	Xue et al. (2017)

a high acetone-butanol-ethanol concentration of 14.26 g l^{-1}, including 4.1 g l^{-1} acetone, 9.3 g l^{-1} butanol and 0.86 g l^{-1} ethanol (Kong et al. 2016).

Acetone does not qualify as biofuel. ABE fermentation with known *Clostridium* species produces acetone in addition to butanol and ethanol which results in net low yield of biofuel solvents. *Clostridium sporogenes*, a non neurotoxigenic counterpart of group 1 *C. botulinum* produces ethanol and butanol without producing acetone in the final mixture, which is advantageous in converting biomass to alcoholic biofuels (Gottumukkala et al. 2013). Gottumukkala et al. (2013) evaluated biobutanol yield of 3.43 g l^{-1} and a total solvent yield of 5.32 g l^{-1} using *Clostridium sporogenes* BE01 from enzymatic hydrolysate of acid pretreated rice straw. Brewer's spent grain (BSG) is a promising lignocellulosic industrial waste, available throughout the year in large amounts at very low cost, for ABE fermentation. Plaza et al. (2017) investigated sulfuric acid pretreatment of BSG at pH 1, 121 °C and different solid loadings (5–15% w/w) followed by enzymatic hydrolysis and ABE fermentation by *Clostrid-*

ium beijerinckii DSM 6422 of non-washed and washed pretreated BSG. A higher titre of biobutanol (75 g biobutanol kg^{-1} BSG) and ABE (95 g ABE kg^{-1} BSG) was obtained when 15% w/w pretreated unwashed BSG was utilized. Fermentation of washed pretreated BSG yielded 6.0 ± 0.5 g l^{-1} of butanol which was lower than that obtained in case of control (7.5 ± 0.6 g l^{-1} butanol, Plaza et al. 2017).

In a study conducted by Rahnama et al. (2014) rice straw was used for its bioconversion to biofuels such as biobutanol. Sodium hydroxide (2%, w/v) pretreatment of rice straw was executed and resulted in 29.87 g l^{-1} reducing sugar after saccharification using *T. harzianum* SNRS3. The sugar hydrolysate was fermented using *Clostridium acetobutylicum* ATCC 824 and yielded 0.27 g ABE yield g^{-1} reducing sugar and 0.16 g biobutanol g^{-1} reducing sugar (Rahnama et al. 2014). A sequential, combinatorial lignocellulose pretreatment procedure for microbial biofuel (ABE) fermentation from sugarcane bagasse was designed to increase sugar yields and reduced generation of microbial growth inhibitors (Su et al. 2015). A series of methods including microwave decomposition, enzyme hydrolysis, ammonia immersion, microbial decomposition, and liquid hot water pretreatment were performed so as to obtain high sugar yields and limited inhibitor production. To assess the effectiveness of sequential, combinatorial lignocellulose pretreatment procedure for biobutanol production through microbial *Clostridium beijerinckii* NCIMB 8052 conversion, two schemes viz. simultaneous saccharification fermentation and separate hydrolysis fermentation were used of which simultaneous saccharification fermentation revealed the highest concentrations of butanol (6.4 g l^{-1}) and total ABE (11.9 g l^{-1}) as compared to that with the separate hydrolysis fermentation method (Su et al. 2015).

The feasibility of producing biobutanol from any LB feedstock also depends on other factors like mild pretreatment approach, detoxification of sugar hydrolysate etc. The detoxification process is an important step need to be carried after pretreatment because most of conventional pretreatment approaches like acid hydrolysis often produce inhibitors like furfural and 5-hydroxymethylfurfural that greatly affects the efficacy of the whole biobutanol fermentation process (Su et al. 2015; Kudahettige-Nilsson et al. 2015).

Kudahettige-Nilsson et al. (2015) studied ABE fermentation of acid-hydrolyzed xylan recovered from precipitate of hardwood kraft black liquor obtained by CO_2 acidification. Activated carbon was used for the detoxification of hydrolysate in order to evaluate the impact of inhibitor removal and fermentation. Mini scale fermentation of semi-defined P2 media and batch fermentation of the hydrolysate using *Clostridium acetobutylicum* ATCC 824 resulted in a total solvent yield of 0.34 and 0.12–0.13 g g^{-1}, respectively, of which 7.3 and 1.8–2.1 g l^{-1} of butanol concentration was obtained. Kudahettige-Nilsson et al. (2015) for the first time studied the process for the production of a biologically-derived butanol-biofuel from xylan recovered directly from industrial kraft pulping liquors as a feedstock and also demonstrates the feasibility of the process. Liu et al. (2015b) evaluated hydrothermolysis pretreatment based butanol production from switchgrass. Non-detoxified hydrolysate showed poor butanol production (1 g l^{-1}) due to the presence of inhibitors. After detoxification with activated carbon the butanol titer increased up to 11 g l^{-1} with a total (ABE) concentration of 17 g l^{-1}.

2.11 Strategical Improvements for Biobutanol Production

Adoption of IL based pretreatment of LB for biobutanol production has received an immense interest during recent years. ILs are the greener solvents which are able to reduce cellulose crystallinity, hemicelluloses and lignin content of biomass (Vaid and Bajaj 2017). ILs increases the surface area of biomass which in turn increasing the enzymatic hydrolysis kinetics, and the yield of fermentable sugars. In addition, low melting points, wide liquid temperature range, high thermal and chemical stability, non-flammability, negligible vapor pressure, consisting of ions (cations and anions) and good solvating properties makes ILs an eminent pretreatment solvent. Ding et al. (2016b) reported fresh and recycled IL [Bmim][Cl] based pretreatment of corn stover hydrolysate for biobutanol fermentation using *Clostridium saccharobutylicum* DSM 13864. A 18.7 and 24.2 g l^{-1} of sugar hydrolysate was produced from pretreated corn stover using ten times recycled and fresh [Bmim][Cl]. Fermentation of corn stover-sugar hydrolysate obtained by fresh [Bmim][Cl] resulted in 7.4 g l^{-1} biobutanol, while 7.9 g l^{-1} biobutanol was achieved in fermentation using hydrolysate pretreated by ten times recycled IL with similar levels of acetone and ethanol (Ding et al. 2016b).

Type of buffer plays an important role in biobutanol production. Eastern redcedar, an invasive softwood feedstock was targeted for butanol production using *Clostridium acetobutylicum* ATCC 824 and *Clostridium beijerinckii* NCIMB 8052. In the acetate buffer medium, both the strains grew well and yielded 3–4 g l^{-1} biobutanol from redcedar hydrolysate as compared to that in citrate buffer medium. After detoxification of inhibitors by activated carbon from redcedar hydrolysate, butanol and total ABE concentration reached up to 13 and 19 g l^{-1} (Liu et al. 2015a). The strength of buffer also has a significant impact on lignocellulosic butanol fermentation (Xue et al. 2016). The effect of various strengths (20–100 mM) of citrate buffer on enzymatic hydrolysis and corn stover feedstock based ABE fermentation was investigated (Xue et al. 2016). The enzymatic hydrolysis is not affected by varied strength of citrate buffer but greatly influenced the production of ABE fermentation using corn stover hydrolysate. With 30 mM citrate buffer the maximum butanol and ABE concentrations of 11.2 and 19.8 g l^{-1} respectively, which was concentrated to 100.4 g l^{-1} butanol and 153.5 g l^{-1} ABE by vapor stripping–vapor permeation process (Xue et al. 2016).

Optimization is an important component for the efficiency of any bioprocess. Optimizing various process variables may help in reducing the cost of whole bioprocess and increasing the product yield (Vaid et al. 2017). Hijosa-Valsero et al. (2017) studied the production of biobutanol from apple pomace after pretreating it using five different soft physicochemical pretreatments viz. autohydrolysis, acids, alkalis, organic solvents and surfactants followed by saccharification. These pretreatments were compared and optimized in a high-pressure reactor using working parameters like temperature, time and reagent concentration. The surfactant polyethylene glycol 6000 (1.96% w/w) based pretreatment of apple pomace released 42 g l^{-1} sugars at relatively mild conditions (100 °C, 5 min) with less production of inhibitors and without detoxification yielded 3.55 g l^{-1} acetone, 9.11 g l^{-1} butanol, 0.26 g l^{-1} ethanol

after fermentation using *Clostridium beijerinckii* CECT 508 in 96 h (Hijosa-Valsero et al. 2017).

Optimized pretreatment protocol was developed for efficient biobutanol production from cornstalks (Tang et al. 2017). Alkali-catalysed organosolv pretreatment of biomass was performed. After optimization of process parameters, about 80% of the total lignin was removed at 110 °C, 4% (w/w dry cornstalk) NaOH, 90 min reaction time, and 60% v/v ethanol, with minimal hemicellulose degradation. A total of 83.7% monosaccharide was obtained after enzymatic hydrolysis which released an ABE concentration of 11.9 g l^{-1} after fermentation with 9.9 g l^{-1} concentration of biobutanol (Tang et al. 2017).

2.12 Lignocellulosic Biomass as Source of Prebiotics

Prebiotics are non-digestible carbohydrates which are capable of mediating alteration in the gut microflora by selectively stimulating the growth and/or activity of certain health benefiting bacteria. Prebiotic supplementation basically involves using carbohydrates of varying chain lengths from diverse sources including polysaccharides from plant cell wall material that resist getting digested in the upper gastrointestinal tract and on reaching the colon are metabolized to form short chain fatty acids comprising mainly of propionic acid, acetic acid, butyric acid. Short chain fatty acids apart from creating a hostile environment for the survival of gut pathogens and can also act as a source of energy for the host (Geigerová et al. 2017).

Annually tons of LB is generated in the form of plant waste from various post-harvest processing activities (Jeske et al. 2017). Thus high yield and rich carbohydrate contents make lignocellulosic biomass an attractive option for production of bioactives, having nutritional and functional value, which could be incorporated into foods. The hemicellulose component consist mainly of xylan, which is a polysaccharide made up of backbone of xylose linked by β-1,4-xylosidic linkages (Biely et al. 2016). Xylan is one of the major structural components of woody tissues of dicots, monocots, some grasses and tissues of cereal grains and could also act as an inexpensive source for retrieving xylan based-xylooligosaccharide (XOS) prebiotics that selectively stimulates the growth of beneficial bacteria (Lin et al. 2016).

Currently non-digestible oligosaccharides falling in the range of di-, oligo-, and poly-oligosaccharides are known to possess prebiotic properties. Plant material like corncobs, straws, bagasse, rice hulls, malt cakes and bran, which are abundant, inexpensive, and renewable biomass, being naturally rich in resistant sugars and polysaccharides like xylan and therefore, can also act as an inexpensive source for retrieving fructan and xylan based prebiotics by applying simple hydrolysis techniques (Moniz et al. 2016). Therefore an extensive research directed at conversion of agro-residues into functional food ingredients from underutilized agro-wastes becomes pertinent and is the need of the hour (Samanta et al. 2015).

Application of probiotics is one of the most promising approaches for averting dysbiosis, and restoring normal gut microbiota. These microorganisms are live micro-

bial feed supplements that improve the intestinal microbial population of the host. The benefits of probiotics include their capacity in controlling intestinal infection, reducing elevated serum cholesterol levels, beneficially influencing the immune system, improving lactose utilization, and having anticarcinogenic activity (Bajaj et al. 2015). The survival of probiotics is suppressed from the environmental stress, such as oxygen, acids and environment of digestive system. Constant efforts are being made to increase the number and/or the activity of beneficial probiotic bacteria in the gut. Therefore, to overcome this challenge prebiotic approach is being employed, which essentially involves the administration of non-viable entity (Noori et al. 2017).

Inulin, fructooligosaccharides, galactooligosaccharides, lactulose and polydextrose are established prebiotics where as isomaltooligosaccharides, xylooligosaccharides and lactitol are emerging ones. Inulin and fructooligosaccharides, are the most dominating prebiotics due to low-calorie, fat-replacement ability, overall texture, mouth-feel and flavor. The most known prebiotics, with the exception of inulin, which is a mixture of fructooligo and polysaccharides, are indigestible oligosaccharides having of 3–10 carbohydrate monomers (Flores et al. 2016). The health benefits associated with the administration of prebiotics are mainly due to an increase in the production of short chain fatty acids. They act as a source of energy and a signaling molecule on the G-protein coupled receptor. Short chain fatty acids play important role in regulating glucose metabolism and energy homeostasis. Metabolism of acetate in human occurs mainly in brain, kidney muscle and heart whereas. Butyrate exerts prodifferentiation, anti-proliferation and anti-angiogenic effects on colonocytes. On the other hand propionate suppresses cholesterol synthesis by acting as a possible gluconeogenic precursor (Valdés-Varela et al. 2017).

2.12.1 Xylooligosaccharides as Prebiotics

Xylooligosaccharides (XOS) are oligomers of two to ten xylose molecules linked by β-1–4 bonds and have substitution of acetyl, phenolic, and uronic acid. They are naturally present in fruits, vegetables, bamboo, honey, milk, onions, garlic, artichoke, chicory etc. (Singh et al. 2015b). XOS are commercially available in the form of a white powder with degree of polymerization (DP) ≤ 20. For food industry applications, XOS chains of 2–4 units are considered. They have been shown to possess pH stability over a wide range (2–8) and withstand low gastric pH. Moreover, XOS show heat resistance and remain stable sterilization. Furthermore, they have low calorie content and are able to achieve appreciable biological effects at a low dietary dose (Singh et al. 2015b).

XOS prebiotics are capable of stimulating the growth of intestinal beneficial bacteria Bifidobacteria. The health benefits associated with XOS are mainly due to their effects on the gastrointestinal flora (Belorkar and Gupta 2016). Results obtained from in vitro as well as in vivo assays have proved that *Bifidobacterium* spp. (*B. adolescentis*, *B. infantis*, *B. longum*, *B. bifidum* etc.) and most *Lactobacillus* spp. are capable of utilizing XOS. Bacteroides can also utilize XOS but to a lesser

extent. Whereas common gut enteropathogens like *Staphylococcus*, *Escherichia coli* and *Clostridium* spp. cannot do the same (Nieto-Dominguez et al. 2017).

The prebiotic potential of XOS is being extensively studied. Nieto-Dominguez et al. (2017) demonstrated the prebiotic potential of XOS mixture produced from birchwood xylan. There was short chain fatty acid production, an increase bifidobacteria population, and beneficial commensals and a decrease in potentially pathogenic bacteria, confirming the prebiotic nature. In another study, XOS produced from sugarcane bagasse supported the growth of bifidobacterial strains, with simultaneous production of short chain fatty acids, under anaerobic conditions (Reddy and Krishnan 2016). Similarly, prebiotic XOS from corn straw resulted in an increased bifidobacteria populations and high short chain fatty acids production (Moniz et al. 2016).

Of all the known oligomeric prebiotics, XOS have garnered much interest in the recent years due to its numerous positive effects viz. increased mineral absorption, immune stimulation, promoting pro-carcinogenic enzymes and have antiallergy, antiinfection, antiinflammatory and antioxidant properties. XOS also stimulates increased levels of bifidobacteria to a greater extent than does fructooligosacchardies and other oligosaccharides are required at lower doses than fructooligosacchardies (De Figueiredo et al. 2017)

2.13 Delignification of Biomass for XOS Production

Lignocellulosic agro-residues, from which XOS are produced by various chemical, biological, or by combination of various processes have xylan as xylan-lignin complex in the biomass and is therefore, resistant to hydrolysis. Therefore, XOS production is carried out in a sequential manner starting from removal of lignin, followed by extraction of xylan, and finally followed by enzymatic hydrolysis for the production of XOS (Rabemanolontsoa and Saka 2016) (Fig. 2.1). Lignin is closely attached to the polysaccharide component through non-covalent and covalent linkages structural linkages thus obstructing the detachment of xylan from biomass and drastically reduces the yield of xylan. The presence of lignin largely affects the efficiency of various xylan extraction techniques by hindering the contact of xylan in raw materials with various xylan solubilizing chemicals making the overall process non-productive. Therefore, delignification of the biomass becomes pertinent lignin removal increases the pore size and makes the surface of the lignocellulosic materials more accessible (Samanta et al. 2015). Furthermore delignification reduces the recalcitrance of the biomass thus making it more amiable to subsequent processing.

Various pretreatment techniques have been employed for delignification. Reddy and Krishnan (2016) employed aqueous ammonia for delignification of sugarcane bagasse and achieved a higher production of XOS from the pretreated biomass. Cassava peel and waste were successfully delignified after pretreatment with 0.5% (w/v) sodium hypochlorite solution for 5 h (Ratnadewi et al. 2016). In another study, various pretreatment strategies such as Fenton, sonocatalytic, and sonocatalytic–synergistic

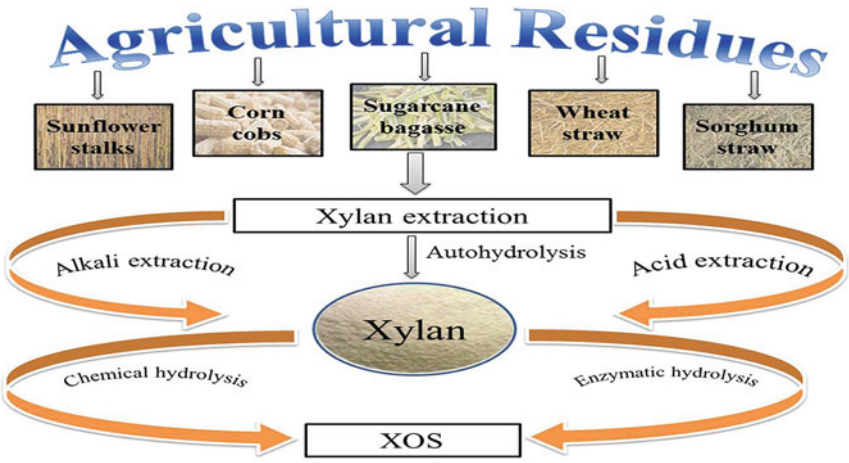

Fig. 2.1 Processes involved in the production of xylooligosaccharides in agro-waste

Fenton were employed to expose lignin content in corncob and enhance the enzymatic XOS production (Kaweeai et al. 2016). A two-stage delignification adopted using calcium hydroxide and peracetic acid was successfully used for kenaf wherein there was appreciable delignification and a high amount of hemicellulose was maintained in the pretreated biomass (Azelee et al. 2014). Bian et al. (2013) employed acidic sodium chlorite solution for delignification of sugarcane bagasse. Sodium hypochlorite pretreatment effectively removes lignin from corncob (Chapla et al. 2012). *Miscanthus* biomass was delignified using a combination of sodium chlorite and acetic acid (Li et al. 2016). Therefore, delignification of biomass is necessary in order to expose the complex structure of lignocellulosic biomass and for making the subsequent treatment steps more effective.

2.14 Xylan Extraction from Lignocellulosic Biomass

The surrounding lignocellulosic components as well as substituents on the xylan backbone restrict the access to xylosic linkages. Moreover, ether bonds are instrumental in linking the hemicellulose with the lignin components via single bonds to an oxygen atom in the biomass. Therefore, for manufacturing XOS from a suitable xylan-rich biomass, the ether bonds of the xylan backbone are targeted, using different approachs, to produce compounds of lower polymerization degree (Rabemanolontsoa and Saka 2016). Xylooligosaccharide production from various feedstocks is a systematic process involving: delignification of native, xylan-containing LB, solubilization and extractiot of xylan from delignified biomass and hydrolysis

of xylan to XOs by steam, dilute solutions of mineral acids, enzymes, etc. (Sun et al. 2016). Therefore, the raw lignocellulosic materials are pretreated with alkali, acids, high temperature autohydrolysis, among others, for the extraction of xylan; the extracted xylan is than subjected to hydrolysis for XOS production.

2.14.1 Alkaline Extraction

Alkaline pretreatment involves dissolution of hemicellulose and saponification of ester and ether bonds as major chemical reactions during extraction process. Alkaline method is an effective method for xylan extraction as it separates structural linkages between hemicellulose and cellulose, avoiding fragmentation of the hemicellulose polymer. The main targets of alkali treatment are ester and ether linkages in hemicelluloses, thus cleaving them down, promotes solubilization of hemicelluloses (Rajagopalan et al. 2017). Furthermore, it causes swelling of LB, which increase its internal surface area decreases the degree of polymerization and crystallinity, thus making hemicellulose more accessible. It also removes acetyl and various uronic acid substitutions on hemicellulose that further, increase the accessibility of hemicelluloses (Kim et al. 2016). Most commonly used alkaline reagents for xylan extraction are NaOH, $Ca(OH)_2$, KOH and Na_2CO_3. Alkaline hydrolysis is carried out at lower temperature and pressure resulting in less sugar degradation. Moreover, as alkali extraction is carried out under ambient conditions, it eliminates the need of specially designed reactors in order to cope up with the severity of the reaction. Recovery of reagents is also possible in some of the alkaline pretreatment methods (Sun et al. 2016).

Rajagopalan et al. (2017) solubilize xylan from pretreated mahogany and mango wood sawdust with NaOH solution (15% w/v) for 24 h. Similarly, Li et al. (2016) extracted xylan from *Miscanthus* biomass with 10% v/v KOH at 25 °C for 16 h. Cassava peel and waste (Ratnadewi et al. 2016) and sugarcane bagasse (Bian et al. 2013) gave a xylan yield of 4.83 and 6.23 and 30% w/v respectively, by carrying out xylan extraction with 10% w/v NaOH for 24 h. Yadav and Hicks (2015) employed an alkaline sodium hydroxide-hydrogen peroxide extraction to obtain arabinoxylans from barley hulls and straws by followed by ethanol precipitation. A yield of 20.51% and 7.41–12.94% was obtained from barley hulls and barley straws, respectively. A xylan yield of 85% w/v was obtained using 12% NaOH in combination with steam application from sugarcane bagasse (Jayapal et al. 2013). Similarly alkaline treatment coupled with steam treatment has been used in other studies (Li et al. 2013; Samanta et al. 2012). Chapla et al. (2012) obtained a xylan yield of 30% w/v from wheat straw and rice straw using dilute alkali treatment (1.25 M NaOH) for 3 h. Similar to the present studies, garlic straw (Kallel et al. 2015) and corncob (Driss et al. 2014; Haddar et al. 2012) were subjected to mild alkali treatment and a high amount of xylan could be extracted from the biomass. The targeted nature of alkaline extraction and high yield of xylan that can be achieved makes it a method of choice for xylan extraction.

2.14.2 Acid Extraction

In acid extraction the susceptibility of the glucosidic bonds of hemicelluloses is exploited to solubilize hemicelluloses from lignocellulosic materials (Sun et al. 2016). Concentrated as well as diluted acids are used to extract xylan from various lignocellulosic materials. Various mineral acids, such as H_2SO_4, HCl, H_3PO_4 and HNO_3 have been commonly used in the process. Concentrated acid pretreatment is less attractive due to the severe degradation of hemicellulose, formation of inhibitors, high toxicity and corrosiveness of the process (Singh et al. 2015c). On the other hand, dilute acid pretreatment promotes hydrolysis of hemicelluloses, resulting in high recovery of hemicelluloses in the liquid fraction and high cellulose content in the solid fraction. However, higher temperature (200 °C) and strong reaction conditions are required to increase the yield of xylan from biomass, thus causing degradation of the amorphous hemicelluloses (Singh et al. 2015c).

Gowdhaman and Ponnusami (2015) subjected corncobs to 0.1% w/v H_2SO_4 treatment at a temperature of 121 °C for 1 h. A xylan yield of 14.7% w/v was achieved with dilute acid treatment. Similar pretreatment with dilute acid (0.1% w/v H_2SO_4) followed by autoclaving for 1 h gave 26.57 g of xylan (Chapla et al. 2012) and 39.2% of xylan (Aachary and Prapulla 2009) from raw dried corncobs. Ruiz et al. (2013) carried out xylan extraction from sunflower stalks by dilute sulfuric acid (1.25% w/v) treatment at 175 °C and 5 min. Liquid fractions showed up to 33 g xylan per 100 g raw material. In another study, Otieno and Ahring (2012) treated lignocellulosic biomasses with 0.1% H_2SO_4 at 145 °C for 1 h. Xylan comprised of more than 20 g dry matter per 100 g of the biomasses. The excessive degradation of biomass associated with using acid makes it a less preferred method for xylan extraction.

2.14.3 Autohydrolysis

Autohydrolysis or hydothermolysis is a non-chemical process of xylan extraction. In this process, xylan is deacetylated in an aqueous medium. Autohydrolysis occurs in presence of hydronium ions [H^+] generated from water and acetic groups released from hemicelluloses. H^+ ions produced by water ionization act as catalysts in higher concentrations and high temperatures thus, providing an effective medium for extraction (Surek and Buyukkileci 2017). The physical disruption of the lignocellulose structure also takes place, since high pressure is involved. This results in decreased crystallinity as well as the degree of polymerization. The hydrolysis liquor obtained thereafter is rich in hemicelluloses or hemicelluloses derived sugars and can therefore be further converted into high value products. Hydothermolysis has become a popular technique for xylan extraction as there is no catalyst required and low inhibitor formation. Moreover low reactor cost is involved thus making the overall process economical (Rabemanolontsoa and Saka 2016). The major drawback of autohydrolysis is the occurrence of several undesirable side-processes resulting in

the accumulation of unwanted compounds like monosaccharides, furfural, and others, thereby making purification necessary and thus increasing the overall cost of the process.

Surek and Buyukkileci (2017) carried out autohydrolysis of hazelnut (*Corylus avellana* L.) shell for obtaining high amount of xylan. Moniz et al. (2016) performed autohydrolysis of corn straw at a temperature of 215 °C and the autohydrolysis liquor obtained afterwards was rich in xylan. *Miscanthus* × *giganteus* hybrids were subjected to autohydrolysis and the hydrolysate obtained after treatment was rich in xylan content (Chen et al. 2016). Hydrothermal pretreatment given to sweet sorghum stems at a high temperature of 170 °C for 0.5 h resulted in high yield of hemicellulose with a relatively low level of xylose and other degraded products (Sun et al. 2015). In another study, water soluble fibers separated from ground corn flour and distillers dried grains with soluble were subjected to autohydrolysis at 180 °C temperature and 20 min hold time resulted in high xylan yield (Samala et al. 2015). Wheat bran samples were subjected to coupled aqueous extraction followed hydrothermal treatment to evaluate their potential as a raw material for obtaining of xylan-derived prebiotics. Hemicellulose rich liquid was obtained after second stage of treatment (Gullon et al. 2014). Rice straw subjected to autohydrolysis at 210 °C yielding a maximum of 40.1 g per 100 g of initial xylan (Moniz et al. 2014). A high yield of hemicellulose was obtained from bamboo culm when it was autohydrolyzed at 180 °C for 30 min (Xiao et al. 2013). Therefore autohydrolysis is a promising technique for conversion of agricultural by-products into useful, high value products, such as prebiotic oligosaccharides.

2.15 Enzymatic Production of Xylooligosaccharides

The current trend of sustainable development has encouraged efforts towards development of environment-friendly techniques for utilization and conversion of xylan component of plants into XOS. Therefore, enzymatic production of XOS, due to its highly specific nature and comparatively lesser amounts of impurities in the products in comparison to thermo-chemical processes, is the method of choice for industries engaged in food and pharmaceutical production (Sun et al. 2016). Enzymatic hydrolysis is preferred over acid hydrolysis for XOS production as acid hydrolysis involves high temperature and always forms xylose, accompanied by toxins, which should be removed leading to high process cost due to downstream processing to obtain highly pure XOS. The enzymatic hydrolysis involves low temperature, and high specificity preventing toxic byproducts formation (Morgan et al. 2017).

Xylanase is the key enzyme for the hydrolysis of xylan producing XOS of variable length ranging from 2 to 10. For XOS production, First endo-β-1,4-xylanase hydrolyze the xylan backbone followed by enzyme like β-xylosidase amd glycosidases for cleaving side chain groups. Xylanase used should have high endo-xylanase activity and low or negligible exoxylanase or β-xylosidase activity as it produces high amount of xylose causing inhibitory effects on the production of XOS. These

enzymes are capable of operating under a wide range of temperature, pH, water activity, and redox potential (Biely et al. 2016).

Liu et al. (2017) produced xylooligosaccharides using *Bacillus amyloliquefaciens* xylanase and xylobiose and xylotriose were the major products, respectively. In another study, xylanase from *Bacillus subtilis* Lucky9 produced xylobiose and xylotriose from beechwood xylan and corncob, respectively (Chang et al. 2017). Reddy and Krishnan (2016) produced high-pure XOS from sugarcane bagasse delignified with aqueous ammonia using a crude xylosidase free xylanase of *Bacillus subtilis*. Presence of XOS including xylobiose, xylotriose and xylotetraose with negligible amount of xylose (0.4%), was confirmed by MALDI-TOF-MS and HPLC analysis. Ratnadewi et al. (2016) used endoxylanase (2.21 U ml^{-1}) from *Bacillus subtilis* of soil termite abdomen for production of XOS. TLC as well as HPLC chromatography confirmed that xylopentose, xylotriose and xylotetrose were present as the major end products, with no xylotriose. A purified alkaline xylanase from *Bacillus mojavensis* A21 was employed for the production of xylooligosaccharides from garlic straw. Xylobiose and xylotriose were the main hydrolysis products yielded from garlic straw, as confirmed by TLC analysis (Kallel et al. 2015). Faryar et al. (2015) utilized an alkali-tolerant endoxylanase for production of XOS. Xylobiose was the predominant oligosaccharide after hydrolysis.

2.16 Strategical Improvements for Production of XOS

Microbes are quite versatile in nature and are capable of producing several enzymes suitable for industrial applications. Xylanases are produced by a variety of organisms, including *Streptomycetes*, *Aspergillus*, *Phanerochaetes*, *Chytridiomycetes*, *Trichoderma*, *Bacillus*, *Fibrobacter*, *Clostridium*, *Ruminococus*, *Thermoascus*, etc. by utilizing various agro-residues (Moreira 2016). Even though the demand for xylanases compatible at industrial level is ever increase, however, low yields and high production costs are the main bottlenecks which are being faced at the industrial level. Therefore statistical optimization is being done to optimized process parameters in order to maximize XOS yields with minimum impurities and unwanted by-products. Moreover incorporation of inexpensive sources which mostly comprises of agricultural refuse in the culture media for enzyme production further help to decrease the production costs (Gupta et al. 2015).

Reddy and Krishnan (2016) optimized culture conditions for production of β-xylosidase-free endo-xylanase from *Bacillus subtilis* KCX006 under solid state fermentation (SSF). Highest xylanase product was supported by wheat bran and groundnut oil-cake at 2158 IU gdw^{-1} and 24.92 mg gdw^{-1} respectively improving xylanase production by 1.5 fold. In another study, Box–Behnken design was used to optimize xylanase production from *Aspergillus candidus*. Parameters optimized were nitrogen source, time of incubation, temperature and moisture content giving maximum xylanase activity of 770 U gds^{-1} (Garai and Kumar 2013). Xylanase production from a newly isolated *Bacillus aerophilus* KGJ2 was statistically optimized using

Plackett–Burman fractional factorial design and Box–Behnken method. Substrate concentration, nitrogen source, moisture content and $MgSO_4 \cdot 7H_2O$ were the significant variables studied, giving a xylanase yield of 45.9 U gds^{-1} (Gowdhaman et al. 2014). Similarly, Plackett–Burman design and Box–Behnken design were used for enhancing xylanases production by *Bacillus mojavensis* A21. Barley bran, NaCl, speed of agitation and cultivation time were the significant variables and statistical optimization resulted in a 6.83 fold increase in xylanase production (Haddar et al. 2012).

Efficiency of XOS production through enzymatic means can also be increased by using immobilized enzymes. Immobilized enzyme can be reused in subsequent batches, as a result of which the amount of enzyme required for the reaction and the reaction time could be effectively reduced. Therefore enzyme immobilization could make commercial production of XOS more affordable (Sun et al. 2016). Sukri and Sakinah (2017) used immobilised xylanase for the production of XOS and obtained xylobiose and xylotriose as the major products of xylan degradation by xylanase. Driss et al. (2014) immobilize *Penicillium occitanis* xylanase on chitosan with glutaraldehyde by covalent coupling reaction. The immobilizated xylanase retained 94.45 ± 3.5% of its activity. In another study, xylanase from *Aspergillus versicolor* were immobilized on glyoxyl-agarose supports. 85% of its catalytic activity was maintained by the immobilized enzyme (Aragon et al. 2013). In another study, an anionic exchange resin via the ionic linkage was used to immobilize an endo-xylanase secreted by the alkaliphilic *Bacillus halodurans,* retaining 80.9% of its activity (Lin et al. 2011).

2.17 Lignocellulosic Biomass for Polyhydroxybutyrate (PHB) Production

Plastic also known as synthetic polymer has become significant because of its properties like durability, mechanical and thermal stability, and resistance to degradation. It is replacing glass, wood and other constructional materials, and also possesses application in industries (Tripathi et al. 2013). Inspite of its widespread usage in day today life, it possess serious drawbacks which includes persistence in the environment leads to deleterious effects on wild life, waterways, quickly fill up landfills and natural areas and greatly affects aesthetic quality of the area (Santimano et al. 2009). The potential hazards generate from synthetic plastic waste incineration and the economy of disposal process makes waste management a problem (Webb et al. 2012). With the increase in population and depletion of non-renewable resources (petroleum products) develops concern not only for energy industry, but also changes the chemical industry. For example, plastic produced annually of 200 million tons and it is predominately derived from petroleum (Du et al. 2012). The shortage of crude oil resources make the production of conventional plastics expensive. It leads to the immense need of using environment friendly substitute and cost effective raw

materials to replace fossil resources. Biopolymer produced using starch, sugars, or cellulose is biodegradable and derived from sustainable biomaterials making it an environmental benign process (Du et al. 2012).

Biodegradable plastics represent a solution to environmental problems generated by the utilization of plastics from petrochemical sources, which have many undesirable properties such as durability and resistance to biodegradation. Biodegradable plastics are plastics that will decompose in natural aerobic and anaerobic environments (Emadian et al. 2017). It can be achieved by enabling microorganisms in the environment to metabolize the molecular structure of plastic films to produce inert humus like material that is less harmful to the environment and allows composting as an additional way for waste disposal (Gouda et al. 2001). Wide range of microorganisms produced polyhydroxyalkanoates (PHAs)—family of biodegradable polymers. During nutrient starving conditions bacteria such as *Ralstonia eutropha* and *Alcaligenes latus* could synthesize PHAs using nutrient non-limiting media (Ojumu et al. 2004). They are usually accumulated as an intracellular energy reserve. According to the chain length of the branching polymers PHAs family can be classified as shortchain-length PHAs possess 3–5 carbon atoms, while medium-chain-length and longchain-length composed of 6–14 and 15 or more carbon atoms, respectively (Altaee et al. 2016).

Polyhydroxybutyrate (PHB) is the most extensively studied member of the PHA family. It was in the mid 1920s, the presence of PHB in *Bacillus megaterium* was first identified (Yu and Stahl 2008). PHB is the oldest known biopolymer used as a biodegradable and biocompatible material for production of bioplastic. It possesses high tensile strength, inertness, high melting point and thermoplastic like properties (Azizi et al. 2017). These features make them suitable for application in the packaging industry and as substitute for hydrocarbon-based plastics. It has wide applications in different areas such as packaging material, long term dosage of drugs, medicines, insecticides, herbicides, fertilizers cosmetic world, disposable items such as razors, utensils, diapers, feminine hygiene products, cosmetics containers, shampoo bottles, cups etc. (Gowda and Shivakumar 2014; Rehm 2006). Studies are progressing for its relevance in medical field for bone replacements and plates, surgical pins, sutures, wound dressings, and blood vessel replacements (Chen and Wang 2013).

In response to imbalanced nutritional conditions, such as an excess of carbon source combined with nutrient limitations, such as oxygen, nitrogen or phosphorus, PHB is synthesized and accumulated by several bacterial species including *Azohydromonas lata*, *Cupriavidus necator* (formerly known as *Ralstonia eutropha*), *Pseudomonas* sp., *Bacillus megaterium*, *Paracoccus denitrificans* and *Protomonas extorquens*, *Aeromonas hydrophila*, *Pseudomonas putida* and recombinant *Escherichia coli* (Castillo et al. 2017). Several microorganisms studied for PHB production utilizing cost effective substrates are shown in Table 2.5.

The major obstruction in the commercialization of PHA and its polymers is their high production cost as compared to synthetic plastic. The process viability of microbial production of PHB is dependent on the development of a low cost process that produces biodegradable plastics with properties close to or surpassing petrochemical plastics (Du et al. 2012). Process economics reveal that the use of renewable carbon

Table 2.5 Organisms producing PHB and other biodegradable polymers using cost effective substrates

Microorganism	Biomass used	Product	Yield	References
Bacillus megaterium B2	Raw glycerol	PHB	1.20 g l^{-1}	Moreno et al. (2015)
Cupriavidus necator	CO_2	PHB	0.26 g g^{-1} cell H^{-1}	Mozumder et al. (2015)
Bacillus megaterium	Glycerol	PHB	4.8 g l^{-1}	Naranjo et al. (2013)
Rhodococcus equi	Crude palm kernel oil	PHB	38%	Altaee et al. (2016)
Bacillus sp.	Soy molasses oligosaccharides	PHAs	90% of CDW	Full et al. (2006)
Bacillus megaterium	Sugarcane molasses and corn steep liquor	PHB	46.2% mg^{-1} CDW	Gouda et al. (2001)
Bacillus megaterium	Sugarcane molasses, urea and trace elements	PHB	1.27 g l^{-1} h^{-1}	Kulpreecha et al. (2009)
Bacillus megaterium R11	Oil palm empty fruit bunch	PHB	9.32 g l^{-1}	Zhang et al. (2013)
Cupriavidus necator PTCC 1615	Brown sea weed *Sargassum* sp.	PHB	3.93 ± 0.24 g l^{-1}	Azizi et al. (2017)
Ralstonia eutropha MTCC 8320 sp.	*P. hysterophorus* and *E. crassipes*	PHB	8.1–21.6% CDW	Pradhan et al. (2017)
Bacillus cereus PS 10	Rice straw hydrolysate	PHB	10.61 g l^{-1}	Sharma and Bajaj (2015c)
Bacillus cereus PS 10	Molasses	PHB	57.50%	Sharma and Bajaj (2015b)
Alcaligenes sp.	Cane molasses and urea	PHB	8.8 ± 0.4 g l^{-1}	Tripathi et al. (2013)
Pseudomonas corrugate	Soy molasses	PHAs	5–17%	Solaiman et al. (2006)
Methylobacterium sp. ZP24	Whey	PHA	2.6–5.9 g l^{-1}	Nath et al. (2008)
Burkholderia cepacia ATCC 17759	Hemicellulosic hydrolysates	PHB	2.0 g l^{-1}	Keenan et al. (2006)
Pseudomonas sp. strain DR2	Waste vegetable oil	PHA	23.5% CDW	Song et al. (2008)

substrates and lignocellulosic biomass in PHA production can cause 40–50% reduction in the overall production cost (Gowda and Shivakumar 2014). Therefore the use of waste residues like starch, whey, molasses, bagasse, soyameal etc., and dairy waste can significantly reduce the substrate cost and in turn downsize the production costs (Du et al. 2012). Hence, the use of waste for production of value adding products not only provides value to the waste but also solve the problem of waste disposal. Other factors which also affect the total production costs are bacterial strains, fermentation strategies and recovery processes (Santimano et al. 2009).

Different biomass and biomass derived products are used for the production of PHB and other polymers to make the process economical. A brown seaweed *Sargassum* sp. biomass was used for production of PHB by *Cupriavidus necator* PTCC 1615. Biomass was pretreated with acid and then enzymatically hydrolysed to release monomeric sugars. Ammonium sulphate (nitrogen source) with hydrolysate resulted in PHB yield of 0.54 ± 0.01 g g^{-1} reducing sugar. NaCl, an external stress factor show positive impact on PHB yield, but increasing concentration of NaCl to 16 g l^{-1} was found to inhibit the PHB production. The highest cell dry weight and PHB concentration were 5.36 ± 0.22 and 3.93 ± 0.24 g l^{-1} respectively of 20 g l^{-1} reducing sugars (Azizi et al. 2017). Furthermore two invasive weeds, viz. *P. hyysterophorus* and *E. crassipes* were used for the production of biodegradable PHB by *Ralstonia eutropha* MTCC 8320 sp. Both the biomass were pretreated with acid and then enzymatically hydrolysed to produce pentose and hexose rich hydrolysates. Sonication was used for the extraction of PHB. Yield of PHB produced was 6.85×10^{-3}–36.41×10^{-3}% of w w^{-1} raw biomass (Pradhan et al. 2017).

Saccharophagus degradans (ATCC 43961) degrade the major components of plant cell walls by readily attaching to cellulosic fibers, and utilize this as the primary carbon source. The minimal media containing glucose, cellobiose, avicel, and bagasse was used for the growth of *S. degradans* to support growth. Lignin in media alone did not support growth, but on addition of glucose support growth. When nitrogen gets depleted, PHA production commences and continues for at least 48 h. This work reveals for the first time, that a single organism can utilize insoluble cellulose and produce PHA (Munoz and Riley 2008).

2.18 *Bacillus* spp. for PHB Production

Bacillus spp. produces variety of enzymes and has been explored for wide range of industrial products including PHB. *Bacillus* spp. comparatively grows faster and has potential to exploit vast range of agro-industrial wastes as substrates (Masood et al. 2012). Furthermore, *Bacillus* spp. acts a model system for heterologus expression of foreign genes including PHA production and other chemicals (Law et al. 2003; Schallmey et al. 2004). Numerous reports are available on the production of PHB by *Bacillus* spp. For the production of PHB granules in cells of *Bacillus megaterium* sugarcane molasses and corn steep liquor were used as sole carbon and nitrogen source respectively. Maximum yield of PHB obtained was 46.2% mg^{-1} cell dry

matter with 2% molasses (Gouda et al. 2001). In another study, Zhang et al. (2013) studied the use of oil palm empty fruit bunch collected from Malaysia palm oil refinery (rich in cellulose and hemicelluloses) for production of PHB by *B. megaterium* R11. From the overall oil palm empty fruit bunch sugar concentration of 45 g l^{-1}, 58.5% of PHB content obtained. On increasing the hydrolysate content to 60 g l^{-1} productivity increases to 0.260 g l^{-1} h^{-1}, reaching the PHB content to 51.6%.

Furthermore, Kulpreecha et al. (2009) studied the homopolymer PHB production by *B. megaterium* BA-019 using renewable and inexpensive substrate sugarcane molasses by fed batch cultivation and urea as a nitrogen source. The optimal feeding conditions require sugar concentration of 400 g l^{-1} and C/N molar ratio of 10 mol mol^{-1} and attained PHB content of 42% of cell dry weight in a short time of 24 h. A maximum of 90% of cell dry mass as PHA was obtained by *Bacillus* sp. strain CL1 isolated from nature capable of fermenting soy molasses and other waste carbohydrates produced. It produced PHA without requiring a nutritional limitation (Full et al. 2006). Albuquerque et al. (2007) described an interesting work of three-step fermentation strategy by producing PHAs from cane molasses. Firstly, molasses were fermented to organic acids. Then, triggered to PHA accumulation and finally, in batch fermentation using the fermented molasses and the PHA-accumulating cultures, PHAs were produced. In another study, PHAs synthesis from fermented molasses was obtained by using a consortium of microorganisms (Pisco et al. 2009 and Bengtsson et al. 2010).

2.19 Strategies for PHB Production

2.19.1 Process Optimization for PHB Production

Many reports were available on production of PHB using crude resources (Du et al. 2012). But to make process efficient and economical, optimization of process parameters is of utmost importance (Singh et al. 2016). Conventional one-variable-at-a-time method was used for the screening of various substrates affecting production. But it is time consuming, laborious and ignores the combined interaction among various variables (Singh and Bajaj 2015). Plackett–Burman design and the central composite design of response surface methodology (RSM) are the statistical tools used for efficient medium optimization and for studying the interaction of various variables (Vaid et al. 2017).

PHB producing strain *B. thuringiensis* IAM 12077 used agro wastes substrates like rice husk, wheat bran, vagi husk, jowar husk, jackfruit seed powder, mango peel, potato peel, bagasse and straw for production. Among all substrates, mango peel yielded the highest PHB of 4.03 g l^{-1}; 51.3% (Gowda and Shivakumar 2014). Also, bacterial isolate *Bacillus cereus* PS10 grow on low cost agro-based residues viz. maize bran, rice husk, wood waste, molasses, whey, walnut shell powder, almond shell powder, corn steep liquor, soy bean bran, mustard cake etc. and accumulated

appreciable amount of PHB. Carbon source molasses support maximum PHB production of 9.5 g l^{-1} after 48 h of fermentation at pH 7 (Sharma and Bajaj 2015a). The isolate *Bacillus cereus* PS10 was then used for statistical optimization of PHB production with crude source molasses. Variables first identified through Plackett-Burman design were molasses, pH and NH_4Cl and then process was optimized through RSM approach resulting in enhancement of PHB yield by 57.5% (Sharma and Bajaj 2015b).

Sharma and Bajaj (2015c) produced bioplastic using the same isolate *Bacillus cereus* PS10. In this study, biphasic-acid-treated rice straw produced hydrolysate was used for fermentation by isolate. Rice straw hydrolysate (RSH) produced more PHB than the refined carbon source glucose. Then the process was optimised using RSM for various process variables were the amount of RSH, NH_4Cl and medium pH and enhanced yield of 23%.

B. megaterium B2 has the ability to accumulate PHB using raw glycerol from biodiesel production as the carbon source. PHB production was statistically optimized to establish key variables and optimal culture conditions by Plackett-Burman and central composite designs. Experimental variables influencing PHB production are temperature, glycerol concentration and Na_2HPO_4 in shake flask with optimized medium produced 0.43 g l^{-1} of PHB with a 34% accumulation in the cells after 14 h of fermentation. The maximum PHB concentration of 1.20 g l^{-1} was reached at 11 h under the same conditions. It corresponds to a 48% and 314% increase in PHB production compared to the initial culture conditions (Moreno et al. 2015). Tripathi et al. (2013) did the optimization by central composite rotatable design for three physical process variables viz; pH, temperature and agitation speed for enhancing PHB production by *Alcaligenes* sp. Cane molasses and urea were used as carbon and nitrogen source. The optimum physical conditions resulted in PHB mass fraction yield of 76.80% on dry molasses substrate. On scale up studies of same optimized media produce maximum yield and productivity of 0.78 and 0.19 g l^{-1} h^{-1}, which was higher than previous reports.

2.19.2 Application of Genetic Engineering Tools

Genetical engineering approaches were also used for maximizing the PHB production. PHA, a bio-based plastic was produced by successful engineering of biomass crop switchgrass (*Panicum virgatum* L.). Engineered crop was able to grow and produce polymer in vitro and glass house conditions. Transformants produce 3.72% dry weight of PHB in leaf tissues and 1.23% dry weight of PHB in whole tillers. First generation of transformants obtained from controlled crosses of transgenic plants also accumulate polymer (Somleva et al. 2008). Halophiles also have potential to produce PHB using agro-industrial wastes. Halophilic bacteria allow the PHA production under continuous mode and unsterile conditions. They are easily manipulated genetically and allow the construction of a hyper-producing strain. For example, both recombinant and wild type *Halomonas campaniensis* LS21 were allowed to grow on

mixed substrates (kitchen wastes) in the presence of NaCl (26.7 g l^{-1}), at pH 10 and temperature of 37 °C continuously, for 65 days, without any contamination. Recombinant produced higher PHB content (70%) than the wild type strains (Kourmentza et al. 2017).

2.19.3 Pretreatment of Biomass

Different methods were used for the pretreatment of biomass to release simple sugars which were easily fermented by microorganisms. Various pretreatment includes mechanical comminution, acid and alkaline hydrolysis, ozonolysis and biological pretreatments. For the pretreatment of biomass switchgrass, radio frequency assisted heating was used to generate hydrolysates and then enzymatically hydrolysed to fermentation with recombinant *Escherichia coli*. Results clearly indicated that hydrolysates obtained through radio frequency pretreatment produced consistently better PHB production. Supplementation of media with yeast extract enhances production under all conditions. In comparison to traditional heating pretreatment process, radio frequency creates harsher conditions for unwinding of biomass structure more and generates more nutrients for fermentation (Wang et al. 2016)

2.19.4 Structural Modifications of PHB

Composition of the PHA during the biosynthesis changes the applications of bioplastic. The representative member of PHA family, namely the homopolyester PHB, possesses high degree of crystallinity and restricted processability of this material. Due to the small difference between the decomposition temperature and melting point provides a little space for processability. This composition can be changed by alternating the building blocks such as (R)-3-hydroxyvalerate or the achiral building blocks 4-hydroxybutyrate and 5-hydroxyvalerate (Koller et al. 2009). Varied techniques were used for the determination of structural and physcio-chemical properties of biopolymers. Thermal properties were determined by TGA, DTG and DSC. ^{1}H NMR results revealed the molecular weight and polydispersity index value. PHB with low polydispersity index value can be used for nanoparticle formation (Sathiyanarayanan et al. 2013). With the advent of genetic engineering techniques, PHA with different compositions and higher productivity has been possible to design (Tsuge 2002). By altering the physical and genetical properties, biopolymers can be produced according to the necessity.

In response to the ever increasing demand, biodegradable plastic may serve as substitute for petroleum derived plastics because of its biodegradable and biocompatible nature, and production from sustainable and agro-waste raw materials which provide independence from fossil fuels. Even though, their manufacturing cost is too high to compare with petrochemical derived plastics but advances in the production

processes using inexpensive substrates makes possible the broad use of bioplastic in future.

2.20 Lignocellulose Biomass for Production of Industrial Enzymes

Enzymes are of great importance for various industries due to their action on specific substrate, resulting in high yield production. They are indispensable ingredients in various processes that are involved in product development. But lot of hurdles is encountered in production of enzyme (Ravindran and Jaiswal 2016) and the major ones are their high production cost and low product yields. The LB is a cheap source for production of enzymes and other valuable products such as bioethanol, organic acids etc. Hydrolytic enzymes likes cellulases, xylanase and pectinase are of utmost importance in valorization of food industry waste (Meng and Ragauskas 2014). Different species of bacteria (*Clostridium*, *Cellulomonas*, *Bacillus, Pseudomonas, Fibribacter*, *Ruminococcus*, *Butyrivibrio*, etc.), fungi (*Aspergillus*, *Rhizopus*, *Trichoderma, Fusarium, Neurospora, Penicillium* etc.), and actinomycetes (*Hermomonospora, Hermoactinomyces* etc.) are involved in the degradation of lignocelluloses due to their extracellular enzyme production attribute (Sajith et al. 2016).

Enzymes are biological catalysts found in all living systems and are proteinaceous in nature with potential to catalyze diverse reactions (Ravindran and Jaiswal 2016). There is a long history of enzyme use for the commercial production of various metabolites, and have been documented to be efficient for industrial scale production. Enzymes are now being genetically manipulated in order to enhance their ability for better results in fermentation media. Such modifications have enabled researchers to use several simple microorganisms with no history of industrial use for production of native enzymes, such as *Escherichia coli* K-12, *Fusarium venenatum* and *Pseudomonas fluorescens* to be successfully utilized as source for expression of industrially important enzymes (Olempska-Beer et al. 2006). Hemicellulase production from filamentous fungi are being developed as one of the best enzyme production systems due to their ability to secrete high quantities of enzymes suitable for industrial applications such as development and commercialization of new products (Gudynaite-Savitch and White 2016).

Since hydrolysis of biomass is essential for generation of fermentable sugars which are then converted to ethanol by microbial action. Thus, alternate enzyme production method using cheaper and abundantly available substrates with higher yield is need of the hour (Ang et al. 2013). Cellulase and hemicellulase production from *Aspergillus niger* KK2 was studied on SSF using different ratios of rice straw and wheat bran biomass. Maximum FPase activity was 19.5 IU g^{-1} in 4 days was found on rice straw. Also, CMCase (129 IU g^{-1}), β glucosidase (100 IU g^{-1}), xylanase (5070 IU g^{-1}) and β-xylosidase (193 IU g^{-1}) activities were concurrently obtained after 5–6 days of fermentation and such enzyme activities are critical for practical

saccharification reaction during bioethanol production (Kang et al. 2004). Ang et al. (2013) utilized untreated oil palm trunk for cellulases and xylanase production by *Aspergillus fumigatus* SK1 under SSF. The cellulases and xylanase activities obtained were 54.27, 3.36, 4.54 and 418.70 U g^{-1} substrates for endoglucanase (CMCase), exoglucanase (FPase), β-glucosidase and xylanase respectively. To bring down the cost of cellulases production, a multifaceted approach using cheap lignocellulosic substrates such as sugar cane bagasse, rice straw and water hyacinth biomass under SSF was utilized. Cellulolytic and β-galactosidase enzymes were produced using SSF on wheat bran as substrate using fungi *Trichoderma reesei* RUT C30 and *A. niger* MTCC 7956, respectively. Kshirsagar et al. (2015) produced cellulases and xylanases from *Amycolatopsis* sp. GDS which were thermostable and active up to 70 °C and were able to function at higher NaCl (2.5 mol l^{-1}) and ionic liquid (10%) concentrations during the pretreatment of biomass. Crude enzymes also resulted in comparable saccharification (60%) of wheat straw as compared to commercial enzymes (64%). The copper dependent lytic polysaccharide mono-oxygenases has been patented by biotech company Novozymes A/S holds patents on the use of these enzymes for the conversion of steam-pretreated plant residues such as straw to free sugars. lytic polysaccharide mono-oxygenases show striking synergistic effect when combined with canonical cellulases enzyme for efficient performance in several large-scale plants for the industrial production of lignocellulosic ethanol (Johansen 2016).

Saratale et al. (2017) isolated lignocellulolytic enzymes using *Streptomyces* sp. MDS cultivated in various agricultural wastes under SSF. The harvested enzyme exhibited good stability at a range of pH (5–8) and temperature (50–80 °C) and efficient activity in the presence of organic solvents, surfactants, and commercial detergents. Novel extracellular endoxylanase and endoglucanase from halo- and thermotolerant *Actinomadura geliboluensis* with molecular mass of 30 and 38 kDa were produced with optimum pH and temperature values of pH 6.0 and 60 °C respectively. These enzymes were strongly inhibited by Hg^{2+} and reducing sugar content was 265.12 mg g^{-1} biomass after incubation with alkali pretreated wheat straw (Adıgüzel and Tunçer 2017). Production of amylolytic enzymes by solid state or submerged fermentations (SmF), followed by purification and evaluation of enzymatic hydrolysis of the polysaccharides of *Spirulina* was carried out. Microfiltration of the crude extracts resulted in an increase in their specific activity and thermal stability at 40 and 50 °C for 24 h, as compared to extracts obtained by SSF and SmF (Rodrigues et al. 2017).

Likewise many other lignocellulosic biomasses have been utilized for commercially important enzyme production. Various microorganism including both bacteria and fungi were used as source of enzyme production in different reaction conditions. A detailed account for such enzymes from various biomasses is given in Table 2.6.

2.21 Conclusion

It may be concluded that though lignocellulosic biomass from agro/forestry and other sources, may have immense potential as a renewable feedstock for production of energy, materials and varierty of other products of commercial importance. But hurdles like pretreatment, apt enzymes for saccharification, efficient fermentation process organisms, and down stream processing need more research attention.

2.22 Future Prospects

The important criterion for the long-term feasibility of any bioprocess is its cost effectiveness. Though LB represents an efficient and abundantly available renewable energy feedstock but its recalcitrance is a big hurdle. It is the need of the hour that effective pretreatment approaches must be developed for LB along with apt enzyme cocktail for saccharification. The fermentation organism must be capable of utilizing the sugars and generate high product yield and concentration. Modified/engineered hydrolases may be developed for efficient saccharification. There is a need to find more innovative methodologies in which the reuse and recycle of pretreatment agents/saccharifying enzymes as well as fermenting organisms can be practiced in an effective manner. The consolidated bioprocesses may be developed in which pretreatment, saccharification and fermentation can be executed in a single vessel using balanced and appropriate combo of biomass, pretreatment agents, enzymes and fermenting organisms that functions synergistically.

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Table 2.6 Different lignocellulosic biomass for commercially important enzymes

Microorganism	Enzyme produced	Substrate used	Mol. mass, pH and temperature	References
Trichoderma reesei RUT C30 and *Aspergillus niger* MTCC 7956	Cellulase and β- glucosidase	Wheat bran	pH 4.8 Temperature 70 °C 43 kDa and 124.0 kDa	Rajeev et al. (2009)
Geobacillus sp. strain WSUCF1	Xylanases	Prairie cord grass and corn stover	pH 5.5–7 Temperature 80 °C 39.5 kDa	Bhalla et al. (2015)
Penicillium echinulatum 9A02S1	Cellulase and xylanase	Elephant grass	pH 4.8 Temperature 50 °C 50 kDa and 80 kDa	Menego et al. (2016)
Aspergillus oryzae P21C3	Xylanase	Sugarcane bagasse	pH 4.8 Temperature 50 °C 35.402 kDa	Braga et al. (2014)
Trichoderma reesei NRRL-6156	Exocellulase, endocellulase, and xylanase	Soybean bran	pH 5.0 Temperature 50 °C 110 kDa	Gasparotto et al. (2015)
Geobacillus sp. strain WSUCF1	Thermostable xylanase	Prairie cord grass and corn stover	pH 6.5 Temperature 70 °C 17 kDa	Bhalla et al. (2015)
Aspergillus niger	Cellulase	Paper and timber sawmill industrial wastes	pH 4–7 Temperature 20–50 °C 17 kDa	Devi and Kumar (2012)
Scytalidium thermophilum	Endoglucanase	Lentil bran and sunflower seed bagasse	pH 4.6 Temperature 45 °C 23 kDa	Ögel et al. (2001)
Coculture of *Trichoderma reesei* and *Aspergillus oryzae*	Cellulolytic Enzyme-β-glucosidase, CBH I, CBH II, EG I and xylanase	Soybean hulls supplemented with wheat bran	pH 4.8 Temperature 30–32 °C 40 kDa	Brijwani et al. (2010)
Gracilibacillus sp. SK1	Alkali-stable cellulase	Corn stover and rice straw	pH 8 Temperature 60 °C 37 kDa	Yu and Li (2015)

(continued)

Table 2.6 (continued)

Microorganism	Enzyme produced	Substrate used	Mol. mass, pH and temperature	References
Aspergillus terreus D34	Exo- and endoglucanases, β-glucosidases, and xylanases	Rice straw and sugarcane bagasse	pH 4.5 Temperature 45 °C 23 kDa	Kumar and Parikh (2015)
Trichoderma reesei Rut C-30	Cellulase	Paper sludge and wood	pH 4.8 Temperature 27 °C 43 kDa	Shin et al. (2000)
Streptomyces viridobrunneus SCPE-09	Cellulase	Wheat bran and corn steep liquid	pH 4.9 Temperature 50 °C 37 and 119 kDa	Da Vinha et al. (2011)
Aspergillus sydowii	Endoglucanase, exoglucanase and β-glucosidase	Lactose	pH 5.5 Temperature 40 °C 95 kDa	Matkar et al. (2013)
Thermoascus aurantiacus RBB1	Cellulase	Wheat bran	pH 4.0 Temperature 70–80 °C 35 kDa	Davea et al. (2013)
Amycolatopsis sp. GDS	Cellulase and xylanase	Paddy straw, sugarcane barboja, corn straw, sorghum husk, water hyacinth and sugarcane bagasse	pH 4.0 Temperature 70 °C 30 kDa	Kshirsagar et al. (2015)
Talaromyces Cellulolyticus	Cellulase	Corn stover	pH 5.0 Temperature 45 °C cellulase III-B (49 kDa), I (61 kDa), and III-A (58 kDa)	Inoue et al. (2014)
Aspergillus awamori	Xylanase, α-L-arabinofuranosidase, β-xylosidase, and β-glucosidase	Sugarcane bagasse	pH 4.0 Temperature 70 °C 30 kDa	De Sousa et al. (2015)
Chrysoporthe cubensis	Cellulolytic cocktails	Sugarcane bagasse	pH 3–4.0 Temperature 55–80 °C 110 and ~38–40 kDa	Dutra et al. (2017)

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Chapter 3
Biotechnological Advances in Lignocellulosic Ethanol Production

Latika Bhatia, Anuj K. Chandel, Akhilesh K. Singh and Om V. Singh

Abstract The worldwide increasing environmental issues owing to fossil fuels and their anticipated shortage in near future have fueled the research towards the search and development of alternative fuels from renewable sources. Recently, the biomass-based transport fuels have turn out to be strategic attention for counties with intention to enhance the sustainability in the terms of bioenergy. Interestingly, bioethanol is an oxygenated biofuel with 35% oxygen content, which can decrease the particulate matter and NO_x emissions produced from fuel combustion. Therefore, gasoline blended with bioethanol can considerably decrease the petroleum consumption along with greenhouse gases emission. Though nearly all the present biofuel ethanol is produced from edible materials like sugars, starch etc., the lignocellulosic biomass has received considerable interest in recent years. Nevertheless, the transformation efficiency and ethanol yield of the biomass varies significantly mainly owing to the difference in lignocellulosic content. In lignocellulosic biomass, the complex lignocellulosic network of cellulose and hemicellulose with lignin is extremely resistant towards depolymerization. Thus, there still remain some obstacles that are to be addressed to make

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lignocellulosic biomass-based bioethanol a commercial reality. Further, the unmet need of cost reduction of lignocellulosic biomass-based bioethanol is progressing towards techno-economic improvements of overall transformation process through efficient biomass pretreatment process as well as recombinant genetic engineering for the microbial strain improvement. Overall, this chapter not only focuses on the current exploitation of different biomasses as substrates but also the biotechnological advances in various bioprocesses leading to improved bioethanol production.

Keywords Lignocellulosic biomass · Pretreatment · Enzymatic hydrolysis Cellulases · Fermentation · Lignocellulosic ethanol

3.1 Introduction

The last decade has seen a massive impetus towards biofuel research because of the depletion of fossil fuel resources for mammoth demands of transportation along with increasing releases of greenhouse gasses (GHGs) like carbon dioxide etc. into the atmosphere. Furthermore, the liberations of GHGs that produced as a result of fossil fuel combustion must undergo reduction significantly and quickly in order to restrict the worldwide climate change to less than 2 °C. Considering these, governments in many countries including the US, Italy, China, Germany and India have invested considerable funds towards biofuel research and also introduced subsidies as well as incentives for vendors, which produce biofuels. For example, the US alone has declared an incentive package of US$100 billion for development of biofuels as eco-friendly, alternative, renewable technology to fossil fuel (Mallick et al. 2016). Besides, there is huge demand to develop strategies for enhancing energy self-sufficiency, minimize import prices and fortify domestic agricultural growth. In this context, biomass-based transport fuels turn into an eye-catching strategy. Remarkably, these biofuels not only minimize vehicle GHGs emissions and increase sustainability but have also been instrumental in a shift to low-carbon fuels, which could substantially encourage sustainability in the transport segment. Many industries like aviation, marine transport and heavy freight have started switching over to biofuels for increasing their sustainability as well as reducing the increasing environmental problems. For instance, based on the growth in travel, it is estimated that aviation industry alone will be responsible for increase in total GHGs emissions by 400–600% between 2010 and 2050. Considering these, the aviation industry took responsibility to put their effort to reduce CO_2 and with this intention they flew their first commercial test flight in 2008 using biofuels (Araujo et al. 2017). Biofuel became a part of half of the jet fuel mixture of approximately 22 airlines in the mid of 2015, with the support of which these airlines had accomplished more than 2000 passenger flights. Recently, about 191 countries have focused themselves to strategically control aviation pollution by signing an agreement, thereby generating an immense scope for continued biofuel adoption.

Amongst biofuel, bioethanol is developed as an important renewable fuel, where the global production of bioethanol enhanced from 50 to 100 million m^3, respectively in 2007 and 2012. Concerning bioethanol, both Brazil and the US accountable for almost 80% of the world supply (Kang et al. 2014). Different reports revealed that approximately all the existing bioethanol is manufactured from materials like sugars, starch, beets, corn/wheat based starch, or root crops such as cassava. Furthermore, bioethanol depicts higher-octane rating with lower energy content per volumetric unit (70%). However, bioethanol improve the combustion features and facilitate the engines to work under comparatively greater compression ratio than the gasoline. Total replacement is rather a bit difficult in most of the regions and hence biofuel can supplement gasoline at roughly 10% only. Improvements are taking place in vehicle designing that would not only assist in their enhanced efficiency but also facilitating towards mid-level blends in the range of 20–40%. Brazil gains an attention in being exception in which their fleet of flex fuel cars works on any mixture of gasoline with ethanol [85% ethanol blend (i.e. E85)] or solely on ethanol. Bioethanol production has an immense potential to substitute 353 gallon (GL) of gasoline (that accounting up to 32% of total worldwide gasoline exploitation) if there is bioethanol use in E85 fuel for a mid-size passenger automobile (Balat 2009). Moreover, the co-product of bioethanol generated from crop residues as well as sugarcane bagasse, has a potential to produce both 458 terawatt-hour (TWh) of electricity (almost 3.6% of worldwide electricity generation) and 2:6 exa joule (EJ) of steam (Kim and Dale 2004). Asia could be the major potential bioethanol producer from crop residues as well as wasted crops that could reach up to 291 gallons (GLs) annually.

3.2 Bioethanol Production: Statistics and Global Overview

Since 1980, biofuel industry has witnessed an outstanding growth in the US ethanol industry when this industry has produced 175 million of GLs of ethanol to combat with its domestic use and worldwide demand. Table 3.1 indicates that this industry in US was leading in 2016 too, when ethanol production reached to 15,330 million of GLs. Approximately 70% of the global biofuel supply was done by Brazil and the United States in 2015, which consisted predominantly of sugarcane- and corn-based ethanol, respectively (M & M 2016). The European Union is among a newer producing region that stresses on bio-diesel production using waste, soy, rapeseed as well as palm. However, Asia has centered itself on sugarcane, corn, wheat, as well as cassava with investment in palm, soybean, rapeseed, and jatropha as far as biodiesel production is concerned. Considering these, it is evident that dealers of European Union as well as Asia represent budding bazaars, which have been developing in the last two decades (Fig. 3.1), but this kind of regional as well as feedstock-based variation is favorable towards the establishment of a global biofuel commodities market (Chandel et al. 2017).

Table 3.1 An overview on global fuel production (Millions of GLs)

Region	2016	2015	2014	2013	2012
United States	15,300	14,806	14,300	13,300	
Brazil	7295	7093	6190	6267	5577
European Union	1377	1387	1445	1371	1139
China	845	813	635	696	555
Canada	436	436	510	523	449
Thailand	322	334	310		
Argentina	264	211	160		
India	225	211	155		

Source Renewable fuel association analysis of public and private data sources (2016)

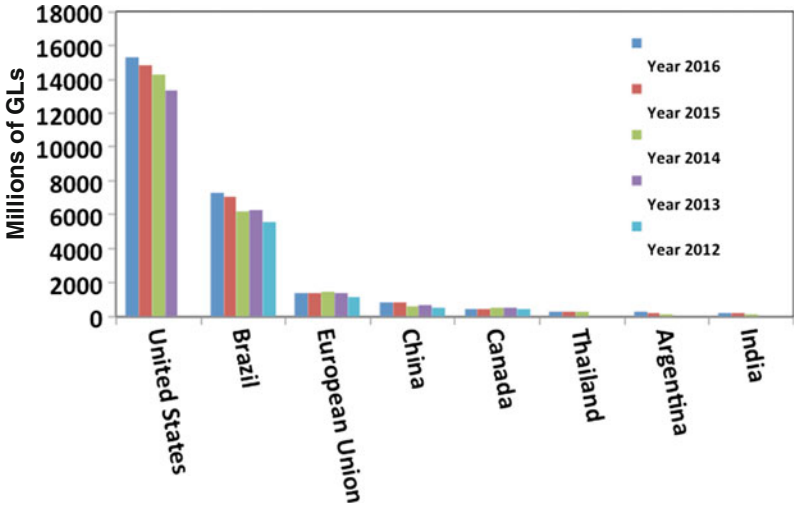

Fig. 3.1 Global production of biofuel (Millions of GLs) (Adapted from Statistical Review of World Energy London, UK, 2016)

3.3 Potential Feedstock, Biomass Composition and Surplus Availability

There are many countries that produce bioethanol from the main crops like corn, barley, oat, rice, wheat, sorghum, and sugarcane. This practice cannot be encouraged in today's scenario as most of the world population is below poverty line and facing extreme health challenges in terms of protein- energy malnutrition. It is an alarming signal to develop strategies and to focus on biofuel production from wasted crop or residues in order to avoid conflicts between human food utilization and industrial exploitation of crops. Thus, in recent years, the non-edible biomass materials

have drawn substantial attention owing to being renewable and sustainable, indirectly assisting CO_2 fixation in the atmosphere, enabling local economy development and stimulation, decreasing air pollution as a result of biomass burning in fields as well as biomass rotting in fields, bringing energy security for countries reliant on imported oil and generating high technology jobs for engineers, fermentation specialists, process engineers and scientists (Greenwell et al. 2012). Amongst biomass materials, lignocellulosic biomass like crop residues and sugarcane bagasse are the attractive alternatives. Asia is rich in rice straw, wheat straw, and corn stover, where these substrates can act as utmost promising bioethanol feedstock. The next utmost prospective region is Europe (69:2 GL of bioethanol), where majority of bioethanol is produced from wheat straw. The foremost feedstock in North America is corn stover that acts as promising platform, where nearly 38:4 GL of bioethanol can be generated annually. Interestingly, rice straw not only contributes globally to generate 205 GL of bioethanol but also recorded the highest quantity amongst single biomass feedstock. Wheat straw is the successive most important promising feedstock that could generate 104 GL of bioethanol (Kim and Dale 2004).

In Brazil, sugarcane is the substrate for ethanol production, while in USA starch crops are exploited (Sanchez 2009). However, these substrates are edible, which has caused controversy for ethanol production (Hahn et al. 2006). China is the highest producer of sweet potato (*Ipomoea batatas*) globally that representing 85% of worldwide production, where the production surpassed 100 million tons in 2005 (Lu et al. 2006). Furthermore, *Ipomoea batatas* considered as an attention-grabbing feedstock towards bioethanol formation (Zhang et al. 2011). On the other hand, Sipos et al. (2009) reported that sweet sorghum bagasse could be transformed effectively to fermentable sugars through Sulphur dioxide catalyzed steam pretreatment with temperature of 190 or 200 °C and incubation period of 10 or 5 min, respectively following enzymatic hydrolysis that cause 89–92% glucan transformation. Hemp and ensiled hemp has been reported to produce ethanol as a result of steam pretreatment carrying out at temperature and incubation period of 210 °C and 5 min, respectively in the presence of 2% Sulphur catalyst followed by immediate saccharification as well as fermentation under loading of high solid (7.5% water insoluble solids). Remarkably, all these processes resulting into the formation of 171–163 g ethanol per kg raw feedstock (Sipos et al. 2010).

There are various industries like forestry, pulp and paper, agriculture and food apart from various wastes from municipal solid waste including animal wastes that produce lignocellulosic wastes in huge amounts (Sims 2003; Kim and Dale 2004; Kalogo et al. 2007; Champagne 2007; Wen et al. 2004). Many environmental concerns raise with the products of agricultural activities like straw, stem, stalk, leaves, husk, shell and peel that were considered as waste in several countries and some developing countries in the past and in present, respectively (Palacios-Orueta et al. 2005). Howard et al. (2003) reported that lignocellulosic residues are important source of value-added substances like biofuels, chemicals as well as animal feed. Valuable constituents like carbohydrate and crude proteins including reducing sugars are found in banana peel and therefore, it can be exploited as a feedstock towards bioethanol generation. Furthermore, banana peels are reasonable and renewable cost-effective raw feedstock (Bhatia and Paliwal 2010; Thakur et al. 2013). Likewise, pineapple

is the subsequent harvest of importance next to bananas, which accounting over 20% of global production of tropical fruits (Coveca 2002). Researchers have produced ethanol from peels of *Citrus sinensis var mosambi, Ananas cosmosus, Litchi chinensis* (Bhatia and Johri 2015; Bhatia and Johri 2016; Bhatia and Johri 2017).

Enormous amount of bagasse is produced in the course of sugarcane processing. Agricultural productivity along with protection of environmental concerns are linked to the bagasse disposal. Recently, promising attempts have been carried out for the effective exploitation of cost-effective renewable agricultural sources like sugarcane bagasse as alternative feedstock towards bioethanol generation (Bhatia and Paliwal 2011). Rice constitutes one of the main crops that cultivated globally with the yield about 800 million metric tons per year that corresponds to the huge generation of rice straw, which lead to apposite lignocellulose feedstock waste towards ethanol generation (Wati et al. 2007). Viability of lignocellulosic feedstock towards bioethanol generation has been studied and accepted globally because of its surplus availability. Utilization of rice husk for ethanol production is an innovative approach towards the utilization of agro-wastes. Internationally, rice husk constitutes one of the highest accessible agricultural wastes in various rice cultivating countries. On average 20% of the rice paddy is husk that contributing a yearly overall production of 120 million tones (Gidde and Jivani 2007). Globally, India is the main producer of rice next to China, where the average annual production of rice is in the range of 100 million metric tons (Moulick 2015). Exploitation of rice husk as fuel in power plants, brick industries and rice mills are well established. Moreover, it is also used as packing material for transport and as thermal barrier (Kumar et al. 2012). This agro-waste is yet to be explored for its potential to produce bioethanol.

3.4 Feedstock Processing to Generate Sugars as Building Block

The major steps towards the transformation of lignocellulose feedstock and other type of starch-based feedstock into bioethanol are well depicted in Fig. 3.2 (Najafi et al. 2009). Pretreatment of the lignocellulosic remains/feedstock is mandatory as degradation of non-pretreated substances is a time taking process, resulting in little generation of value-added product. Enhancements in pore size as well as reduction in the crystalline region of cellulose are the main outcomes of pretreatment methods (Dawson and Boopathy 2007). Approachability of cellulosic material towards the cellulolytic enzymes is also enhanced after its pretreatment, thereby reducing enzyme needs along with the price of bioethanol generation. In addition to enhancing the bio-hydrolysis of the waste materials towards bioethanol formation, it also causes improvement of the recalcitrant bio-degradable substances, leading to increased ethanol production from the wastes (Mosier et al. 2005; Sun and Cheng 2007; Yang and Wyman 2008; Dashtban et al. 2009). Figure 3.3 exhibits the main process steps for biomass processing into ethanol or biochemical.

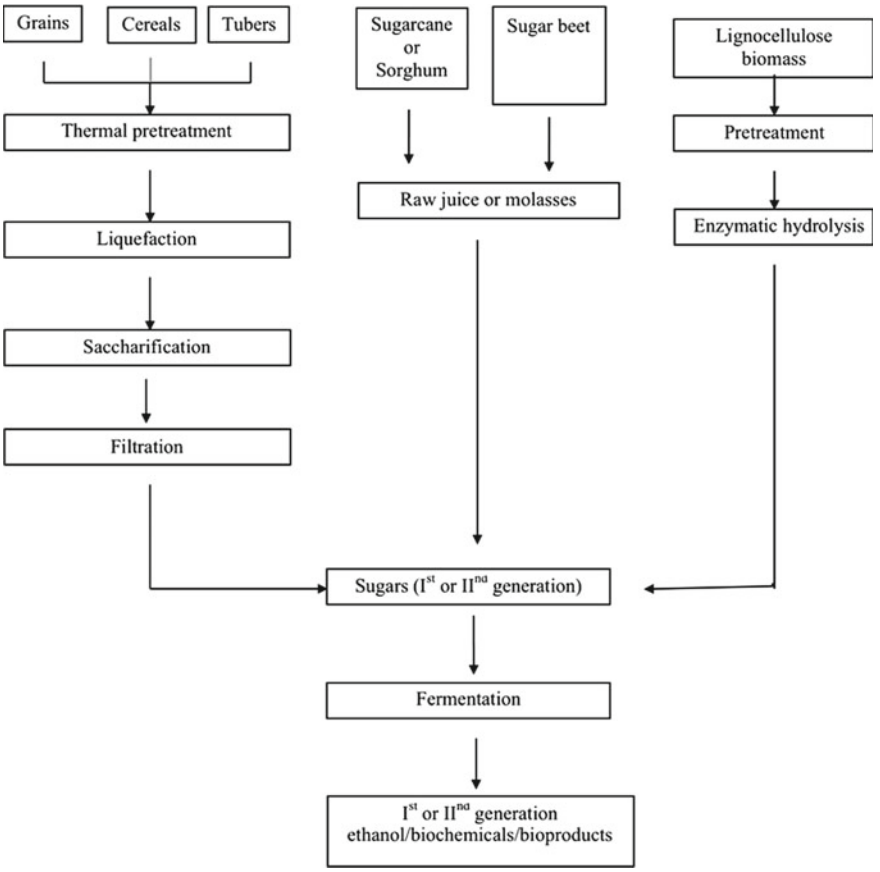

Fig. 3.2 Basic technology and main steps for bioethanol production from lignocellulosic biomass and starch-based feedstock

3.5 Types of Pretreatment Methods

3.5.1 Physical Pretreatment Approaches

Physical pretreatments techniques can be employed towards hydrolysis of lignocellulosic materials with restricted success. Examples of such methods are ball milling and grinding This method is one of the promising fields for future investigation as this pretreatment is one on which comparatively small investigation has been carried out in spite of being a cost-effective and eco-friendly process. Furthermore, waste materials can be comminuted by a combination of chipping, grinding and milling to reduce cellulose crystallinity. After chipping and milling/grinding, the size of the materials is usually in the range of 10–30 and 0.2–2 mm, respectively. Vibratory ball

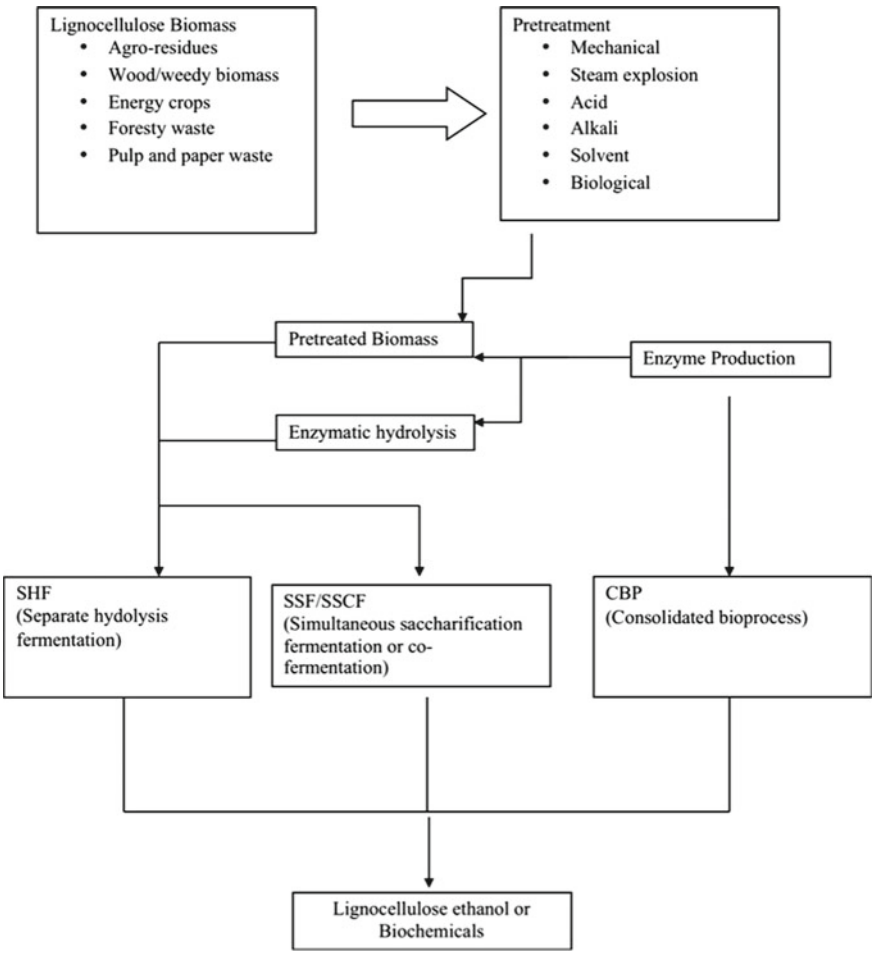

Fig. 3.3 Schematic representation of stages that result in the transformation of lignocellulose feedstock into bioethanol

milling aids in an effective hydrolysis of the cellulose crystallinity of spruce as well as aspen chips, thereby increasing the degradability of the biomass feedstock compared to conventional ball milling (Millet et al. 1976). The final particle size and the waste biomass characteristics are the determining factors of the power requirement of mechanical comminution of agricultural materials (Cadoche and Lopez 1989). Lignocellulosic materials can also be pretreated by pyrolysis (Kilzer and Broido 1965; Shafizadeh and Bradbury 1979).

The effectiveness of ultrasound towards the processing of vegetal substances is established (Vinatoru et al. 1999). Improvement is observed in the extractability of hemicellulose feedstocks (Ebringerova and Hromadkova 2002), cellulose (Pappas et al. 2002) and lignin (Sun and Tomkinson 2002) so as to obtain clean cellulose

fiber from exploited paper (Scott and Gerber 1995), when lignocellulosic biomass has been treated with ultrasound. It was found out that ultrasound supports saccharification processes (Rolz 1986). Sonication not only found to reduce cellulose enzyme needs by one third to half but also enhance bioethanol formation from blended waste office paper by almost 20% (Wood et al. 1997). The impact of ultrasound fragmentation of Avicel (acid treatment mediated production of microcrystalline cellulose) is equivalent to that of the enzymes for small incubation intervals (Gama et al. 1997). The time duration required towards ultrasonic treatment might be decreased with enhancement in irradiation power (Imai et al. 2004).

3.5.2 Chemical Pretreatment Methods

3.5.2.1 Alkaline Pretreatment

Bases like NaOH, KOH, $Ca(OH)_2$ as well as NH_4OH are exploited towards the alkaline lignocellulosic feedstock pretreatment. This pretreatment lead to hydrolysis of ester as well as glycosidic side chains and thereby, causing structural modification of lignin, cellulose distension, limited decrystallization of cellulosic material (Ibrahim et al. 2011) and restricted solvation of hemicellulosic materials (Sills and Gossett 2011). The NaOH damages the lignin assembly of the biomass, thereby enhancing the approachability of cellulase as well as hemicellulose enzymes (Zhao et al. 2008). The physical conditions for usually mild for alkaline pretreatment. It could be carried out at ambient physical conditions, nevertheless, prolonged pretreatment time durations are needed compared to higher temperatures. Most frequently employed alkali in the alkali pretreatment processes involve NaOH as well as $Ca(OH)_2$ (Aswathy et al. 2010; Hamelinck et al. 2005). However, pretreatment involving $Ca(OH)_2$ is preferred over NaOH as it is less expensive, more safer and could be effortlessly recovered from the hydrolysate through reaction with carbon dioxide (Mosier et al. 2005).

3.5.2.2 Acid Pretreatment Methods

Concentrated and diluted acids are used in acid pretreatment to hydrolyze the rigid assembly of the lignocellulose feedstock. Amongst acid pretreatment, dilute sulphuric acid, is the most commonly used acid that has been commercially exploited for the pretreatment of a wide-range of biomasses such as switchgrass, corn stover, spruce (softwood), and poplar (Taherzadeh and Karimi 2008). Strong acid facilitates towards the thorough hydrolysis of the constituents of biomasses into sugars, however, also needs huge quantity of concentrated sulfuric acid that could lead to the generation of an inhibitory byproduct such as furfural (Goldstein and Easter 1992). On the other hand, dilute acid enables decreased acid concentrations, nevertheless, needs higher temperatures and once more lead to furfural formation. Also, once

the acid pretreatment is done, succeeding enzyme mediated hydrolysis stage is occasionally not needed because the acidic environment causes the hydrolysis of biomass into fermentable sugars (Zhu et al. 2009). A blend of sulfuric acid with CH_3COOH led to 90% saccharification (De Moraes-Rocha et al. 2010). Hemicellulosic as well as lignin feedstocks are solubilized with marginal hydrolysis, and the hemicellulosic material is transformed into sugars as a result of pretreatment of acid. However, the main disadvantage associated with acid pretreatment approaches involve the price of acid as well as the need for neutralization of the acid after treatment.

3.5.2.3 Wet Oxidation

Wet oxidation exploits oxygen as a platform for the oxidization of substances dissolved in water. In this process drying and milling of lignocellulosic biomass (2 mm) followed by addition of water and biomass in the proportion of 1:6, respectively. This approach leads to fractionation of lignocellulose feedstock via dissolving hemicellulosic material as well as eliminating lignin content. This approach is reported to be efficient for pretreatment of a different types of biomasses like wheat straw, corn stover, sugarcane bagasse, cassava, peanuts, rye, canola, faba beans, and reed to obtain glucose as well as xylose after enzyme mediated degradation (Martin et al. 2008; Banerjee et al. 2009; Ruffell et al. 2010). During the process of wet oxidation, lignin undergo decomposition leads to CO_2, water and carboxylic acids formation. Wet oxidation helps in removing a dense wax covering bearing silica as well as protein on the biomass like straw and reed including other cereal crop remains (Schmidt et al. 2002; Azzam 1989). Bjerre et al. (1996) synergized wet oxidation and alkaline hydrolysis of wheat straw (20 g straw per liter, 170 °C, 5–10 min), and achieved 85% conversion yield of cellulose to glucose.

3.5.3 Physicochemical Pretreatment Methods

3.5.3.1 Steam-Explosion

Steam-explosion pretreatment involves physicochemical approaches for the hydrolysis of the structure of lignocellulose feedstock (McMillan 1994). In this technique, substance are initially exposed under high pressures as well as temperatures for a small time duration followed by quick depressurization of the system that lead to hydrolysis of cellulose micro-fibrils structure. With the disruption of the microfibrils, accessibility of the cellulose to the enzymes increases in the course of breakdown/degradation (Ballesteros et al. 2006).

3.5.3.2 Liquid Hot Water

Liquid hot water pretreatment approach exploits water under higher temperature as well as pressures for retaining its liquid state so as to provoke breakdown along with separation of the lignocellulose matrix. In this approach, the temperature may fluctuate from 160 to 240 °C over time in the range of a few minutes to an hour. Furthermore, it is the temperature that regulate the kinds of sugar generation, while time duration controlling the quantity of sugar production (Yu et al. 2010). The present approach depicts benefit pertaining to price standpoint, where no additives like acid catalysts are needed.

3.5.3.3 Ammonia Fiber Explosion (AFEX)

The AFEX approach is also based on physicochemical process analogous to steam explosion pretreatment technique. In this strategy, the biomasses are exposed with liquid anhydrous ammonia at higher pressures as well as modest temperature followed by rapid depressurization. This process is cost-effective as the moderate temperatures in the range of 60–100 °C are employed, therefore, facilitating lesser energy input with decrease in total price (Chundawat et al. 2007).

3.5.3.4 Ammonia Recycle Percolation

In ammonia recycle percolation strategy, aqueous ammonia with the concentration in the range of 5–15% (wt%) is allowed to move over a packed bed reactor holding the lignocellulose feedstock at a rate of approximately 5 ml/min. It is far better than AFEX as it is able to eliminate a huge amount of lignin content (that reaching up to 75–85%) and dissolve more than half of the hemicellulosic material (50–60%) while retaining higher cellulosic material (Kim and Lee 2005). With this process, about 60–80 and 65–85% lignin content found to remove from corn stover and switchgrass, respectively (Iyer et al. 1996).

3.6 Biological Pretreatment Methods

Microbial enzymes are exploited in biological pretreatment for removal of lignin content from lignocellulose feedstocks and found to be advantageous as it is eco-friendly, demands low-energy and minimizes the waste generation. In this approach, microbes like brown-, white- as well as soft-rot fungi are utilized to hydrolyze the lignin along with the hemicellulose content of biomass feedstocks. White-rot basidiomycetes like *Penicillium chrysosporium* found to non-specifically hydrolyze lignin as well as carbohydrate (Anderson and Akin 2008). Considering this, *P. chrysosporium* was effectively exploited towards the pretreatment of cotton stalks as a result of

solid state cultivation (SSC), where it was observed that the fungal organism allows the biomass transformation to bioethanol (Shi et al. 2008). Cellulose acts as substrate for brown-rots, whereas white- and soft-rots capable of exploiting cellulose and lignin as substrates. Remarkably, white-rot fungal species are the utmost efficient basidiomycetes towards pretreatment of lignocellulosic biomasses. Furthermore, *Phlebiaradiata*, *P. floridensis* and *Daedalea flavida* etc. are other basidiomycetes that specifically hydrolyze lignin content of wheat straw and are good alternatives towards removal of lignin content from lignocelluloses. *Ceriporiopsis subvermispora*, nevertheless, failed to produce cellulases but capable to synthesize manganese peroxide as well as laccase that specifically hydrolyze lignin content of various wood species. The benefits of this bio-pretreatment process involves requirement of lower-energy as well as mild environmental conditions. Associated demerit is that the rate of hydrolysis in most biological pretreatment processes is very low (Sun and Cheng 2007; Yang and Wyman 2008).

Parawira and Tekere (2011) reported that the non-selectivity of acid treatment leads to the generation of not only complex sugars but also other substances, which have inhibitory effect on the microbes towards bioethanol formation. Xylose constitutes the main fraction, while arabinose, mannose, galactose, and glucose constitute the minor fractions apart from inhibitors of microorganisms in the course of the depolymerization of hemicellulose as a result of chemical process (Chandel et al. 2013). Such inhibitors were classified into following catogories: (i) organic acids like acetic, formic and levulinic acids, (ii) furan derivatives such as furfural and 5-hydroxymethylfurfural (5-HMF)] and (iii) phenolic compounds. Overall, cell physiology is affected by these inhibitors, which often results in decreased viability along with bioethanol yield as well as productivity (Chandel et al. 2007). These toxic components affects the rate of sugar uptake that ultimately hinder the microbial growth with instantaneous degradation of product formation. The physiology of microbial cells are also affected with these inhibitors as they disturbs the role of biological membranes affecting the growth of organisms that lingers towards prolonged incubation time with reduced generation of metabolite. Nevertheless, the yield content might remain the unchanged (Chandel et al. 2013). Furfurals (sugar derived inhibitors) inactivates the cell replication, which decreases the rate of growth as well as the cellular mass production on ATP including volumetric growth rate as well as specific productivities. Under aerobic condition, furfurals have been found toxic to *Pichia stipitis*, while the cultivation of *Saccharomyces cerevisiae* was influenced by little extent under anaerobic environment as a result of transforming into furoic acid. Furans, i.e. furfurals as well as 5-HMF in association with CH_3COOH found to be extremely appropriate towards the cultivation of *P. stipitis* and *Pachysolen tannophilus* including *E. coli* (Martinez et al. 2000). The ethanol producing microbial species found to depict promising capability towards hydrolyzing some inhibitors (Mussatto and Roberto 2004). Among the specific detoxification approaches that were studied previously, the ion exchange resins, active charcoal, enzymatic detoxification utilizing laccase, alkali treatments as well as overliming with $Ca(OH)_2$ are comprehensively investigated (Fonseca et al. 2011).

3.7 Biotechnological Advancements

3.7.1 Microbial Production of Cellulases and Enzymatic Hydrolysis of Pretreated Substrates

Enzymes are the bioproduct of prime importance in many areas such as industrial, environmental and food technology. Currently, the development and advances in biotechnology resulting into novel applications pertaining to enzymes. Filamentous fungi are better producers of commercially important enzyme when compared with those obtained from yeast and bacteria. *Aspergillus niger* and *Tricoderma viridae* are the most important and safe organisms for industrial use and potent producers of cellulases. These organisms are the important source of extracellular enzymes with homologous and heterologous proteins due to their high capacity of protein secretion machinery. Hyphal development of filamentous fungi allows them to effectively colonize and penetrate the solid substrate and hence these fungi play a pivotal role in solid state fermentation. Remarkably, *A. niger* is a potent producer of specialized enzymes like pectinases, cellulases, hemicellulases and xylanases (de Vries et al. 2002; de Vries 2003) in its natural environment.

Enzymatic hydrolysis is an economically feasible process that converts cellulose to easily fermentable low-cost sugars (Kotchoni et al. 2003). Naturally available cellulosic material is an linear homopolysaccharide composed of D-glucose residues, which are connected through β-1,4-glucosidic linkages. The smallest repeating monomeric unit in cellulose is cellobiose that made up of two glucose residues. Cellulose is regarded as a valuable resource largely because it can be hydrolysed into soluble cellobiose and glucose sugars when β-bonds are broken. The aerobic fungi *T. reesei, T. viride, T. koningii, Penicillium pinophinum,* etc. are the important producers of cellulase (Murashima et al. 2002). Cellulase is also produced by some thermophylic aerobic fungi like *Chaetomium thermophile, Humicola insolens, Thermocola moidea,* etc. and mesophilic anaerobic fungi represented by *Neocallimastix frantalis, Paromonas communis,* etc. (Mach and Zeilinger 2003).

So far, the cost of cellulase production is one of the major hurdle for exploitation of cellulose. High yielding cellulolytic organisms, locally available cheap raw material and optimizing culture conditions are the solutions to combat above mentioned problem, as these strategies could increase the productivity of cellulase production. With the advances in industrial biotechnology, it is now possible to economically utilize the agro-industrial residues, particularly those originating from tropical regions such as sugarcane bagasse, cassava bagasse, wheat bran, rice bran, sugar beet pulp and apple pomace, etc. These agro-wastes are proved to be the best substrates for solid state fermentation (SSF) processes (Pandey 1999). Overall, SSF involves the growth of microorganisms on moist solid substrates in the absence or near absence of free-flowing water (Ellaiah et al. 2004). Solid substrate resembles natural habitat of micoorganisms where they may be able to produce certain enzymes, metabolites, proteins and spores more efficiently than in submerged fermentation. Wheat bran is

a familiar as a complete medium for producing cellulases, amylases and xylanase in solid substrate fermentation (Smits et al. 1996; Roussos et al. 1991).

3.7.2 Hydrolysis of Pretreated Biomass

With the help of cellulases and hemicellulases, the liberated cellulose as well as hemicelluloses after pretreatment are converted into hexoses and pentoses, respectively. This process is a main obstacle towards biofuel generation and innovative biotechnological advancements are required for enhancing their efficacy that could make this process cost-effective towards ethanol generation. Corrosion problems are eliminated by the application of enzyme (cellulase) over acid and, therefore enabling smaller maintenance prices along with mild processing environments towards higher productions. Nevertheless, the biomass recalcitrance of plant cell walls and insufficient quantities of one or more lignocellulolytic enzymes needed towards effective transformation of lignocellulose feedstocks into fermentable sugars produced by fungal strains are rate-limiting steps for the biotransformation of lignocellulose feedstocks into bioethanol (Himmel et al. 2007). These constitute one of the major noticeable bottlenecks towards the generation of economical cellulosic bioethanol. Thus, enhancing the fungal mediated hydrolysis of lignocellulose feedstocks together with exploring stable enzymes with ability to withstand harsh environments has been turn out to be a main concern in various recent investigations.

3.7.2.1 Fungal Extracellular Cellulases

Extensive research has been carried out by various researches on the enzyme mediated hydrolysis of various lignocellulose feedstocks like sugarcane bagasse, corncob, rice straw, etc. by cellulases towards biofuel generation (Kuhad et al. 2010). Interestingly, bacterial as well as fungal strains/species are the promising synthesizer of glucanases (cellulases), which degrade lignocellulose feedstocks. These microbial strains/species may be aerobic or anaerobic as well as mesophilic or thermophilic. The important genera of bacteria that are potent cellulase producers are *Clostridium*, *Cellulomonas*, *Bacillus*, *Thermomonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, *Acetovibrio*, *Microbispora*, and *Streptomyces*. Anaerobic bacterial strains/species like *Clostridium phytofermentans*, *Clostridium thermocellum*, etc. are anaerobic producers of cellulases that associated with high specific activity. Although, the most industrial/commercial level production of glucanases (cellulases) is carried out with *Trichoderma ressei*, while the production of β-D-glucosidase enzyme is carried out with *A. niger* (Kaur et al. 2007). However, amongst the fungi, *Trichoderma* strains/species are comprehensively investigated towards the production of cellulase enzyme.

3.7.2.2 Fungal Hemicellulases

Several different enzymes are needed to hydrolyze hemicelluloses, due to their heterogeneity. Xylan is the major component of hemicellulose, which contributes over 70% of its structure. The β-1,4 linkages of xylan are hydrolysed by xylanases and give rise to oligomers that are further degraded to xylose in the presence of β-xylosidase enzyme. Nevertheless, supplementary enzymes like β-mannanases, arabinofuranosidases or α-L-arabinases are required when the hemicellulosic feedstock associated with ample amount of mannan or arabinofuranosyl. Furthermore, analogous to cellulases, majority of the hemicellulase enzymes are glycosyl dehydrolases (GHs). However, several hemicellulase enzymes that fit into the group of carbohydrate esterases (CEs) found to carry out the hydrolysis of ester bonds of acetic or ferulic acid side groups (Shallom and Shoham 2003). It has been bserved that a mixture of hemicellulase or pectinase enzymes with cellulase enzymes exhibits a substantial enhancement in the extent of cellulosic feedstock transformation. Several species of fungi like *Trichoderma*, *Penicillium* and *Aspergillus* are found to synthesize considerable quantities of extracellular cellulase and hemicellulase enzymes.

3.7.2.3 Fungal Ligninases

Fungal strains/species are able to hydrolyze lignin content through liberating enzymes that are known as 'ligninases'. These involve two ligninolytic families: (i) phenol oxidase (laccase) and (ii) peroxidases (lignin peroxidase as well as manganese peroxidase) (Martinez et al. 2005). Interestingly, the *Coriolus versicolor*, *P. chrysosporium* and *T. versicolor*, which are the white-rot basidiomycetes have been found to be the most efficient lignin-degrading microorganisms.

3.7.2.4 Ethanologenic Microorganisms

The *S. cerevisiae* is not only the yeast of historic evidence but also preferred one for most ethanol fermentation, where ethanol production reach as high as 18% of the fermentation broth. Simple sugars like glucose as well as the disaccharide sucrose are needed for this yeast to grow. *Saccharomyces* is ideal for producing alcoholic beverages as it is usually accepted as safe as a food additive for human consumption. Furthermore, this organism does not depict many of the bottlenecks similar to bacterial strains/species. The only limitation with *S. cerevisiae* is that it is not able to ferment xylose. To solve this limitation, metabolic engineering of xylose fermentation in *S. cerevisiae* is an attention-grabbing strategy (Sonderegger and Sauer 2003). On the other hand, *Zymomonas mobilis*, is not only safe, but also depict simple nutritional needs. Some researchers reported it as superior over *S. cerevisiae*. Despite a potent ethanologen, *Z. mobilis* is not well suited towards all of the biomass feedstocks transformation as it has the potential to ferments merely glucose and fructose including sucrose. However, *Z. mobilis* prefers glucose over fructose/sucrose. Thus,

the specific rates of glucose utilization as well as bioethanol generation are found to be highest on glucose medium as sole carbon source (Lin and Tanaka 2006).

Apart from aforementioned organisms, the yeast *Pichia stipitis* is also one of the known pentose fermenting organisms and is most promising for industrial applications. The *P. stipitis* is one of the finest explored xylose-fermenting organism that have a substrate range involving all the monomeric sugars of lignocellulosic feedstock (Watanabe et al. 2011). *P. stipitis* is not only able to utilize CH_3COOH but also decrease the furan ring of furfural as well as HMF, which generates an opportunity for this organism to remove some toxic substances during transformation of cellulose feedstock. Among yeasts, *P. stipitis*, *Candida shehatae* and *Pachysolen tannophilus* are potential organisms that capable to ferment xylose. Yeasts capable to exploit xylose using xylose reductase, which transforms xylose into xylitol, followed by xylitol dehydrogenase that transform xylitol into xylulose. Furthermore, the resulting xylulose metabolized as a result of phosphorylation through the pentose phosphate route. Amongst the wild type yeasts fermenting xylose, *P. stipitis* was found to be most promising (Agbogbo and Coward-Kelly 2008) as it has a xylose reductase with capability to exploit both NADPH as well as NADH as cofactor. Thus, xylose fermentation is carrying out with *P. stipitis* using NADH under anaerobic environments.

Kluyveromyces marxianus depicts several advantages and, therefore, drawn a special attention when an organism is chosen for ethanol production, as this organism not only transform a variety of biomass feedstocks together with xylose into bioethanol but also effectively utilize range of feedstocks under high temperature. Thermotolerant enzymes like cellobiohydrolase, endoglucanase and β-glucosidase encoding genes were expressed in combination with *K. marxianus*. Reports indicated that *K. marxianus* was genetically engineered to show *T. reesei* endoglucanase II as well as *Aspergillus aculeatus* β-glucosidase on the cell surface (Yanase et al. 2010). Likewise, Sanchez et al. (1999) has developed a xylose fermenting yeast, i.e. *Pachysolen tannophilus* for industrial level ethanol production. In this investigation, UV light-induced mutants of *P. tannophilus* were isolated, which found to grow quicker on xylose. On the other hand, Sharifia et al. (2008) have explored the potential of the fungus *Mucor indicus* for faster ethanol production with an average productivity of 0.90 g/l h from glucose, fructose and inverted sucrose. This production was found to be far better than the filamentous form with an average productivity of 0.33 g/l h.

3.8 Biotechnological Advancements

3.8.1 Strategies Used to Improve Fungal Enzyme Production

For the production of commercial/industrial level of bioethanol, the fermentative microbial strain/species requires to be robust. The economical production of ethanol depends on the effective exploitation of all the sugars produced as a result of hydroly-

sis of lignocellulose feedstocks. The conventional ethanol fermenting yeast (*S. cerevisiae*) or bacterium (*Z. mobilis*) have a limitation that they cannot convert multiple sugar substrates to ethanol. Furthermore, development of a suitable microbial strain towards the fermentation of a blend of sugars like glucose, xylose, arabinose, and galactose for the cost-effective and commercial production of bioethanol is another challenge that needs to be addressed. Over the last 25 years, efforts are directed towards developing a number of recombinant microbial strains/species like *E. coli*, *Klebsiella oxytoca* and *Z. mobilis* including *S. cerevisiae* with an aim to ferment blended sugars into bioethanol. Saha and Cotta (2011) has produced a recombinant *E. coli* (strain FBR5), which is capable of fermenting mixed multiples sugars to ethanol. They have engineered, the plasmid pLOI297 into this strain that carries genes of pyruvate decarboxylase (pdc) as well as alcohol dehydrogenase (adh) from *Z. mobilis* that are essential towards effective transformation of pyruvic acid into bioethanol. Based upon the recent biotechnological advances, it is evident that new technologies need to introduce along with further improvement in the existing technologies for effective biological transformation of lignocellulose feedstocks into bioethanol and other value-added products, so as to make biofuels price cost-effective over other energy resources like fossil fuels. Such recent biotechnological advances occurring in strategies like mutagenesis, co-culturing and heterologous gene expression of cellulases with the intention to improve in the production/activity of fungal enzymes, which are discussed below.

3.8.2 Mutagenesis

Site-directed mutagenesis has a pivotal role towards the characterization as well as improvement of cellulases together with their putative catalytic as well as binding residues. With the use of site-directed mutagenesis, it was detected that Glu 116 and 200 are the catalytic nucleophile and acid-base residues, respectively in *Hypocreaje corina* (anamorph *T. reesei*) Cel12A. In this investigation, mutant enzymes were developed in which Glu was substituted with Asp or Gln at each location (E116D/Q and E200D/Q). The specific activity of these mutants was decreased by over and above 98%, signifying the important function of these two residues in the catalytic function of the enzyme. In another study, the thermostable endo-1,4-β-xylanase (XynII) mutants from *T. reesei* were further mutated to resist inactivation under higher pH by using site-directed mutagenesis. All mutants were found to resistant towards thermal inactivation under alkaline pH. For instance, thermotolerance for one mutant (P9) at pH 9 was improved around 4–5 °C, which depicting enhanced activity in sulphate pulp bleaching over control (Fenel et al. 2006). Also, the catalytic effectiveness as well as optimal pH of *T. reesei* endo-β-1,4-glucanase II were enhanced as a result of saturation mutagenesis followed by random mutagenesis and two rounds of DNA shuffling. The pH optimum of the variant (Q139R/L218H/W276R/N342T) was shifted from 4.8 to 6.2, whereas the enzyme activity was enhanced over and above 4.5-fold (Qin et al. 2008). Besides, the stability of *T. reesei* endo-1,4-β-xylanasesII

(XynII) was improved by engineering a disulfide bridge at its N-terminal region. In fact, two amino acids (Thr-2 and Thr-28) in the enzyme were replaced with cysteine (T2C:T28C mutant) that causing a 15 °C enhancement in thermostability.

3.8.3 Co-cultivation

Fungal co-culturing is an effective approach that not only increase degradation of lignocellulose feedstocks, but also increases product exploitation. This approach reduces the requirement of supplementary enzymes in the biotransformation phenomenon. Occurrence of all the three constituents of enzyme (EG, CBH and β-glucosidase) in large amounts is mandatory for the effective degradation of cellulose. There are many hurdles to fulfill this demand as the best mutants of fungal strains are not capable to generate ample amounts of the enzymes at the same time. Interestingly, *T. reesei* is an efficient producer of CBH and EG, however, its β-glucosidase activity is rather low. Reverse of this condition associated with *A. niger* that can efficiently produce huge quantities of β-glucosidase but has restricted EG constituents (Kumar et al. 2008). Care also has to be taken pertaining to hemicellulose hydrolysis when lignocellulose feedstocks are exposed for biomass transformation. The pretreatment approaches play a pivotal role under these conditions. An alkali pretreatment method efficiently removes lignin and thus, hemicellulosic feedstock has to be hydrolysed using hemicellulases, while in acid-catalyzed pretreatment, the hemicellulosic layer will undergo hydrolysis (Hahn et al. 2006). Fungal strains differ from each other with respect to their efficiency to produce either hemicellulolytic/cellulolytic enzymes together with efficiency to hydrolyze hemicellulosic/cellulosic regions. It is feasible to convert both cellulose as well as hemicellulose hydrolytic products in a single step by co-culturing two or more compatible microbial strains/species with the capability to exploit these feedstocks. This consortia of lignocellulolytic microorganisms works naturally to degrade lignocellulosic residues.

3.8.4 Metabolic Engineering

Metabolic engineering is an effective approach for the improvement, redirection, or generation of new metabolic reactions or entire routes in microbial strains/species. Undesirable pathway(s) could be blocked by modifying metabolic flux and/or the metabolic flux of desirable pathway(s) can be enhanced. For instance, the application of homologous recombination approach resulted into enhanced synthesis of *T. reesei* β-glucosidase I by exploiting xylanase (xyn3) as well as cellulase (egl3) promoters that increased β-glucosidase activity by 4- and 7.5-fold than the parent, respectively. This will facilitate fungus like *T. reesei* to be more effective towards the breakdown of cellulosic feedstock into glucose that enhance the yield and consequently, minimize the overall production price (Rahman et al. 2009). Similarly, the engineering

of *S. cerevisiae* strain resulted into development of recombinant strain with ability to exploit L-arabinose (pentose sugar) for growth followed by ethanol fermentation (Becker and Boles 2003). Economically viable biomass feedstock into ethanol fermentation is only possible if the feedstock fermentation choice of *S. cerevisiae* is expanded to involve pentoses. *Thermoanaerobacterium saccharolyticum*, which is a thermophilic anaerobic organism with potential to generate bioethanol by utilizing xylan and biomass-derived sugars, was engineered by Shaw et al. (2008).

3.8.5 Heterologous Expression

Improvement in the generation of enzymes along with the activity of an enzyme is two powerful outcomes of heterologous expression. Depending upon the requirement towards a functional cellulase system, various fungal cellulases associated with higher and/or specific activity have been cloned and expressed. This will form a robust lignocellulolytic fungal strain. For instance, thermostable β-glucosidase (cel3a) belonging to fungal strain *T. emersonii* was expressed in *T. reesei* RUT-C30 by employing a strong *T. reesei* based cbh1 promoter. The expressed enzyme was not only extremely thermostable with optimal temperature of 71.5 °C but had high specific activity too (Murray et al. 2004).

3.8.6 Immobilization

Technical and economic advantages are the two major outcomes of immobilizations of microbial cells and enzymes over free cell system. Exploiting immobilized enzymes not only generates a pure product but is also a cleaner process that generates cost-effective and recoverable. The immobilized biocatalysts have been extensively studied during last few decades. The generation of cellulosic ethanol was mediated by some researchers using immobilized cellobiase enzyme system for the enzymatic hydrolysis of biomass (Das et al. 2011). Similarly, immobilized lipase using porous kaolinite particle as a carrier was employed for production of alcohol as well as biodiesel fuel from triglycerides. Immobilization of yeast cell system towards fermentation of alcohol is an attention-grabbing and thrust investigation field as it provides additional technical as well as economic benefits over free cell system. The development of a protective layer and/or specific adsorption of ethanol by the support might assist to reduce end product inhibition as it reduces the ethanol concentration in the direct environment of the microorganism. Immobilized yeast cell systems are able to function with high productivity at dilution rates surpassing the maximal specific growth rate that result in enhance production of ethanol with cellular stability. Reduction in process expenditures owing to the cell recovery as well as reuse are another advantage of this process (Lin and Tanaka 2006). There are four categories of promising approaches for yeasts immobilization: Firstly, yeast

can be attached or adsorbed to solid surfaces like wood chips, delignified brewer's spent grains, DEAE cellulose, and porous glass. Secondly, yeast can be entrapped within a porous matrix of calcium alginate, k-carrageenan, polyvinyl alcohol, agar, gelatine, chitosan, and polyacrylamide. Thirdly yeast can be mechanically retained behind a barrier of microporous membrane filters, and microcapsules. Fourth way is of self-aggregation of the cells by flocculation (Ivanova et al. 2011).

3.9 Bioethanol Recovery from Fermented Broth

The recovery of water-free ethanol after the fermentation of cellulosic sugars is one of the most important factor that affects the overall cost and economics of ethanol. A number of innovative distillation approaches like per-evaporation, supercritical solvent extraction, mechanical vapor compression, membrane assisted or molecular sieve adsorption might be the finest technologies towards recovery of ethanol from a fermented cellulosic sugar solution (Widgren et al. 2008). According to Keller and Bryan (2000) distillation is still a 'formidable competitor' as a main separation technique even although considerable investigation has been carried out on its alternatives. The first option of industry regarding separation of a liquid mixture is distillation, especially simple distillation; other approaches together with complex distillation, e.g., azeotropic distillation are in use merely if simple distillation is considered to be precisely non-viable or economically non-feasible owing to usually three huge stainless steel distillation towers, stainless steel heat exchangers and expenditure of stainless up 400% in last six years, high operating prices as 280 million metric British thermal units energy is consumed (100 million gallons per year ethanol). Mole sieve drying contributing towards energy prices and that is why energy prices up considerably with cost of crude oil. In some conditions, retrofitting of a persisting process can be reasonably far more feasible over developing a new process, particularly when the monetary capitals are inadequate and/or when short term requirements are to be come across in a tight time limit. The modeling as well as optimization of the process employing MINLP tools revealed 12% savings towards the production prices considering a 32% enhancement in membrane area with the decrease in both reflux ratio as well as ethanol concentration in the distillate of the column (Szitkai et al. 2002).

3.10 Conclusion

It is anticipated that cost-effective lignocellulose feedstock will turn out to be the major platform towards bioethanol generation in the near future. Also, supplementary waste materials could be screened as well as exploited as feedstocks so as to meet the requirements pertaining to large- scale bioethanol production. In addition, biotechnological methodologies together with systems biology as well as com-

putational tools are anticipated to be promising platforms for overcoming problems related to bioethanol yield and productivity. Furthermore, future inclinations concerning decrease of prices should involve development of more effective and competent biomass pretreatment approach, improvement of recombinant microbial strains/species towards efficient utilization of all the sugars liberated in the course of the pretreatment including hydrolytic processes, and further development of co-generation system. Unquestionably, process strengthening as a result of incorporation of various phenomena with unit operations and the application of amalgamated bioprocessing of various substrates to bioethanol, which needs the production of tailored recombinant strains/species will provide the most noteworthy results in the course of the exploration of the effectiveness in bioethanol generation. These will pave the way towards qualitative improvement towards the commercial generation of fuel bioethanol in the future.

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Chapter 4
Sustainable Production of Biofuels from Weedy Biomass and Other Unconventional Lignocellulose Wastes

Anurup Adak, Surender Singh, A. K. Lavanya, Anamika Sharma and Lata Nain

4.1 Introduction

Sustainable energy availability is one of the most critical factors for world's economic growth. Most of the countries in the world rely on various fossil fuel sources to meet their energy demand. However, with ever increasing urbanization, transportation, industrialization and depleting fossil fuel resources the global energy demand has increased tremendously leading to energy crisis. Moreover, gradual increase of air pollution followed by global warming over recent years reignited public interest to exploit renewable sources such as lignocellulosic biomass. In addition, global economic downturn offers an opportunity to cultivate the green technology while costs are lower and green growth is the only desired future for growth and progress of human society. This world energy crisis has shifted the global attention towards development of sustainable technologies including solar energy and biofuels (Weldemichael and Assefa 2016; Liu et al. 2015). One of the most commercially available bioenergy strategies is production of bioethanol using sugar crops such as sugarcane, sugar beet and grain crops, such as corn, wheat etc. (Pimentel and Patzek 2005; Tiffany 2009; Dziugan et al. 2013). However, exploitation of these grains or fodder crops for bioenergy purpose may create global food crisis (Li and Khraisheh 2010). With the concern of the growing world population and to avoid *food vs fuel* controversy, some alternatives such as weedy lignocellulosic (LC) biomass or non-fodder agricultural waste biomass are seen as alternative feedstocks for biofuel production (Al-Hamamre et al. 2017; Zabed et al. 2016).

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India is amongst the rapidly expanding economies and is facing a formidable challenge to meet its energy needs to support its growing population. India needs to generate two to three fold more energy than the current output (Gopinathan and Sudhakaran 2009) to meet its demand in the future. Throughout the last decade, ethanol consumption grew from 1.8 billion litres to 2.4 billion litres in 2016, and will continue to increase in 2017 to 2.5 billion litres. The consumption basket will comprise 700 million litres for fuel ethanol and 1.8 billion litres for the industrial and chemical sectors (Aradhey 2016). At present, sugar and starch based raw materials along with cereal grains are used for the production of bioethanol. In India, population has already reached a billion and thus food security is a national priority and hence India cannot afford to use food resources for ethanol production as is commonly done in other biofuel promoting countries like Brazil, Europe and USA. So, the available sources are plant biomass which is an abundant and renewable source of energy alternate which can be efficiently converted by microbes into biofuels of which, bioethanol is widely produced on an industrial scale today.

In search for the expansion of bioethanol production without compromising food security, the use of lignocellulosic materials such as surplus crop residues has been encouraged. Globally India ranks first in the production of jute and second in rice, wheat, sugarcane, cotton and ground nut. Thus, because of the agricultural strength of the country, crop residues production in the country is also huge that may serve as a feedstock for the production of second generation biofuels (Gupta and Verma 2015; Dashtban et al. 2009; Guerriero et al. 2016). Around 500–600 MT of lignocellulosic biomass is generated annually in India of which 40–50% is estimated as surplus for bioenergy generation (Hiloidhari et al. 2014). Among the crop residues, the fibre crop biomass may also serve as a sustainable lignocellulosic resource for biofuel production. Fibre crops like cotton, jute, mesta, sun hemp are widely grown in India. These fibre crop biomasses have high cellulosic content coupled with low lignin that fit well in biofuel production systems. The net residue availability of fibre crop residues for biofuel production is about 20.5 MT (Ravindranath et al. 2011). Because of its high biological efficiency, high biomass content and wide ecological adaptability these fibrous crops have high growth rate, low lignin and wide ecological adaptability that can supplement the feedstock supply for second generation bioethanol production.

In addition to conventional crop biomass, waste biomass in the form of noxious invasive weeds could be the potential feedstock for sustainable biofuels production. With high fecundity, these plants produce huge biomass within a short period of time. Recently weedy lignocellulosic plants such as *Eichhornia crassipes*, *Lantana camara*, *Saccharum spontaneum*, *Prosopis juliflora*, *Ricinus communis* and *Parthenium hysterophorus* has been explored as promising and cheaper biomass for fuel ethanol (Chandel et al. 2009; Singh et al. 2014). These plant species have infested millions of hectares of arable and degraded (or infertile) land, leading to enormous monetary losses due to reduction in crops and forage yields. The actual biomass produced by these noxious weeds is in the range of 15–20 tons per hectare. Nonetheless, these biomasses can form feedstock for bioethanol due to their significant holocellulose content, which can be hydrolysed to produce fermentable monomeric sugars.

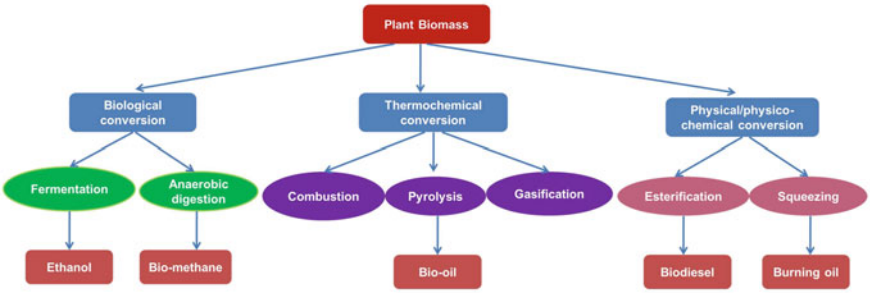

Fig. 4.1 Technological options for efficient fuel production from plant biomass

4.2 Technological Option and Limitations of Biofuel Production

A number of research groups around the world including India are continuously searching alternatives to meet the ever increasing energy demand. The abundant biomass available can be used to produce various types of fuels by using different technologies (Fig. 4.1). However, the renewable sources like lignocellulosic biomass have a major limitation of lack of continuous supply of feedstock throughout the year, as the resources are discrete in nature. Moreover most of the sources are over-estimated for this purpose. In India jathopha was considered as one of the most promising resources for biodiesel production, but later found unsuitable due to its high production cost (Swain 2014). Today USA and Brazil are the leading countries where renewable fuel technology has been successful. However most of countries have not such stipulation on biofuel production. In India bioethanol production is largely dependent on sugarcane molasses which are unable to meet 5% blending with gasoline while it was estimated that at the end of the year 2017, India can meet its ambitious target of 20% ethanol blending programme (EBP) (Aradhey 2016). Based on different technology platform world fuel ethanol production are gradually increasing and have capacities in the range 250–1500 tonnes biomass/day. However, the technologies deployed in biofuel production suffer from three major issues for a country like India: (a) the recommended scales of economy, i.e. biomass processing/day are in excess of 500 tonnes/day and thus are too large for India, where biomass production is mixed and unorganized (b) most technologies are feedstock-specific and will need pilot scale trials for different feedstocks (c) the capital costs are too high (Rs. 15–20/l ethanol over and above the variable cost of production, which is in the range Rs. 30–35/l). Alternative source of biomass like nonconventional fibrous lignocellulosic and noxious weedy biomass may be exploited to explore their potential as feedstock for bioethanol. Apart from technological aspects transportation of these biomass to the site of bioethanol production is the major bottleneck because of high transportation cost associated that makes the process economically unreliable.

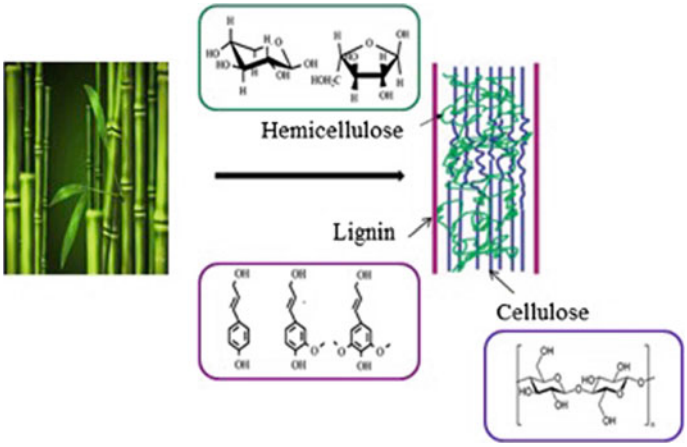

Fig. 4.2 Structural components of plant cell wall

4.3 Lignocellulosic Biomass Composition of Weedy Biomass

The primary building block of all the plants cell wall is cellulose, lignin, and hemicellulose (xylan, glucuronoxylan, arabinoxylan, or glucomannan), along with smaller amounts of pectin, protein, extractives (soluble non-structural materials such as sugars, nitrogenous material, chlorophyll, and waxes) and ash (Jørgensen et al. 2007). The cellulose fibrils are embedded in a network of hemicellulose and lignin. The cross-linking of this network results in the elimination of water from the wall and the formation of a hydrophobic composite that limits the accessibility of hydrolytic enzymes and is a major contributor to the structural characteristics of secondary walls. The composition of these constituents can vary from one plant species to another and the main component of the lignocellulosic biomass which can be used for biofuel production is cellulose and hemicellulosic portion (Fig. 4.2). In addition, the ratios between various constituents within a single plant vary with age, stage of growth, and other condition (Pérez et al. 2002).

4.3.1 Cellulose ($C_6H_{10}O_5)_n$

The main constituent of fibrous plant residues is cellulose, which is a structural polysaccharide that consists of a linear chain of glucose units joined by β (1–4) linkages and is responsible for conferring structural rigidity and strength to the cell wall. The individual glucan chains of cellulose are composed of 2000 to >25,000 glucose residues and is linked by a number of intra and inters molecular hydrogen bonds and is insoluble in water and most organic solvents. Because of the alternat-

ing spatial configuration of the glycosidic bonds linking adjacent glucose residues, the repeating unit in cellulose is considered to be cellobiose, a disaccharide. The individual glucans that make up the microfibril are closely aligned and bonded to each other to make a highly ordered (crystalline) ribbon that excludes water and is relatively inaccessible to enzymatic attack. Cellulose in biomass is present in both crystalline and amorphous forms. The major proportion of cellulose is crystalline form, whereas as small percentage of unorganized cellulose chains form amorphous cellulose. Amorphous form cellulose is more susceptible to enzymatic degradation (Béguin and Aubert 1994). In order to increase the digestibility of cellulose, large amounts of hemicelluloses must be removed as they cover cellulose fibrils limiting their availability for the enzymatic hydrolysis.

4.3.2 Hemicellulose $(C_5H_8O_4)_n$

It is the second most common carbohydrate constituents in fibrous plant located in secondary cellwall containing heterogeneous branched biopolymers, pentoses (xylose, arabinose), hexoses (mainly mannose, less of glucose and galactose) and uronic acids (glucuronic, methyl galacturonic and galacturonic acids). Mannose and xylose are the dominant pentose sugars in soft wood, hard wood and agricultural residues. The most important biological role of hemicelluloses is to strengthen the cell wall by interaction with cellulose and, in some walls, with lignin. They are relatively easy to hydrolyze because of their amorphous and branched structure (with short lateral chain) as well as their lower molecular weight. The pretreatment parameters such as temperature and retention time must be controlled to avoid the formation of unwanted products such as furfurals and hydroxyl methyl furfurals which later inhibit the fermentation process.

4.3.3 Lignin $[C_9H_{10}O_3(OCH_3)]_n$

It is the third most common aromatic heterogeneous polymer in fibrous plant residue. It is the integral component of the cell walls of plants and its content increases as the plant ages. The phenylpropane is the major constituent of lignin that consists primarily of syringyl, guaiacyl and p-hydroxyphenol linked together by a set of linkages to make a complicated matrix. As lignin forms in the wall, it displaces water from the matrix and forms a hydrophobic network that tightly bound to cellulose and prevents wall enlargement and acts as structural strength to the biomass fibres, reduces the susceptibility of walls to attack by pathogens and act as a barrier to enzymes or solutions (Pérez et al. 2002). The most important characteristic of lignin is that it is most recalcitrant component of lignocellulosic biomass (Hamelinck et al. 2005).

4.4 Available Bioresources for Sustainable Biofuel Production

4.4.1 Agricultural by Products

Better understanding of agro residue availability and utilization of the surplus crop residue has given a route to conquer the food-fuel controversy over the fuel ethanol production. The production of renewable energy from this biomass through the enzymatic hydrolysis route could complement these as well other known eco-friendly energy production strategies. The major agricultural feedstocks used for bioethanol production are mainly from sugarcane, wheat straw, rice straw, corn stover etc. (Kim and Dale 2004). The current annual availability of biomass in India is estimated around 500 million metric tons, which includes agricultural and forest biomass (MNRE). The biomass generated from major agricultural crops of India is listed in Table 4.1. However, as of now only small amount of the lignocellulosic biomass is effectively used and rest goes as waste. The residues generated after harvesting is left in the field are usually burnt by farmers causing environmental pollution that can be diverted to bioethanol refineries.

4.5 Weedy Lignocellulosic Biomass

The most common biomass used for biofuel production are from monoculture crops grown on fertile soil such as sugarcane, corn, switchgrass and hybrid poplar along with other agricultural waste. Recently weedy lignocellulosic such as *Eichhornia crassipes, Lantana camara, Saccharum spontaneum, Prosopis juliflora, Ricinus communis* and *Parthenium hysterophorus* has been explored as promising and cheaper biomass for fuel ethanol (Pandiyan et al. 2014; Singh and Poudel 2013). The morphology of the common weed plants is depicted in Fig. 4.3. The characteristics like high growth rate, wider climatic adaptability and minimum nutrient requirement makes these weedy species a potential renewable source of lignocellulosic biomass for ethanol production. In the following section, general description of the common weeds and their potential availability for biofuel production has been described.

Lantana camara

Lantana camara, commonly known as red sage, is the member of Verbenaceae family mainly found in tropical regions of Central and South America which are being considered as an ornamental plant. The shrub's taxonomic position is defined as belonging to the class of magnoliopsida, order lamiales, family verbenaceae and genus *Lantana*. The weed possesses a strong root system and the roots even after repeated cuttings give new flush of shoots (Priyanka and Joshi 2013). It has infested millions of hectares land of cropping or degraded lands around the world including

Table 4.1 Biomass generated from agricultural crops in India [*Source* Murali et al. (2007), Kumar et al. (2015)]

Crop residues	Residue type	Biomass potential (kt/yr)
Coconut	Fronds, husk, pith, shell	10,463.6
Sugarcane	Tops and leaves	12,143.9
Maize	Stalks, cobs	26,957.7
Areca nut	Fronds, husk	1000.8
Banana	Residue	11,936.5
Pearl millet	Stalks, cobs, husk	15,831.8
Cotton	Stalk, husk, bollshell	52,936.5
Wheat	Stalks, panicle	112,034
Paddy	Straw, husk, Stalks	169,965.1
Mesta leaves	Stalks, leaves	1645.5
Pigeon pea	Stalks, husk	5734.6
Black gram	Stalks, husk	924.9
Green gram	Stalks, husk	762.5
Oilseeds	Stalks	1143.1
Castor seed	Stalks, husk	1698.6
Soyabean	Stalks	9940.2
Chick pea	Stalks	5440.6
Lentil	Stalks	600.3
Sesame	Stalks	1207.7
Other Pulses	Stalks	1390.4
Sorghum	Cobs, stalks, husk	24,207.8
Groundnut	Shell, stalks	15,120.4
Rubber	Primary and secondary wood	2492.2
Tapioca	Stalks	3959
Coffee	Husk, pruning and wastes	1591
Tea	Sticks	909.8
Sunflower	Stalks	1407.6
Small millets	Stalks	600.1
Potato	Leaves, stalks	887.3
Safflower	Stalks	539.3
Mustard	Stalks, husk	8657.1
Ragi	Straw	2630.2
Barley	Stalks	563.2
Others	Stalks, husk	2395.3
Total		511,041.5

Fig. 4.3 Morphology of some common weed and fibre crops. (I) *Lantana camara*, (II) *Saccharum spontaneum*, (III) *Prosopis juliflora*, (IV) *Ricinus communis,* (V) *Arundo donax*, (VI) *Mikania micrantha*, (VII) *Eichhornia crassipes*, (VIII) *Parthenium hysterophorus*, (IX) Cotton, (X) Jute, (XI) Mesta and (XII) Sunn hemp

India (Hiloidhari et al. 2014). It has established and spreaded over 60 countries with some 650 varieties which overlay the structure and floristics of natural communities (Prasad and Williams 2009). At some places, infestations have been so unrelenting that it has completely mired the regeneration of the rain forest over long periods. In Australia, since its introduction as ornamental plant in the 1840's it was estimated that about four million hectares land is covered by this invasive weeds (www.weeds.org.au). It can also affect the agricultural productivity in number of ways. It may affect the economical viability of crops such as coffee, oil palm, cotton etc. (Day et al. 2003). In India, the weed has invaded most of the tropical and subtropical region of the country and approximately 15–17 tonnes/ha/year biomass can be produced which implies its availability for bioethanol programme (Sukumaran et al. 2010). Therefore, the abundance of this biomass is likely to offer a potential renewable source for biofuel production.

Saccharum spontaneum

Saccharum spontaneum, also known as kans grass or wild sugarcane is a perennial, herbaceous, rhizomatous grass mostly native to Indian subcontinent. However, *Saccharum* genus has approximately 40 species and also widespread in North Africa and South Mediterranean regions (Clayton and Renvoize 1986). This grass can grow up to three meter in height with spreading rhizoid root. It has solid stem with polished, silky below panicles and minutely silky below upper leaf-insertions. In India, the growing season normally starts in August after rainy season and it is fully mature during January to February. Elsewhere, this grass colonizes via seed and its propensity for aggressive rhizomatous spread is so quick that it can cover a wide range of land within a short period of time. Even, it is considered a weed, in the country like India, Pakistan and Thailand where it is native in nature (Panje 1970; Yadav et al. 2007). It was estimated that with sufficient water availability *S. spontaneum* can produce up to 37.86 ton/ha of solid biomass (Cosentino et al. 2015). The weed biomass is rich in carbohydrate and fibre in its cell wall. It was estimated that about 60–70% of carbohydrate present in its cell wall which make it suitable lignocellulosic biomass for bioethanol production (Chandel et al. 2009; Scordia et al. 2010).

Prosopis juliflora

Prosopis juliflora is the member of Fabaceae family, a kind of shrub or small tree, native to Mexico, South America and the Caribbean. However with time of invasion, it becomes established as an invasive weed in Asia, Africa, Australia and elsewhere. The tree can grows up to 12 m in height and has emerges a trunk with a diameter of about 1.5 m. The root of the plants can also grow to a great depth in search of water. Several *Prosopis* species have been successfully introduced in many countries around the world. However, the main concerned member of this family is *P. juliflora* (Witt 2010). This is regarded as invasive tree in countries like India, Pakistan, Sudan, Ethiopia, Kenya, Somalia, and elsewhere. In South Africa, the invasive *Prosopis* species now occupy more than 1.8 million hectares (equivalent to 173,000 ha at 100% canopy cover) of land, mainly in the Northern Cape Province of South Africa (Versfeld et al. 1998). It was first introduced in India in 1877 where it has become invasive. At the moment *P. juliflora* provides approximately 75% fuel wood needs

of rural people in arid and semi arid region of India. Using *Prosopis* species to make biofuels is another perceived potential benefits which can be achieved by employing this biomass.

Ricinus communis: *Ricinus communis* is a perennial herbaceous flowering plant species of Euphorbiaceae family. It is the only species in the monotypic genus family and the main producer of castor oil from its seeds. The plant is indigenous to the south-eastern Mediterranean Basin, Eastern Africa, and India, but is wide spread throughout tropical regions. It is a fast-growing, suckering shrub that can reach the size of a small tree of around 12 m. The glossy leaves are 15–45 cm long stalked, alternate and palmate with 5–12 deep lobes with coarsely toothed segments. In Brazil, almost 4 million ha land is occupied for the cultivation of this plant which are capable to produce 1.5 tons of seed/ha for biodiesel production (Freitas and Fredo 2005). In India, it is distributed mostly in arid region of the country along with wasteland and roadside. India follows Brazil and China as a major producer of castor seeds and is chief producer of castor oil, contributing about 60% of the total global castor oil production (Dias et al. 2009). Castor oil is also known for blending with jatropha and palm oil for biodiesel application (Ogunniyi 2006; Lavanya et al. 2012; Zuleta et al. 2012). The biomass along with high oil content also has 42–50% cellulose which can be employed for bioethanol production (Mukhopadhyay et al. 2011).

Arundo donax

Arundo donax, also known as giant reed, is a tall perennial rhizomatous cane growing in damp moderately saline soils. The grass belongs to the subfamily Arundinoideae of the Poaceae family. It has cane like appearance similar to bamboo and can reach a height up to 8 m. It is native to Middle East Asia and some parts of Africa, however it is distributed around the world and has number of applications. Due to its seed sterility, it needs to be established by vegetative propagation. Its dynamism makes *A. dxona* an effective potential competitor for other plant species. Once established, it tends to cover large areas with dense clumps, compromising the presence of native vegetation not able to compete (Pilu et al. 2012). The potential productivity of giant reed can reach up to 100 tonne/ha/year in the second or third growing period under optimal conditions, what corresponds to 3–37 tonne/ha biomass, a bit higher of what sugar cane produces, 5–23 tonne/ha (Lewandowski et al. 2003). In several experimental studies, it was found that it contain about 65–70% holocellulose depending on the climatic condition (Scordia et al. 2010; Borah et al. 2016). Due to its rapid growth and its ability to grow in different soil types and climatic condition, it is seen as a good candidate for use as renewable biofuel feedstock source (Lewandowski et al. 2003; Lemons e Silva et al. 2015).

Mikania micrantha

Mikania micrantha is a perennial tropical plant in the Asteraceae family that grows in orchards, forests, along rivers and streams in disturbed areas, and roadsides (Kong et al. 2000). The species is native to the sub-tropical zones of North, Central, and South America in areas with high humidity, light and soil fertility. It has ribbed stems that grow up to 6 m in length with 4–13 cm long leaves that have a heart-shaped base and a pointed apex. 4.5–6.0 mm white flowers grow in clusters (Day et al.

2016). There are about 273 species of *Mikania*, most of which are native to tropical America and many are serious weeds (Wang et al. 2000). In open areas *M. micrantha* produces cushiony growth with twining roots up to 30 cm thick, while in forests and orchards it grows more than 20 m up and forms a heavy mat smothering the canopy. *Mikania micrantha* have good combustion as well as fuel properties and can be used as substitute for fuel wood or an alternative fuel in rural areas (Singh and Poudel 2013). In India, it also use for green manure production for rice cultivation (Raj and Syriac 2016). Recent study also demonstrate that this weedy lignocellulosic can be used for the production of bioethanol (Borah et al. 2016). The research is further going on for utilization of this huge biomass for other biofuel option.

Eichhornia crassipes

Eichhornia crassipes, commonly known as water hyacinth, is an aquatic plant native to tropical and subtropical America. This aquatic plant emerges as invasive species outside its native place and often highly problematic. This free floating plant has broad, thick, glossy, ovate leaves, which may rise as much as 1 m in height above the surface water. It has long, spongy bulbous stalks that support a single spike of 7–10 flowers. It is one of the fastest growing aquatic plants which reproduce by the way of runner or stolen. Moreover, each plant can produce thousands of seeds which are viable up to 20 years (Sullivan and Wood 2012). Though it was native to South America, it has been widely introduced in North America, Europe, Asia, Africa, Australia and New Zealand. In India, it is a prevalent aquatic weed which makes it a source of biomass for various uses (Sharma 1971; Nigam 2002). It was estimated that it can produce about 0.26 ton of dry biomass per hectare in all seasons (Nigam 2002). The composition analysis confirmed that it contains almost 16–20% of cellulose, 48–55% of hemicelluloses and 3–5% of lignin. This high amount of hemicellulose can be used for bioconversion to biofuel (Mishima et al. 2008; Yan et al. 2015). Instead of terrestrial plant, aquatic plant like *E. crassipes* may be a next promising renewable resource for biofuel. It is also used as feed stock for biogas production (Verma et al. 2007; Ighodalo et al. 2011). The water hyacinth being an aquatic plant has many advantages such as growing on and in bodies of water without competing against most grains and vegetables for arable land; they have also been used for water purification to extract nutrients and heavy metals.

Parthenium hysterophorus

P. hysterophorus is a belligerent noxious annual herbaceous weed of Asteraceae family. It has been considered as one of the world seven most devastative weeds responsible for number of health related problems in men and animals besides loss of crop productivity (Patel 2011; Kaur et al. 2014). *P. hysterophorus* generally behaves as an annual herb with deep tap root and an erect stem that gradually becomes semi-woody with age. It can grow and reproduce itself any time of the year. However, low temperature considerably reduces plant growth, mainly flowering and seed production. *Parthenium* is a prolific seed producer; a single plant can produce 25,000 seeds per plant in a highly infested field (Sharma 2003; Kumar 2009). It is native to north-east Mexico and endemic in American tropics. However, this weed of global significance occurring in Asia, Africa and Australia, has invaded as many as 30 countries around

the globe (Adkins and Shabbir 2014). Countries like South Africa, Ethiopia, Kenya, Mozambique, Zimbabwe, Mauritius; Madagascar has become very much affected by this invasive weed. It has been believed that *Parthenium* was introduced in India along with food grain from North America and spread alarmingly all over the country. However, in India, it was first pointed out in Maharashtra during 1955, as stray plants on rubbish heaps. Since then, this invasive weed is widely prevalent in India and now it has been estimated that about 35 million hectares of land is infested with this herbaceous menace (Dhileepan and McFadyen 2012). Eradication of this weed from the environment is a major challenge for us. Nowadays, management of this noxious weed through its utilization for different purpose is going on. The *Parthenium* biomass was used as substrate production of xylanase and laccase enzymes (Adak et al. 2016; Dwivedi et al. 2009). This weed biomass contain about 40–45% cellulose, 18–25% hemicellulose and 15–22% lignin (Pandiyan et al. 2014; Bharadwaja et al. 2015). The presence of about 70% holocellulose in the biomass prompted the researchers to use this renewable feed stock for biofuel production (Rana et al. 2013; Tiwari et al. 2015).

4.5.1 Fibre Crop Biomass

The major feedstocks used for bioethanol production are mainly from sugarcane, wheat straw, rice straw, corn stover and waste wood (Kim and Dale 2004). Apart from these, the fibre crops like cotton, jute, mesta, sunn hemp (Fig. 4.3) can also be used as raw material for ethanol production in future to avoid conflicts between human food use and industrial use of crops. The lignocellulosic composition of these fibre and weedy biomasses are depicted in Table 4.2. These fibre crops contain good amount of cellulose as well as hemicellulose that can be exploited for biofuel production. Moreover, the residues generated after harvesting of fibre from the stalks is left in the field are usually burnt by farmers causing environmental pollution; the alternative way is to use it as a feedstock for biofuel production. In Indian perspective, area of the farming land and production yield of the fibre crop is depicted in Table 4.3.

Cotton

It belongs to the genus *Gossypium*, family Malvaceae. Most commercially cultivated cotton is derived from two species, *G. hirsutum* (upland cotton, 90% of world area) and *G. barbadense* (long staple cotton). Two other species, *G. arboreum* and *G. herbaceum*, are indigenous to Asia and Africa and are popularly referred as desi cotton in India. The new world cottons in India are popularly known as American (*G. hirsutum*) and Egyptian (*G. barbadense*) cottons. Commercially it is grown as an annual crop and only reaches to a height of 1.2 m. It consists of an erect main stem and a number of lateral branches, the main stem carry branches and leaves but no flowers.

Cotton plant is a soft fluffy staple fibre producing shrub native to tropical and subtropical regions around the world, including the Americas, Africa, and India.

Table 4.2 Biochemical composition of common fibrous crop residues and weedy lignocellulosic biomass

Crops	Cellulose (%)	Hemicellulose (%)	Lignin (%)	References
Cotton	45–50	14–20	24–28	Silverstein et al. (2007), Rowell and Stout (1998)
Jute	40–47	18–25	20–25	Sur and Amin (2010)
Mesta	65–70	10–14	08–10	Ghosh and Chakraborty (1970)
Sunn hemp	70–92	18–22	03–05	Kamireddy et al. (2013)
Coir pith	26–30	03–07	30–40	Rowell and Stout (1998)
Sugarcane bagasse	26–50	25–28	23–25	Pandey et al. (2000), Guo et al. (2009)
Corn cobs	33–37	31–40	06–10	Sharma et al. (2017)
Rice straw	30–40	15–18	09–13	Guo et al. (2009), Wati et al. (2007)
Wheat straw	32–40	20–25	8–14	Saha (2003)
Lantana camara	30–35	18–25	12–20	Kuila et al. (2011), Day et al. (2003)
Ricinus communis	35–42	15–18	15–20	Mukhopadhyay et al. (2011)
Bambusa bambos	40–48	12–15	18–25	Kuila et al. (2011)
Saccharum spontaneum	38–45	20–25	15–22	Chandel et al. (2009), Scordia et al. (2010)
Jatropha curcas	25–35	10–15	08–12	Jingura et al. (2010)
Prosopis juliflora	40–50	15–20	22–30	Witt (2010)
Water hyacinth	16–20	42–50	03–06	Mishima et al. (2008), Yan et al. (2015)
Parthenium hysterophorus	40–45	18–25	15–22	Pandiyan et al. (2014); Adak et al. (2016)

Table 4.3 Area of the cropping land and production yield of some major fibrous crops in India [*Sources* Anonymous (2016), Annual Report (2015)]

Crop	Area ('000 hectares)	Production ('000 bales)	Fibre yield (kg/ha)	Total biomass (million tonnes)
Cotton	13,083	35,475	461	17.7
Jute	749	10,934	2627	2.8
Mesta	59	515	1567	2.8
Sunn hemp	31.5	18.8	600	1.5

Cotton fibres (lint and fuzz) are made from unicellular hairs that grow out from the surface epidermal cells on seeds immediately after fertilization and known as "white gold". Some cells continue to lengthen while others stop growing after a time formed by twisted ribbon like shaped fibres. The former is known as the lint and the latter is the fuzz. The hairs are twisted into usable threads which are tough and strong. The total length of hairs in a single cotton boll may exceed 300 miles. Fibre is extracted by the process of ginning. Fibre quality traits such as length, fineness and strength are important as spinning are dependent on these characteristics.

The greatest diversity of wild cotton species is found in Mexico, followed by Australia and Africa. It is the major fibre crop of India and about 96 lakh hectares under cultivation accounting for one-fourth of the global cotton area and it contributes to 16% of the global cotton produce. It is widely available and cheap agricultural residue lacking economic alternatives. India has the sole distinction of growing all the four cultivated species of cotton. In India, cotton is grown in three distinct agro-ecological zones, viz., Northern (Punjab, Haryana and Rajasthan), Central (Gujarat, Maharashtra and Madhya Pradesh) and Southern zone (Andhra Pradesh, Tamil Nadu and Karnataka). It is also grown in small area in the eastern region in sundarbans of West Bengal and in north-eastern states. In India the net cotton residue available for biofuel production is about 18 million tonnes (Ravindranath et al. 2011). Most of the leaves of the cotton plant fall on the ground before harvesting. The remaining stalks, roots are woody in nature and are commonly buried in soil by tillage operations or used as house hold fuel. In this instance, higher energy is required for tillage and often results in the degradation of the soil structure. This raises the possibility of using cotton stalks in producing energy, which would otherwise be wasted.

Jute

Jute (*Corchorus capsularis* and *Corchorus olitorius*) is a long, soft, shiny vegetable fibre produced from the plants of Malvaceae family. It is one of the most affordable natural fibres after cotton. The jute fibre is off white to brown in colour, 1–4 m long and mainly composed of high percentage of cellulose and lignin. The stem contains very high volume of cellulose which is synthesized within 4–6 months.

The bast fibre is obtained from the inner bast tissue of the bark of the plants stem. The fibres lie beneath the bark and surround the woody central part of the stem (Satya and Maiti 2013). The fibre strands nearest the bark generally run the full length of the stem. The fibre is separated from the stem by process of retting in pool

of stagnant water. The period of retting depends upon the nature of the water, the kind of fibre, and climatic conditions. It varies from two to twenty-five days. It has very long golden and silky shine fibre from six to ten feet in length, are quite stiff, as they are considerably lignified and hence called "golden fibre". The spinnable filament or strand is extremely coarse by comparison with most other spinnable commercial fibres and has no staple length; however, it is characterized by high strength and low elasticity (Maity et al. 2012).

The fibre obtained from jute is 100% bio-degradable, recyclable, environmental friendly and is in great demand because of its low cost, softness, strength, length, lustre and uniformity of its fibre. It is the most important bast fibre of Bangladesh and India. The production and cultivation of jute is restricted mainly to the state that lies along the Ganga-Brahmaputra delta in West Bengal and in Assam, Bihar and Orissa. The share of Ganga delta contributes for about 85% of the global jute cultivation. In recent years, jute cultivation has also been extended to the states of Meghalaya, Tripura, Tamil Nadu, Maharashtra and Uttar Pradesh. Out of two cultivated species of jute, tossa jute (*C. olitorius*) yields more fibre per unit area as compared to white jute (*C. capsularis*). The residue availability for biofuel production is about 2.8 million tonne (Ravindranath et al. 2011).

Mesta

Mesta is an annual or biennial herbaceous plant in the Malvaceae family also called Deccan hemp and Java jute. It is common word used for both *Hibiscus cannabinus* and *H. sabdariffa* which produces good fibre of commerce. The plants can gain height up to 1.5–4.0 m long with a woody base and 1–2 cm diameter. Generally, the plants grows best in the hot and humid regions between the latitude of 300 N to 300 S. It does, however, grow wild or is successfully cultivated at latitudes much farther from the equator for instance on the Southern shores of Caspian sea in Southern Russia, in Manchuria and Korea. In India, Mesta can, however, be grown even in those areas where jute is not grown. It was reported that Mesta is grown in an area of more than 26 lakh hectares with a production of more than 2,13,600 tonne fibre biomass (http://assamagribusiness.nic.in/mesta.pdf).

The stem consists of two parts, outer part known as bast fibres (30% of the total dry weight of the stalk) and inner part is core (70% of the total dry weight of the stalk) fibre (Paridah et al. 2011). The pattern of orientation of the fibre bundles is similar to that of jute, but mesta varies in the structure and form of the fibre bundle surface (Maiti 1979). Furthermore, the intensity of reticulation determines the quality of the fibre. The fibre cells formed inside normally do not mature physiologically. At this stage most of the fibre cells are thin walled state and are liable to be lost during extraction. This reduces the yield. Therefore, it is necessary to wait for these fibres are fully mature and are extracted by the process of retting. The quality of the kenaf fibre is good except its fineness and semi-meshy structure. The fibre strands are more irregular than jute. An attractive feature of kenaf is that up to 40% of the stalk yields usable fibre, roughly twice that of jute, hemp or flax, which makes the fibre quite economical. The mesta fibre in India is of poor quality and is not exported, but blended with jute for local burlap manufacture. So this high cellulose containing fibre

biomass may be exploited as feed stock for lignocellulosic biofuel production. The residue availability for biofuel production is about 0.04 MT while gross productivity of about 0.11 MT/year (Suresh et al. 2017).

Sunn hemp

Sunn hemp (*Crotalaria juncea*), a plant of sub-order Paplionaceae of order Leguminosae is an annual shrub cultivated as multipurpose legume especially for its fine fibre in many countries including India. It is a shrubby, herbaceous, sub-tropical annual legume that grows about 3–9 feet in height. It is one of the earliest and most distinct fibres of India, has great potential as an annually renewable, multipurpose fibre crop (Sarkar et al. 2015). It is most widely grown green manure crops throughout the tropics and also serves as a good fodder crop in many parts of India.

It is grown in almost all states of India either as a fibre crop, green manure or fodder crop (Gupta and Prakash 1969). Besides India, the crop is cultivated in many other countries like China, Korea, Pakistan, Bangladesh, Romania, Russian countries. India contributes about 23% of production with 27% of world's area under cultivation. China produces highest fibre yield in the entire world. The states of Bihar, Madhya Pradesh, Maharashtra, Rajasthan, Orissa and Uttar Pradesh grow this crop mainly for fibre. These states cover nearly 87% of the total area under cultivation of sunn hemp. Among these states, Orissa alone produces 26% of the total sunn hemp produced in the country. Average productivity of sunn hemp in the country is around 3.4 bales/ha. The actual proportion of bast fibre in dry stalks ranges from 6.4 to 10.5% (Kundu 1964). Basal diameter and plant height are significantly correlated with the fibre yield (Maiti and Chakravarty 1977; Mahapatra et al. 2012).

The most important properties of the fibre are extensibility, fatigue property, torsional rigidity, fineness and density. Strength is one of the most important properties which largely determine the quality of yarn. Sunn hemp has a fibre content of 2–4% on the basis of weight of green stem to 8–12% in terms of dry weight. With improved cultivation practices under irrigated conditions, it is possible to get about 0.8–1.0 tonnes of fibre/ha. By taking into consideration of properties all these fibre crops and the residue generated by them in the field which has no further use and can be employed as feedstock for bioethanol production.

4.6 Biofuel Production Process

The lignocellulosic biomass when used for biofuel production needs three major steps:

i. Delignification through pretreatment
ii. Saccharification
iii. Fermentation.

4.6.1 Overview of Pretreatment Methods

The pretreatment is the critical step for transformation of lignocellulosic biomass to ethanol. Due to the complex lignocellulosic structure, the pretreatment is required for the removal of lignin, partial to total hydrolysis of the hemicellulose, decreasing the fraction of crystalline cellulose and increase the porosity of the lignocellulosic material, thus improving accessibility of the remaining cellulose to enzymatic attack. Pretreatment can also strongly influence downstream costs by reducing fermentation toxicity, increasing enzymatic hydrolysis rates, increased enzyme loadings, and other process variables (Mosier et al. 2005). Pretreatment methods can be divided into following categories: physical (milling and grinding), physicochemical (steam pretreatment/auto hydrolysis, CO_2 explosion and ammonia fibre explosion), chemical (alkali, dilute acid, ozonolysis and organic solvents) and biological, or a combination of these. Table 4.4 represents the different pretreatment methods employed for conversion of fibrous and other weed biomass to fermentable monosaccharide.

4.6.1.1 Mechanical Treatment

The methods employed in physical pretreatment reduce the size of lignocellulosic biomass to facilitate subsequent treatments. Reduction of biomass size to 1 mm reduces the cellulose crystallinity, increase the digestibility of cellulose and hemicellulose in the lignocellulosic biomass (Karunanithy and Muthukumarappan 2011). Major methods followed are chipping, grinding, milling, heating, mixing and shearing resulting in physical and chemical modifications (Chinnadurai et al. 2008; Tassinari et al. 1980). It disrupts the lignocellulose structure and increases the accessibility of carbohydrates to enzyme attack.

4.6.1.2 Physical Treatment

The combined chemical and physical treatment helps in improved accessibility of the cellulose for hydrolytic enzymes, dissolving hemicellulose and alteration of lignin structure (Hendriks and Zeeman 2009).

Steam explosion
In this method, biomass is treated with high-pressure saturated steam, and then the pressure is suddenly reduced, which makes the material to undergo an explosive decompression. It operates at a temperature of 160–260 °C and corresponding pressure of 0.69–4.83 MPa for seconds to few minutes before the material is exposed to atmospheric pressure. Due to high temperature the treatment causes hemicellulose degradation and lignin transformation, thus increasing the rate of cellulose hydrolysis but retain most of insoluble lignin in the pretreated biomass (Carvalheiro et al. 2008; Sannigrahi et al. 2008). It is regarded as an economical and environmentally

Table 4.4 Comparison of different pretreatment methods studied for delignification of fibrous and weedy lignocellulosic substrates for biofuel production

Substrate used	Pretreatment method	Salient findings	References
Chemical pretreatment			
Cotton stalk	Alkali treatment (NaOH)	Hydrolysis efficiency 85.1%, Ethanol yield 0.48 g/g	Silverstein et al. (2007)
Cotton gin trash	Acid treatment (H_2SO_4)	Xylose recovery in pretreated liquors (87% theoretical), sugar recovery 89%, Ethanol yield 70%	McIntosh et al. (2014)
Kapok fibre (*Ceibapentandra* L.)	KOH treatment	Hydrolysis efficiency 64.5%, Ethanol yield 80%	Yoon et al. (2016)
Sunn hemp	Dilute acid pretreatment	Crystallinity index increased, Hydrolysis efficiency 68 wt%	Kamireddy et al. (2013)
Jute fibre	NaOH + liquid ammonia treatment	Increased crystallinity of cellulose	Mannan (1993)
Cotton waste	Ozone treatment	Hydrolysis efficiency 53 mg/ml, Ethanol yield 19.82 mg/ml	Kaur et al. (2014)
Hemp hurd	Organosolv treatment	>75 % of total hemicellulose and 75 % of total lignin removal, Hydrolysis efficiency 60%	Gandolfi et al. (2014)
Jute fibre	Peracetic acid (PAA)	Increased crystallinity of cellulose	Duan et al. (2017)
Hemp (*Cannabis sativa*), Sisal, Jute, Kapok fibre	NaOH treatment	Decreased crystallinity index of hemp fibre while sisal, jute, and kapok fibres showed a slight increase in crystallinity at NaOH (conc. 0.8–30%)	Mwaikambo and Ansell (2002)
Hemp (*Cannabis sativa*)	NaOH treatment	Hydrolysis efficiency was 72%	Ouajai and Shanks (2005)
Kenaf (*Hibiscus cannabinus* L.)	Ammonium oxalate + sodium hydroxide + acidic chlorite	Showed the greatest viscosity and lower kappa number values	Keshk et al. (2006)
Mesta	KOH	70–80% of hemicellulose removal	Neto et al. (1996)

(continued)

Table 4.4 (continued)

Substrate used	Pretreatment method	Salient findings	References
Mesta *(Hibiscus cannabinus)*	Dilute H_2SO_4 treatment	Higher glucose conversion (25.3%) when the process was conducted for 60 min at 180 °C, Crystallinity increased from 46.6 to 70.0%	Nur Aimi et al. (2015)
Water hyacinth (*Eichhornia crassipes*)	NaOH/H_2O_2-pretreated water hyacinth; 1.5% v/v H_2O_2 and 3% (w/v) NaOH at 25 °C	Reducing sugar yield of 223.53 mg/g dry biomass with reduced cellulose crytallinity	Yan et al. (2015)
Parthenium hysterophorus	1% w/v, NaOH at room temperature for 30 min	Value added APPL recovered 7.53 ± 0.5 mg/g biomass Reducing sugar yield 513.1 ± 41.0 mg/g dry substrate	Pandiyan et al. (2014)
Physico-chemical pretreatment			
Cotton stalk	Steam explosion	Hydrolysis efficiency 82.13 ± 0.72%, Ethanol yield 0.44 g/g	Keshav et al. (2016)
Jute	Steam explosion	Hydrolysis efficiency 48%	Wei et al. (2016)
Ramie (*Boehmeria nivea*)	Steam explosion donax	Hydrolysis efficiency 27%	
Mesta	Steam explosion	Hydrolysis efficiency 37%	
Hemp (*Cannabis sativa* L.)	Electron beam irradiation	450 kGy resulted in better enzymatic hydrolysis	Shin and Sung (2008)
Arundo donax	Microwave assisted NaOH pretreatment. Dilute H_2SO_4 with autoclaving for an hour	Glucose is the main monomeric sugar, 31.99 g/100 g biomass. Xylan associated monosaccharide yield: 201 mg/g Ethanol yield 109 mg/g	Scordia et al. (2010), Komolwanich et al. (2014)
Parthenium hysterophorus	Autoclaving for 30 min with 1% v/v, H_2SO_4	Total fermentable sugar released 39.77 g/100 g raw biomas	Singh et al. (2014)

(continued)

Table 4.4 (continued)

Substrate used	Pretreatment method	Salient findings	References
Saccharum spontaneum	Autoclaving with different alkali concentration. Aqueous ammonia (15%) at 50 °C for 24 h followed by enzymatic hydrolysis (5–35 FPU/g dry biomass)	70.75% lignin removal for 0.5% w/v NaOH treatment at 120 °C with total reducing sugar yield in enzymatic saccharification: 350 mg/g. A max. sugar yield of 631.5 ± 3.25 mg/g with 89.38% hydrolytic efficiency (HE) after enzymatic hydrolysis of aqueous ammonia pretreated biomass	Chandel et al. (2009), Kataria and Ghosh (2014)
Biological pretreatment			
Cotton stalks	*Phanerochaete chrysosporium*	Lignin degradation of 19.38% and 35.53% under submerged and solid state fermentation respectively, Hydrolysis efficiency 14.94%, Ethanol yield 0.027 g/g	Shi et al. (2009)
Banana fibre	Laccase and xylanase enzyme treatment	Laccase treatment: higher lignin removal, Xylanase treatment: higher hemicellulose removal	Vishnu Vardhini and Murugan (2017)
Bamboo fibre	White rot fungi *(Echinodontium taxodii)*	Structural alterations of lignin during pretreatment can decrease the thermal stability	Yu et al. (2009)
Lantana camara	Laccase treatment followed by SSF, Chlorite pretreatment	Maximum bioethanol production 6.01% v/v using mutant strain of *S. cerevisiae* Chlorite pretreatment improve saccharification, it reach about 86–92%	Kuila et al. (2011), Gupta et al. (2011)

(continued)

Table 4.4 (continued)

Substrate used	Pretreatment method	Salient findings	References
Parthenium hysterophorus	*Trametes hirsuta* ITCC136 Ligning degrading micromycete fungus *Myrothecium roridum* LG7	Delignification of substrate to greater extent within 7 days with higher lignin recovery (1.92 fold). Pretreated biomass yield 485.64 mg/gds sugar upon enzymatic hydrolysis. Within 7 days of incubation, the fungus led to high amount of lignin removal (5.8–6.98 mg/gds), released 455.81–509.65 mg/gds sugar in enzymatic hydrolysis	Rana et al. (2013), Tiwari et al. (2013)

friendly treatment that greatly reduces biomass particle size and extracts cell wall polymers (Jørgensen et al. 2007; Alvira et al. 2010).

CO_2 explosion

Utilization of CO_2 as a supercritical fluid (Zheng et al. 1998) for the pretreatment can help in effective removal of lignin and increasing substrate digestibility. It is believed that CO_2 reacts with water to form carbonic acid, thereby improving the hydrolysis rate and helpful in hydrolysing hemicellulose as well as cellulose. Moreover, the low temperature prevents any appreciable decomposition of monosaccharides by the acid. Upon an explosive release of the CO_2 pressure, the disruption of the cellulosic structure increases the accessible surface area of the substrate for hydrolysis. Because CO_2 explosion is operated at low temperature, it does not cause degradation of sugars unlike steam explosion (Zheng et al. 1995).

Ammonia fibre explosion (AFEX)

The fibrous biomass treated with anhydrous ammonia at 60–120 °C and high pressure for varying periods of time and then the pressure is suddenly reduced (Dale and Moreira 1982; Holtzapple et al. 1991). High pressure and temperature results in swelling, physical disruption of biomass fibres and partial decrystallization of cellulose. AFEX treatment also alters the structure of the cellulose and lignin, which results in modification and redistribution of lignin (Lau et al. 2008). The AFEX process is very similar to steam explosion. In a typical AFEX process, the dosage of liquid ammonia is 1–2 kg of ammonia/kg of dry biomass, the temperature is 90 °C, and the residence time is 30 min. The use of high pressure treatment raises the cost of equipment which is the major drawback of this method.

4.6.1.3 Chemical Treatment

Chemicals like oxidizing agents, alkali, acids and salts can degrade lignin, hemicellulose and cellulose from the biomass. Powerful oxidizing agents such as ozone and H_2O_2 effectively remove lignin; does not produce toxic residues for the downstream processes and the reactions are carried out at room temperature and pressure (Sun and Cheng 2002).

Alkali treatment

Alkali pretreatment is regarded as an efficient pretreatment method for removing lignin from lignocellulosic biomass. The hydroxides of sodium, calcium and ammonium are suitable for the treatment process. NaOH and $Ca(OH)_2$ are the most extensively studied bases for pretreatment. Due to its ability to absorb enzymes, lignin is known to have adverse effect on efficiency of cellulases. Alkali disrupts the ester bonds between lignin and the carbohydrate polymers, thus increasing the porosity of biomass and increased access for cellulase enzymes (Silverstein et al. 2007; Asgher et al. 2013). This cause changes in the gross crystallinity index due to removal of amorphous regions (lignin and hemicellulose) of the biomass, rather than to structural change in the cellulose fibres. This is the reason that the gross crystallinity index of treated biomass often rises after pretreatment. The only disadvantage of the process is its long residential time for pretreatment and effluent generation. Alkaline processes are known to cause less sugar degradation than acid pretreatment. Moreover hemicellulose solubilization is less in comparison with acid and thermal pretreatment. This method is more effective on agricultural residues than on wood materials.

Acid treatment

Pretreatment with acid hydrolysis can result in improvement of enzymatic hydrolysis of fibrous biomass to release fermentable sugars. Concentrated or diluted acid like sulphuric acid (H_2SO_4), hydrochloric acid (HCl) or phosphoric acid (H_3PO_4) can be used for pretreatment of biomass and pulverized at low temperature and pressure. Commonly used concentration of sulphuric acid is 65–86% w/v, hydrochloric is 30–40% and phosphoric acid is 55–85% w/w (Chen et al. 2012), but utilization of concentrated acid is less preferred due to the formation of inhibitory compounds, equipment corrosion, and high operational and maintenance costs (Yang and Wyman 2008). Moreover, during concentrated acid pretreatment glucose and xylose degrades to furfural, HMF and 2-furfuralaldehyde (Dias et al. 2009; Bozell and Petersen 2010).

In case of dilute acid method there are two types of acid hydrolysis (i) High temperature and continuous flow process for low substrate loading, (ii) low temperature and batch process for high substrate loading. High hydrolysis is obtained with dilute acids (Mirahmadi et al. 2010) which often results in the higher content of acid-insoluble lignin than that of the initial material (Sannigrahi et al. 2008; Pingali et al. 2010). The major drawback of this method is the removal of hemicellulose fraction (C5 sugars) which prevents the efficient conversion of all the sugars of biomass to biofuel and requirement of corrosion resistant reactors which increase the cost of the process.

Ozonolysis
Ozone is a powerful oxidant that reacts preferably with lignin than carbohydrates, promoting biomass destruction and shows high delignification efficiency and the process is carried out at room temperature and normal pressure. This result in an increase of in vitro digestibility of the treated material, and unlike other chemical treatments, it does not produce toxic residues. Ozonolysis pretreatment has an advantage of low generation of inhibitory compounds, and especially no generation of furfural, HMF (which might hinder following downstream stages) and the reactions are carried out at room temperature and normal pressure. A drawback of ozonolysis is that a large amount of ozone is required, which can make the process expensive (Travaini et al. 2016).

Organosolv process
Pretreatment using organic/aqueous solvent mixtures like methanol, ethanol, acetone, ethylene glycol and tetrahydrofurfuryl achieves high lignin removal (Zhao et al. 2009) and minimum cellulose loss (Pan et al. 2006). The pretreatment is typically performed at around 200 °C, but if acid catalysts are used the process can be run at lower temperatures. The process in which cellulose is partially hydrolysed into smaller fragments those still remain insoluble in the liquor, hemicellulose, is hydrolysed mostly into soluble components, such as oligosaccharides, monosaccharide, and acetic acid. Lignin is hydrolysed into low molecular weight fragments that dissolve in the aqueous ethanol liquor. The solvents must be removed from the system to avoid inhibition of enzymatic hydrolysis and fermentation, and should be recycled to reduce operational costs.

4.6.1.4 Biological Treatment

An alternative to harsh chemicals and high temperature is the microbial pretreatment employing microorganisms, mainly white and soft-rot fungi, actinomycetes, and bacteria which degrade lignin, the most recalcitrant polymer in biomass, through the action of lignin degrading enzymes such as peroxidase and laccases. *Phanerochaete chrysosporium* has been the model organism for studies of lignin degradation by white rot fungi (Millati et al. 2011). Brown rot fungus attacks cellulose while white and soft rot fungi attacks both cellulose and lignin (Prasad et al. 2007). Among all these fungi, white rot fungi belonging to the basidiomycetes is be most effective with highest delignification efficiency owning to its complex lignolytic systems (Salvachúa et al. 2011). The extracellular lignolytic enzymes, mainly lignin peroxidase, manganese peroxidase and laccase are responsible for delignification by white rot fungi (Wan and Li 2012). In biological pretreatment, particle size, moisture content, pretreatment time and temperature could affect lignin degradation and enzymatic hydrolysis yield. Search for efficient white rot fungi with selective lignin degradation capabilities is still going on. In authors lab, *Myrothecium rodidum* LG 7 and *Trametes hirsuta* were found to be highly efficient in biological delignification

of paddy straw as well as holocellulose enrichment in the treated substrates (Rana et al. 2013; Tiwari et al. 2013; Saritha et al. 2012).

4.6.2 Enzymatic Hydrolysis of Lignocellulosic Biomass

Enzymatic hydrolysis is an environmentally friendly alternative that uses carbohydrate degrading enzymes (mainly cellulases) to hydrolyse lignocellulose into fermentable sugars. This process is also called as saccharification. Saccharification is one of the important steps for producing sugars, such as 6-C (glucose, galactose, and mannose) and 5-C (xylose, mannose and rhamnose) from complex polysaccharide of cellulose and hemicellulose, respectively. These sugars can be further metabolised and fermented into ethanol. The process can be mainly carried out in two ways; (i) enzymatically by using cellulolytic and xylolytic enzymes (biological); (ii) Non enzymatically by using acids hydrolysis (chemical). However, enzymatic hydrolysis is becoming a method of choice due to several benefits like high product yield, less chemical requirements, less energy and mild environment conditions, and generation of fewer fermentation inhibitor products.

Enzymatic hydrolysis of cellulose

Enzymatic hydrolysis of cellulose is typically carried out by cellulases. Hydrolysis is usually conducted at a pH of approximately 4.8 and at a temperature of 45–50 °C. The hydrolysis temperature is well above the growth temperature of most microorganisms. Cellulases are a complex system of three enzymes that act synergistically to hydrolyze cellulose. The three enzyme components are: 1,4-β-D-glucan glucanohydrolase (EC 3.2.1.3), 1,4-β-D-glucan cellobiohydrolyase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) (Sun and Cheng 2002; Ladisch et al. 1983). These enzymes are commonly referred to as endoglucanase, exoglucanase and cellobiase, respectively. Cellulases are produced by several fungal genera like *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium.* These organisms use cellulose as a primary carbon source and are of industrial interest for their potential to convert waste woody cellulosic materials to biofuels and various bacterial genera such as *Clostridium*, *Cellulomonas*, *Streptomyces* and *Bacillus* have also been reported for enzyme production. However, due to advancement in commercial cellulase preparation most of the large scale bioethanol production employs commercially available high titre enzyme cocktails obtained from genetically engineered strains of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium*. Some commercially available cellulase enzyme preparations are listed in Table 4.5.

Enzymatic hydrolysis of hemicellulose

Enzymatic hydrolysis of hemicellulose is carried out by hemicellulases. Complete hydrolysis of xylan involves three main enzymes: endo-β-1-4-xylanase, exoxylanase and β-xylosidase. These enzymes are primarily involved in depolymerization and while some of them are responsible for cleaving side-groups like α-L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, ferulic acid

Table 4.5 List of some commercially available cellulase enzyme preparation [*Source* Ju et al. (2014)]

Commercial Enzyme	Company	Cellulase activities	
		FPU/ml	FPU/mg
Cellic® Ctec2	Novozymes (Franklinton, NC)	119	0.46
Accelerase® 1500	Dupont (Genencor) (Rochester, NY)	57	0.50
Cytolase CL	DSM (Seclin, France)	117	0.82
Cellic® Ctec3	Novozymes (Franklinton, NC)	196	0.75
Novozyme 188®	Novozymes (Franklinton, NC)	487 (CBU/ml)	N/A

esterase, and *p*-coumaric acid esterase (Saha and Bothast 1999). Microorganisms like *Penicillium capsulatum* and *Talaromyces emersonii* have been identified that have complete enzyme systems to degrade xylan (Belancic et al. 1995). Other microorganisms that have been reported as sources for hemicellulose degrading enzymes are *Aureobasidium pullulans* (Christov et al. 1997) and several *Fusarium* sp. (Saha 2003). While the number of enzymes required for xylan hydrolysis is much greater than for cellulose hydrolysis, accessibility to the substrate is easier since xylan does not form tight crystalline structures (Gilbert and Hazlewood 1993).

4.6.3 Fermentation and Distillation

Both fermentation and distillation are vital steps for lignocellulosic biofuel production. Several microorganisms are used for fermentation of saccharified biomass, but industrial application of lignocelluloses for bioethanol production is impeded by the lack of ideal microorganisms that can ferment both pentose and hexose sugars efficiently. To make the process of ethanol production commercially viable, an ideal microorganism should have a broad substrate utilization range, high ethanol yield and productivity, ability to withstand high concentrations of ethanol and high temperature and also tolerance to inhibitors present in the hydrolysate. Genetically modified microorganisms are used to achieve complete utilization of sugars in hydrolysate and better production benefits. Various fermentation strategies are followed in industries to achieve high ethanol production with low production cost.

Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) processes are usually involved in the fermentation of lignocellulosic hydrolysate. SSF is superior to conventional SHF process for ethanol production since it can improve ethanol yields by removing end product inhibition and eliminate the need of separate reactors. It is also cost effective but the optimum

temperature conditions of saccharifying enzyme and fermentation differ and this poses some limitations (Hamelinck et al. 2005; das Neves et al. 2007) that can be removed by using thermo-tolerant microorganisms like *Kluyveromyces marxianus* which has been developed to withstand the higher temperatures needed for enzymatic hydrolysis.

Apart from SHF or SSF, consolidated bioprocessing (CBP) and simultaneous saccharification and co-fermentation (SSCF) are the available alternatives (Cardona and Sánchez 2007). Cellulase production, biomass hydrolysis and ethanol fermentation are all together carried out in a single reactor in CBP. Mono-or co-culture of microorganisms is generally used for the fermentation of cellulose directly to ethanol. CBP process does not require any capital investment for purchasing enzymes or its production. A report which showed that the white rot fungus, *Trametes hirsuta* is capable of assimilating a broad spectrum of carbon sources and has the potential to convert the lignocellulosic biomass into bioethanol directly through consolidated bioprocessing. *Clostridium thermocellum* is a potential biocatalyst in CBP for the direct conversion of plant biomass-derived material into cellulosic ethanol due to its efficient degradation and utilization of cellulose under anaerobic conditions.

Genetic engineering has been employed for the improvement of various aspects of fermentation from higher yield and broad substrate utilization to increased recovery rates. Many improvements have been made for the fermentation of xylose and arabinose to ethanol and other products such as lactic acid. However, bioconversion of pentoses to ethanol still presents a considerable economic and technical challenge (Chandel et al. 2009; Bharadwaja et al. 2015). Numerous technologies have been employed for the strain development which can ferment xylose readily and efficiently which includes (i) optimization of xylose-assimilating pathways, (ii) perturbation of gene targets for reconfiguring the metabolism, and (iii) simultaneous co-fermentation of xylose and cellobiose (Kim et al. 2011). Glucose transporters mediate xylose uptake, but this competes with glucose uptake; this indicates that they have common transport components. No transporter specific for xylose has yet been identified. Glucose transporters exhibit lower affinity for xylose than for glucose; therefore glucose and xylose consumed simultaneously only under glucose limited conditions. The most commonly used strains of *S. cerevisiae* (PE-2 and CAT-1) in Brazilian fuel ethanol industry were genetically modified for xylose fermentation. The strain PE-2 fermented xylose faster than CAT-1 strain, but produced considerable amount of xylitol also. The deletion of aldose reductase *GRE3* resulted in reduced level of xylitol with an increased production of ethanol, 0.47 g/g of total sugars, which was 92% of the theoretical yield.

The efficient microorganisms for fermentation can be developed in three ways: (a) making *C. shehatae*, *P. stipitis* and recombinant *E. coli* more resistant to inhibitors (b) genetic engineering of *S. cerevisiae* or *Z. mobilis* for xylose fermentation (c) metagenomics of natural genes to develop an efficient fermentation process. For sustainable generation of biofuels, exploring modern genetic engineering tools to produce tailor-made perennial plants and trees with increased amounts of biomass and to develop microbes which ferment both hexose and pentose sugars is an unavoidable necessity.

4.7 Conclusion

In future, it is desirable to explore the unconventional lignocellulosic biomass, improve breeding of energy plants, better enzymes, and specialized fermentation yeasts. Sustainable feedstock supply is the most important requirement for any bioethanol production programme. Fibrous crop and weed biomass may be explored as a feedstock source for biofuel production to provide a higher degree of national energy security in an environment friendly, cost-effective and sustainable manner. Weedy and fibrous crop biomass can augment the supply of feedstocks during lean periods of crop biomass availability. Weedy biomass with low production cost and high productivity is rich in holocellulose and low in lignin that can substitute or supplement the crop biomass for bioethanol production. The promising alternative sources of fibrous crop residues are cotton, jute, mesta and sunn hemp which can be explored for biofuel production. The transformation of such biological resources as energy rich crops requires the conditioning or pretreatment of the feedstocks for fermenting organisms to convert them into bioethanol. The conditions employed in the chosen pretreatment method affect various substrate characteristics which in turn govern the susceptibility of the substrate to hydrolysis and the subsequent fermentation of the released sugars. However the data about fibrous crop and weedy biomass production, its availability and supply chain management options including transportations is still lacking. Moreover many potential weedy biomasses like *Parthenium, Eichhornia, Mikania, Arundo, Saccharum* and *Lantana* are mostly growing on community or degraded lands which make it difficult to collect the biomass for any commercial purposes. Supply chain management including collection, transportation and storage are the biggest challenge in utilization of these resources especially weedy biomass due to their discrete availability. Establishment of biomass collection cooperative societies at district or block level may help in solving the issues related to supply chain management of these lignocellulosic wastes.

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Chapter 5
Technological Aspects of Lignocellulose Conversion into Biofuels: Key Challenges and Practical Solutions

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Abstract Biofuels produced from crops have been the driving force in renewable energies since many years. In the first decade of the 21st century, there was a major focus on the debate of food versus fuel. Reports made by various national and international agencies, concluded that the food commodity prices were being impacted by consumption for the production of biofuels. Lignocellulosic biomass is an attractive renewable resource for future fuel. Efficiently and cost-effectively production of bioethanol from various lignocellulosic biomass, not only depends on the development of a suitable pretreatment system but also on other technological aspects with engineered feedstock. The aim of this chapter is to summerize and critically review on existing pretreatment method which is highly efficient due to engineering the feedstock as well as effectively using biocatalytic hydrolysis of various lignocellulosic biomass materials. The success behind this lignocellulosic bioethanol is depend on the modern technological development of pretreatment technologies as well as advanced conversion processes within the line of process intensification strategies.

keywords Lignocellulose · Biofuels · Fermentation · Heterogeneous catalysis Engineered biomass · Biorefinery

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5.1 Lignocellulose Biomass Recalcitrance: Physico-Chemical Characteristics of the Plant Cell Wall

The lignocellulosic biomass is characterized by a natural resistance of the plant cell wall to microbial and enzymatic degradation, due to its rigid and compact structure, known as "biomass recalcitrance" (Himmel et al. 2007). This property is closely related to the chemical and physical features of the plant cell wall, which is a matrix of cross-linked polysaccharide networks, glycosylated proteins, and lignin. Several aspects contribute in building the lignocellulose's recalcitrance, such as epidermal tissue and chemicals, chemical compositions, physical structure of the cell wall, cellulose structure, and pre-treatment-induced causes (Zhao et al. 2012). In particular, Himmel et al. have provided a list of the natural factors supposed to play a part in constructing the recalcitrance of lignocellulosic feedstocks to chemicals or enzymes, that includes: (i) the epidermal tissue of the plant body, particularly the cuticle and epicuticular waxes; (ii) the arrangement and density of the vascular bundles; (iii) the relative amount of sclerenchymatous (thick wall) tissue; (iv) the degree of lignification; (v) the structural heterogeneity and complexity of cell-wall constituents such as microfibrils and matrix polymers; (vi) the challenges for enzymes acting on an insoluble substrate; and (vii) the inhibitors to subsequent fermentations that exist naturally in cell walls or are generated during conversion processes (Himmel et al. 2007). These chemical and structural characteristics affect liquid penetration and/or enzymes accessibility and activity, resulting in increased conversion costs.

Lignocellulosic biomass is mainly composed of three polymers: cellulose, hemicellulose and lignin along with smaller amounts of pectin, protein, extractives and ash (Bajpai 2016), which do not participate significantly in forming the structure of the material (Harmsen et al. 2010).

Depending on the type of biomass, these polymers are organized in complex non-uniform three-dimensional structures to different degrees and varying relative compositions, as illustrated in Table 5.1, for various lignocellulosic feedstocks.

As can be seen from Table 5.1 cellulose is the major structural component of cell walls, and it provides mechanical strength and chemical stability to plants. Hemicellulose is a copolymer of different C5 and C6 sugars. Lignin is a polymer of aromatic compounds produced through a biosynthetic process that forms a protective layer for the plant walls (Harmsen et al. 2010). Their internal structures will be described in detail in the follwing paragraphs. From a structural point of view, the plant cell wall is a complex matrix typically composed of three types of layers, namely the middle lamella, the primary and the secondary wall (Fig. 5.1), that provide support and strength essential for plant cell survival. The main functions of the cell wall include the conferral of resistance, rigidity and protection to the cell against different biotic or abiotic stresses, but still allowing nutrients, gases and various intercellular signals to reach the plasma membrane (Ochoa-Villarreal et al. 2012).

Primary and secondary cell walls are microfibril-based nanocomposites that differ in the arrangement, mobility and structure of matrix polymers, the higher-order organization of microfibrils into bundles and discrete lamellae, their rheological and

Table 5.1 Composition of representative lignocellulosic feedstocks (Menon and Rao 2012)

Feedstocks	Carbohydrate composition (% dry wt)		
	Cellulose	Hemicellulose	Lignin
Barley hull	34	36	19
Barley straw	36–43	24–33	6.3–9.8
Bamboo	49–50	18–20	23
Banana waste	13	15	14
Corn cob	32.3–45.6	39.8	6.7–13.9
Corn stover	35.1–39.5	20.7–24.6	11.0–19.1
Cotton	85–95	5–15	0
Cotton stalk	31	11	30
Coffee pulp	33.7–36.9	44.2–47.5	15.6–19.1
Douglas fir	35–48	20–22	15–21
Hardwood stems	40–55	24–40	18–25
Rice straw	29.2–34.7	23–25.9	17–19
Rice husk	28.7–35.6	11.96–29.3	15.4–20
Wheat straw	35–39	22–30	12–16
Wheat bran	10.5–14.8	35.5–39.2	8.3–12.5
Grasses	25–40	25–50	10–30
Newspaper	40–55	24–39	18–30
Sugarcane bagasse	25–45	28–32	15–25
Sugarcane tops	35	32	14
Pine	42–49	13–25	23–29
Poplar wood	45–51	25–28	10–21
Olive tree biomass	25.2	15.8	19.1
Jute fibres	45–53	18–21	21–26
Switchgrass	35–40	25–30	15–20
Grasses	25–40	25–50	10–30
Winter rye	29–30	22–26	16.1
Oilseed rape	27.3	20.5	14.2
Softwood stem	45–50	24–40	18–25
Oat straw	31–35	20–26	10–15
Nut shells	25–30	22–28	30–40
Sorghum straw	32–35	24–27	15–21
Tamarind kernel powder	10–15	55–65	–
Water hyacinth	18.2–22.1	48.7–50.1	3.5–5.4

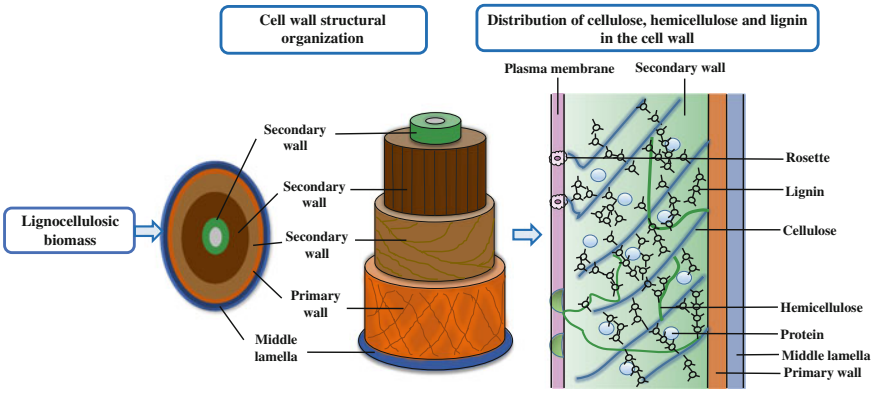


Fig. 5.1 Cell wall structure

mechanical properties, and their roles in the life of the plant (Cosgrove 2012). In the primary wall, the basic structure is a skeleton of cellulose cross-linked with glycans; according to the cross-link types present, there are two types of primary walls: (i) Type I walls that are found in dicotyledonous plants and consist of equal amounts of glucan and xyloglucan embedded in a matrix of pectin; (ii) Type II walls, present in cereals and other grasses, having glucuronoarabinoxylans as their cross-linking glucans, but lacking of pectin and structural proteins (Zhao et al. 2012). The secondary wall usually consists of three sub-layers, which are termed as S1 (outer), S2 (middle), and S3 (inner) lamellae, respectively. The cellulose microfibrils of secondary wall are embedded in lignin, being like steel rods embedded in concrete, but with less rigidity. Cellulose, hemicellulose, and lignin have different distribution in these layers. In wood fibers, it has been found that cellulose concentration is increased from middle lamella to the secondary wall. S2 and S3 lamellaes have the highest cellulose concentration. Hemicellulose has a similar tendency of distribution in the cell wall to cellulose, and most of the hemicellulose distributes in the secondary wall. Lignin is found to be the dominant composition in the outer portion of the compound middle lamellae. The percentage of lignin in the lignocellulosic matrix decreases with increasing distance into the middle lamella; that means that the percentages of lignin in the primary wall and in the S1 layer of the secondary wall are much higher than those in the S2 and S3 sections (Zhao et al. 2012).

5.1.1 Cellulose

Cellulose is a linear homopolymer composed of D-glucopyranose units linked by β-1,4-glycosidic bonds. The chemical formula of cellulose is $(C_6H_{10}O_5)n$; n, called the degree of polymerization (DP), represents the number of glucose groups, ranging from hundreds to thousands or even tens of thousands. In the twentieth century, it

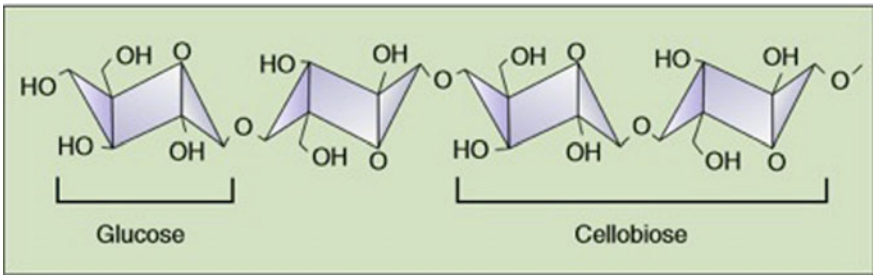


Fig. 5.2 Molecular chain structure of cellulose

was proved that cellulose consists of pure dehydrated repeating units of D-glucoses (as shown in Fig. 5.2), and the repeating unit of the cellulose is called cellobiose (Chen 2014).

The cellulose chains (20–300) are grouped together to form microfibrils, which are bundled together to form cellulose fibers. The long-chain cellulose polymers are linked together by hydrogen and van der Waals bonds, which cause the cellulose to be packed into microfibrils, that in most conditions, are covered by hemicellulose (dry matter accounting for 20–35%) and lignin (dry matter accounting for 5–30%) (Bajpai 2016). Natural cellulose has 10,000 glucose units and the fibril contains approximately 60–80 cellulose molecules. It is insoluble in water, dilute acidic solutions, and dilute alkaline solutions at normal temperatures and is found in both the crystalline and the non-crystalline structure (Harmsen et al. 2010). Indeed, study of the supramolecular structure of natural cellulose showed that the crystalline and non-crystalline phases intertwine to form the cellulose. The noncrystalline phase assumes an amorphous state when tested by X-ray diffraction because most hydroxyl groups on glucose are amorphous. However, large amounts of hydroxyl groups in the crystalline phase form many hydrogen bonds, and these hydrogen bonds construct a huge network that directly contributes the compact crystal structure.

5.1.2 Hemicellulose

The term hemicellulose is used to represent a family of polysaccharides such as arabino-xylans, gluco-mannans, galactans, etc. that are present in both the primary and the secondary cell walls, and in a small amount also the middle lamella region. They have different composition and structure depending on their source and the extraction method. The most common type of polymers that belongs to the hemicellulose family of polysaccharides is xylan (Harmsen et al. 2010). Xylans are a diverse group of polysaccharides with the common backbone of β-(1,4)- linked xylose residues, with side chains of α-(1,2) linked glucuronic acid and 4-O-methyl glucuronic acid residues. Composition and distribution of the substitutions is wide

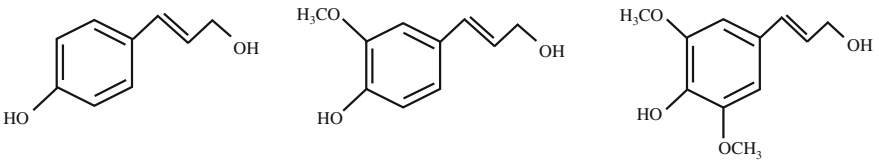
variable according to the plant cell species. Xylans usually contain many arabinose residues attached to the backbone which are known as arabinoxylans and glucuronoarabinoxylans (Ochoa-Villarreal et al. 2012). The β-(1,4)-linked polysaccharides rich in mannose or with mannose and glucose in a nonrepeating pattern are the glucomannans and galactoglucomannans. Hemicellulose extracted from plants possesses a high degree of polydispersity, polydiversity and polymolecularity (a broad range of size, shape and mass characteristics). However, the degree of polymerization does not exceed the 200 units whereas the minimum limit can be around 150 monomers (Harmsen et al. 2010). Hemicellulose is insoluble in water at low temperature. However, its hydrolysis starts at a temperature lower than that of cellulose, which renders it soluble at elevated temperatures.

5.1.3 Lignin

Lignin is the most complex natural polymer. It is present in the primary cell wall and functions as the cellular glue which provides compressive strength to the plant tissue and the individual fibres, stiffness to the cell wall and resistance against insects and pathogens (Isikgor and Remzi 2015). It is an amorphous three-dimensional polymer with phenylpropane units nonlinearly and randomly linked as the predominant building blocks; the most commonly monomers encountered are p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Fig. 5.3).

The coupling modes between each basic unit include “β-O-4, “β-5, “β-1, and so on. Ether bonds in lignin include phenol-ether bonds, alkyl-ether bonds, dialkyl bonds, diaryl ether bonds, and so on. About two thirds to three quarter phenylpropane units of lignin are linked to the adjacent structural units by ether bonds; only a small part is present in the form of free phenolic hydroxyl (Chen 2014).

Lignin is synthesized by polymerization of these components and their ratio varies between different plants, wood tissues and cell wall layers. Dividing higher plants into two categories, hardwood (angiosperm) and softwood (gymnosperm), it has been identified that lignin from softwood is made up of more than 90% of coniferyl alcohol with the remaining being mainly p-coumaryl alcohol units. Contrary to softwoods,



(a) p-coumaryl alcohol (b) Coniferyl alcohol (c) Sinapyl alcohol

Fig. 5.3 Basic structural units of lignin (Srndovic 2011)

lignin contained in hardwood is made up of varying ratios of coniferyl and sinapyl alcohol type of units (Harmsen et al. 2010).

5.2 Chemical Interaction Between Components

The types of bonds identified within the lignocellulosic structure are four: ether, ester, carbon-to-carbon and hydrogen bonds. They can be divided into intrapolymer and interpolymer linkages (Table 5.2); the former refer to linkages within the individual components of the lignocellulose, while the latter to the connections among the different components to form complexes.

As shown in Table 5.2 the bonds types linking the molecules in the structure of the lignin are ether bonds and carbon-to-carbon bonds; the ether bonds may occur between allylic and aryl carbon atoms, or between aryl and aryl carbon atoms, or even between two allylic carbon atoms. The total fraction of ether type bonds in the lignin molecule is around 70% of the total bonds between the monomer units. The carbon-to-carbon linkages form the remaining 30% of the total bonds between the units. They can also appear between two aryl carbon atoms or two allylic carbon atoms or between one aryl and one allylic carbon atom (Harmsen et al. 2010). In the cellulose's polymer, the glucose units are connected together by a 1-4 β D-glucosidic bond, that can be considered as an ether bond, since it is in fact the connection of two carbon atoms with an elementary oxygen interfering. The other main type of bond present in the cellulose is the hydrogen bond that is responsible for its crystalline fibrous structure. In fact, every glucosyl ring of cellulose has three active hydroxyls: one primary hydroxyl group and two secondary hydroxyl groups. Thus, cellulose may have a series of chemical reactions related to hydroxyl. However, these hydroxyl groups also can form hydrogen bonds between molecules, which has a pro-

Table 5.2 Different types of bonds identified in the lignocellulose (Harmsen et al. 2010)

Bonds with different components (intrapolymer linkages)	
Ether bond	Lignin, (hemi)cellulose
Carbon to carbon	Lignin
Hydrogen bond	Cellulose
Ester bond	Hemicellulose
Bonds connecting different components (interpolymer linkages)	
Ether bond	Cellulose-lignin Hemicellulose lignin
Ester bond	Hemicellulose-lignin
Hydrogen bond	Cellulose-hemicellulose Hemicellulose-lignin Cellulose-lignin

found influence on the morphology and reactivity of cellulose chains, especially the intermolecular hydrogen bond formed by oxhydryl at C3 and oxygen at an adjacent molecule ring. These hydrogen bonds not only can enforce the linear integrity and rigidity of the cellulose molecule but also make molecule chains range closely to form a highly ordered crystalline region. The accessibility of cellulose refers to the difficulty of the reagents to arrive at the cellulose hydroxyl. In heterogeneous reactions, the accessibility is mainly affected by the ratio of the cellulose crystalline regions to the amorphous regions. The reactivity of cellulose is the reactive capability of the primary hydroxyl and the secondary hydroxyl at the cellulose ring. Generally, because of the smallest steric hindrance, the reactivity of the primary hydroxyl groups is higher than for the secondary hydroxyl groups, so the reactivity of hydroxyl at C6 with a bulky substituting group is higher (Chen 2014). In addition, it was noted that, carboxyl groups are also present in cellulose in a fraction of 1 carboxyl per 100 or 1000 monomer units of glucose. With respect to the structure of the hemicellulose, it can be stated that its molecule is formed mainly by the ether type bonds, such as the fructosic and glucosidic one. The main difference with cellulose is that the hydrogen bonds are absent and that there is significant amount of carboxyl groups. The carboxyl groups can be present as carboxyl or as esters or even as salts in the molecule (Harmsen et al. 2010).

The interpolymer linkages, namely those connecting the different polymers of the lignocellulose complex, can be determined by breaking down the lignocellulose and separating the individual components. Their separation is commonly achieved by methods that cause the alteration of their original structure. Therefore, the results obtained about the connecting linkages between the polymers are not definite. However, it has been identified that there are hydrogen bonds connecting lignin with cellulose and with hemicellulose, respectively and the existence of covalent bonds between lignin and polysaccharides. In particular, it is known that hemicellulose connects to lignin via ester bonds and that there are ether bonds between lignin and the polysaccharides. It is still not clear though whether the ether bonds are formed between lignin and cellulose, or hemicellulose (Harmsen et al. 2010).

5.3 Lignocelluloses Feedstock Biorefinery

Biorefinery represents the sustainable processing of biomass into a spectrum of marketable products and energy, as defined by International Energy Agency (IEA) Bioenergy Task 42 (Van Ree and Van Zeeland 2014; Morais and Bogel-Lukasik 2013). In biorefinery, all the types of biomass feedstocks can be exploited, including products, byproducts, residues and waste provided from different sectors: forestry (wood, logging residues, trees, shrubs and wood residues, sawdust, bark, etc.), agriculture (dedicated crops and residues), aquaculture (algae and seaweeds), industries (process residues and leftovers) and households (municipal solid waste and wastewaters) (De Jong and Jungmeier 2015). The biomass feedstock can be converted into dif-

ferent classes of bio-products, via combinations of different technologies, including mechanical/physical, (bio)chemical and thermochemical processes (de Wild 2015).

Among the possible biomass raw materials, the lignocellulose is one of the most promising feedstock for biorefineries, as the availability of the input material is relatively high and input material prices are low (Uihlein and Schebek 2009). The input material, used in lignocelluloses feedstock (LCF) biorefinery, can be obtained from: forestry residues and wood waste (including residues from harvest operations left in the forest after stem wood removal: branches, foliage, roots, etc.), agricultural residues (e.g. husks, chaff, cobs, bagasse), energy crops (crops specifically bred and cultivated at low-cost, on marginal land not suitable for food crops production), and municipal paper waste (Demirbas 2009).

The LFC biorefinery is classified as "phase III biorefinery"; three different types of biorefinery, known as phase I, II and III, have been described by Kamm and Kamm, and van Dyne et al. The phase I and II biorefinery use only one feedstock such as corn and wheat. The difference is that phase I biorefinery has the capability to produce a single major product by single process, while phase II biorefineries is capable of producing various end-products and has far more processing flexibility (Van Dyne et al. 1999). In Europe, there are many phase I biorefineries producing biodiesel from vegetable oil (rapeseed oil), through transesterification process. The Novamont plant in Italy is, indeed, an example of a phase II biorefinery that use corn starch to produce several chemical products, such as biodegradable polyesters (Origi-Bi) and starch derived thermoplastics (Master-Bi) (Clark and Deswarte 2015). The phase III biorefineries use various types of feedstocks and processing technologies to produce a variety of products (Van Dyne et al. 1999; Clark and Deswarte 2015). In LCF biorefinery, lignocellulosic feedstocks are fractioned into intermediate outputs (cellulose, hemicellulose and lignin) that are further processed into a multitude of products and bioenergy, (such as biofuels, fine chemicals, advanced polymer materials, steam/heat, and electricity), by jointly applying several technological processes (de Jong and Gosselink 2014). A number of commercial technologies are available today for the pretreatment of lignocelluloses all around the world. Some of these technologies have already been commercialized and are well known, whereas others are still at lab scale. The most relevant commercial technologies are given in following Table 5.3:

The general scheme for lignocellulose bioconversion involves multi-step processes. The first step, following feedstock selection, is the lignocellulosic biomass pretreatment that is a necessary upstream process to reduce the size of biomass and to fractionate, solubilize, hydrolyze and separate cellulose, hemicellulose, and lignin components (Capolupo and Faraco 2016). As described before pretreatments methods can be classified into different categories (Table 5.4): physical, physiochemical, chemical, biological, electrical, or a combination of these (Amelio et al. 2016).

Physical (mechanical) pretreatment increases the surface area by reducing the size of the biomass and improves the flow through biorefinery processes (Arens and Liu 2014). The physico-chemical methods requires high temperature and pressure; it is therefore necessary a high control of operating conditions. Steam explosion is the most commonly physico-chemical method used for pretreatment of lignocellulosic

Table 5.3 Pretreatment technologies commercially available

Process	Company	Characteristic
Steam explosion	Beta Renewables	Low xylose yield
		High enzyme loading
Single-stage dilute acid	Abengoa	High xylose yield
		Moderate enzyme loading
Two-stage dilute acid	Poet-DSM	High xylose yield
		Low enzyme loading
Ammonia & Steam	Dupont	Require high enzyme loading

biomass (Verardi et al. 2016). It combines mechanical forces and chemical effects. The mechanical effects cause separation of lignocellulose matrix in individual fibers (hemicelluloses, cellulose and lignin) with minimal loss of material. The chemical effects promote the hydrolysis of acetyl groups included in hemicellulose (Verardi et al. 2015).

Chemical methods remove and/or dislocate hemicelluloses and lignin, loosening the structural of lignocellulosic matrix (Capolupo and Faraco 2016). Biological pretreatment methods use cellulolytic, hemicellulolytic, and ligninolytic systems synthetized from microorganisms, such as fungi, bacteria, and actinomycetes, in order to degrade lignin, cellulose, and hemicellulose (Sindhu et al. 2016). Electrical method, such as pulsed-electric field (PEF) pretreatment, exposes the cellulose content in the biomass through the formation of pores in the cell membrane, allowing the entry of agents necessary to break the cellulose into constituent sugars (Kumar and Sharma 2017).

Following pretreatment, the biomass components (lignin, cellulose, hemicellulose and residues) are subject to a combination mainly of thermochemical and biochemical processes in order to convert lignocellulosic feedstock into valuable products (FitzPatrick et al. 2010). Thermochemical conversion includes processes as combustion, pyrolysis, gasification, and liquefaction. The combustion process, performed at 800–100 °C, allows to transform biomass into energy by oxidation of carbon and hydrogen-rich biomass to CO_2 and H_2O. This method, used for the production of electricity and heat, is similar to fossil-fuel fired power plants and can produce high NO_x emission.

A variety of value-added chemicals can be obtain from main biomass constituents, hemicellulose, cellulose and lignin, by pyrolysis that consists of a thermal degradation, without oxidizing agent, of solid lignocellulosic biomass into gases and liquids (Table 5.5) This thermal decomposition starts at 350–550 °C and goes up to 700–800 °C (de Wild 2015).

Gasification means the conversion of lignocellulosic biomass into a combustible gas mixture, called producer gas, consisting of carbon monoxide (CO), hydrogen (H_2), and traces of methane (CH_4), at 700–1600 °C. After cleaning, producer gas can be used directly as an engine fuel or upgraded to liquid fuels or converted into chem-

Table 5.4 Methods for lignocellulosic biomass pretreatment

		Operating conditions	Advantages	Disadvantages
Physical	Chipping Grinding Milling	Room temperature Energy input <30Kw per ton biomass	Reduces cellulose critallinity	Power consumption higher than inherent biomass energy
Physio-chemical	Steam pretreatment	160–260 °C (0,69–4,83 MPa) for several second (~15 min in the range 200–230 °C)	Causes hemicellulose auto hydrolysis and lignin transformation; cost-effective for hardwoods and agricultural residues	Destruction of a portion of the xylan fraction; incomplete distruption of the lignin-carboydrate matric; generation of compounds inhibitory; less effective for softwoods
	AFEX (Ammonia fiber explosion method)	90 °C for 30 min. 1–2 kg ammonia/kg dry biomass	Increases accessible surface area, removes lignin and hemicellulose;	Do not modify lignin neither hydrolyzes hemicellulose
	ARP (Ammonia recycle percolation method)	150–170 °C for 14 min Fluid velocity 1 cm/min	Increases accessible surface area, removes lignin and hemicellulose;	Do not modify lignin neither hydrolyzes hemicellulose;
	CO2 explosion	4 kg CO_2/kg fiber at 5.62 MPa 160 bar for 90 min at 50 °C under supercritical carbon dioxide	Do not produce inhibitor for downstream processes. Increases accessible surface area, does not cause formation of inhibitory compounds	It is not suitable for biomass with high lignin content (such as woods and nut shells) Does not modify lignin neither hydrolyze hemicelluloses
	Ozonolysis	Room temperature	Reduce lignin content; does not produce toxic residue	Expensive for the ozone required;

(continued)

Table 5.4 (continued)

		Operating conditions	Advantages	Disadvantages
	Wet oxidation	148–200 °C for 30 min	Efficient removal of lignin; Low formation of inhibitors; low energy demand	High cost of oxygen and alkaline catalyst
Chemical	Acid hydrolysis: dilute-acid pretreatment	Type I: T > 160°, continuous-flow process for low solid loading 5–10%,)-Type II: T < 160 °C, batch process for high solid loadings (10–40%)	Hydrolyzes hemicellulose to xylose and other sugar; alters lignin structure	Equipment corrosion; formation of toxic substances
	Alkaline hydrolysis	low temperature; long time high; concentration of the base; For soybean straw: ammonia liquor (10%) for 24 h at room temperature	removes hemicelluloses and lignin; increases accessible surface area	Residual salts in biomass
	Organosolv	150–200 °C with or without addition of catalysts (oxalic, salicylic, acetylsalicylic acid)	Hydrolyzes lignin and hemicelluloses	High costs due to the solvents recovery
Biological		Several fungi (brown-, white- and soft-rot fungi	Degrades lignin and hemicelluloses; low energy requirements	Slow hydrolysis rates
Electrical	Pulsed electrical field in the range of 5–20 kV/cm,	~2000 pulses of 8 kV/cm	Ambient conditions; disrupts plant cells; simple equipment	Process needs more research

Table 5.5 Main chemicals from lignocellulosic biomass pyrolysis (de Wild 2015)

Biomass constituent (thermal degradation range)	Pyrolysis products
Hemicellulose (150–300 °C)	Acetic acid; Furfural
Cellulose (200–400 °C)	Levoglucosan, Hydroxyacetaldehyde,
Lignin (150–600 °C)	2-Methoxyphenols (e.g. guaiacol), 2,6-Dimethoxyphenols (e.g., syringol), Catechols, Phenol, Alkyl phenols, Methanol,
Whole biomass (100–600 °C)	Extractives (e.g., terpenes), Charcoal, Pyrolysis oil, Gases (e.g., CO, CO_2, CH_4)

ical feedstocks by several methods, as biological fermentation or catalytic upgrading through the Fischer-Tropsch process. Hydrothermal liquefaction is the thermochemical conversion of lignocellulosic biomass into liquid fuels, at 280–370 °C and 10–25 MPa, by processing in a hot, pressurized water environment (Rajvanshi 2014).

Biochemical conversion involves breaking down biomass into sugars, which can then be converted into potential fuel blend stocks and other bioproducts, including renewable gasoline, ethanol and other alcohols, and renewable chemical products, through the use of microorganisms and catalysts.

The most common types of biochemical processes are fermentation and anaerobic digestion (Zhao and Bai 2009). Fermentation is one of the oldest technologies in the world, mainly based on bioethanol synthesis from plant biomass. The fermentation process, in the presence of oxygen, is carried out by microorganisms, including bacteria, yeasts, and fungi. The most commonly used microbe is yeast, mainly *S. cerevisiae* (Zhao and Bai 2009). Several fungal species belonging to genera *Fusarium, Rhizopus* (Hahn-Hägerdal et al. 2007), *Monilia* (Gírio 2010), *Neurospora* (Xiros and Christakopoulos 2009), and *Paecilomyces* (Sommer et al. 2004) were able to ferment monomeric sugars. Bacteria used to produce bio-alcohols (ethanol) from fermentable sugars include *Zymomonas mobilis*, *Bacillus macerans*, *Bacillus polymyxa*, *Klebsiella pneumoniae*, *C. acetobutylicum*, *Aeromonas hydrophila*, *Aerobacter sp.*, *Erwinia sp*., Leuconostoc sp., and *Lactobacillus sp*. (Thatoi et al. 2014). Another bacterial resource is engineering *E. coli* (Srichuwong et al. 2009). Microbial culture types used in fermentation can be classified into five different categories: pure culture, consisting of only one type of organism developed from a single cell (e.g., *S. cerevisiae*); co-culture, containing growths from two distinct cell types (e.g., Aspergillus niger and *S. cerevisiae*); mixed culture, consisting of more than two organisms (*Paenibacillus sp.* and four strains of *Z. mobilis*); immobilized culture; and a co-immobilized culture made by entrapping microorganisms within a given matrix (Thatoi et al. 2014).

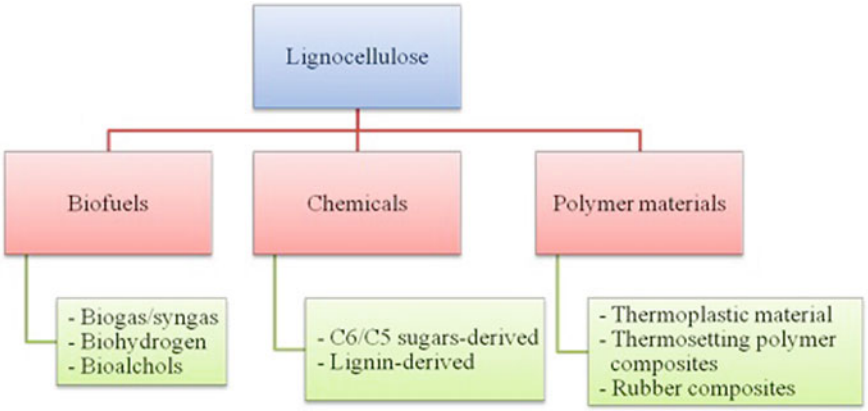


Fig. 5.4 High value bio-products from lignocellulose biomass

Lignocellulosic biomass can be also biochemically degraded by anaerobic digestion. The process is carried out from micro-organisms able to break down organic matter (liquid and solid) in the absence of oxygen and to produce biogas containing mostly methane and carbon dioxide for use as a source of renewable energy. Moreover, anaerobic digestion can be used as an biological pretreatment of lignocellulosic feedstocks, easing the subsequent fractionating of such biomass into its constituent sugars (glucose, galactose, xylose, arabinose, and mannose) and/or short chain fatty acids (acetic, propionic, and butyric acids), which can be further converted into valuable chemicals and biofuels (Surendra et al. 2015).

5.4 High Value Bio-products from Lignocelluloses Feedstock Biorefinery

The products derived from LCF biorefinery, such as biofuels, fine chemicals and advanced polymer materials (Fig. 5.4), might replace petroleum-based products (Cherubini 2010, Cheng and Zhu 2009) The progressive replacement of petroleum refinery oil with lignocellulosic feedstock biorefinery is generally regarded as necessary step for the development of a sustainable industrial society, energy independence, and for the effective management of greenhouse gas emissions (FitzPatrick et al. 2010).

5.5 Biofuels from Lignocelluloses

The conversion of lignocellulose material into several kinds of biofuel, such as biogas/syngas, biohydrogen, and bioalcohols, offers primarily a way to develop renewable and environmental friendly alternatives to substitute fossil fuel with net zero carbon dioxide (CO_2) emission; the CO_2 emitted during fuel combustion is indeed captured during the growth of the feedstock.

Biogas and syngas are complex mixtures composed mainly of methane (CH_4), carbon dioxide (CO_2), hydrogen (H_2), and carbon monoxide (CO) (Awe et al. 2017). The process used to produce the two gasses' mixtures is different: biogas is produced trough anaerobic digestion which involves different groups of facultative or obligatory anaerobic microorganisms (Sárvári Horváth et al. 2016); syngas is created by gasification process that causes the partial combustion of biomass (Samiran et al. 2016).

Biohydrogen production can be obtained with low cost from biomass via hydrolysis and fermentation processes. During combustion process, hydrogen produces only water as its environment-friendly product, receiving widespread attention from researchers in the world (Jiang et al. 2016).

Bio-alcohols, such as bioethanol, biobutanol (or biogasoline), and propanol, can be obtained through the biomass fermentation by the action of aerobic and anaerobic micro-organisms. Today, biological ethanol and butanol are the most commonly produced alcohol fuels: in fact, they can be used directly as substitutes for gasoline, or mixed with gasoline in any ratio (Amelio et al. 2016). The bioconversion process of lignocellulose biomass to ethanol or butanol includes several stages: the pretreatment of feedstock, hydrolysis and fermentation steps, and recovery of products (Verardi et al. 2015). On the contrary, propanol, or isopropyl alcohol, is rarely used as alcohol fuel: it is produced through fermentation of carbohydrates from Escherichia coli to be commonly used as a solvent (Ibrahim 2013).

5.6 Chemicals from Lignocellulose

Besides biofuels production, the lignocellulose biomass holds a great potential for sustainable production of other value-added chemicals. Examples of some chemicals that have been obtained from lignocellulose biomass are given in Fig. 5.5.

The efficient cellulose and hemicellulose depolymerization in hexose (C6) and pentose (C5) sugars is of critical importance for the further development of valuable chemicals. Glucose is the only simple sugar produced by cellulose decomposition. On the other hand, the hemicellulose degradation results in formation of both C6 (glucose, mannose, galactose, and rhamnose), as well as C5 (xylose and arabinose) monosaccharides.

The glucose and xylose can be dehydrated, respectively, into 5-hydroxymethylfurfural (HMF) and furfural (2-furaldehyde), which can further

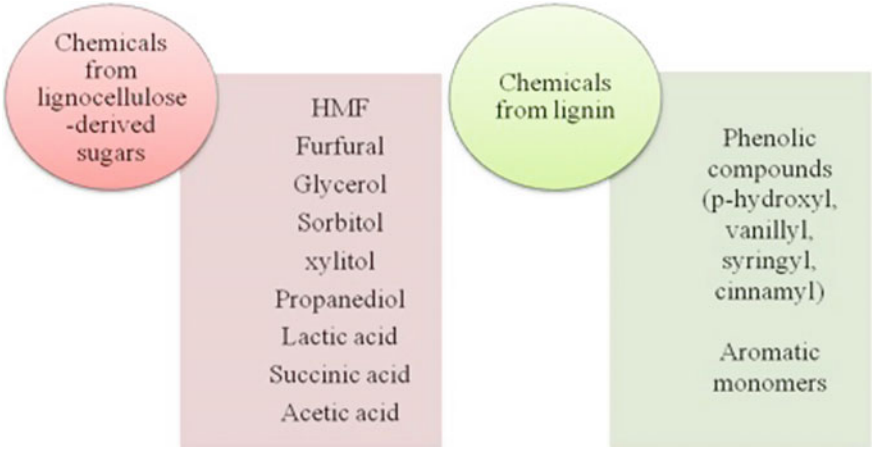


Fig. 5.5 Examples of Chemicals from lignocellulose biomass

be converted into various value-added compounds through feasible chemical transformations (Pereira et al. 2015).

The oxidation of HMF provides an efficient route to synthesis of 2,5-furandicarboxylic acid (FDCA), and 2,5 diformyl-furan (DFF). Currently, FDCA is the most famous HMF derivative: it has attracted much attention recently as potential substitute for terephtalic acid, a petroleum-derived monomer primarily used to produce poly-ethylene-terephthalate (PET) (Han et al. 2017).

Oxidation of HMF to FDCA is a multi-stages process which requires the primary oxidation of HMF to 2,5-diformylfuran (DFF) intermediate, and its sequential oxidation to 5-formyl-2-furancarboxylic acid (FFCA); therefore, FDCA is obtained by further oxidation of FFCA (Zheng et al. 2017).

HMF can also be reduced into 2,5-dihydroxymethylfuran (DHMF), 2,5-dihydroxymethyltetrahydrofuran (DHMTHF), and 2,5-dimethyltetrahydrofuran (DMTHF), or used as intermediate for the production of 2,5-dimethylfuran (DMF), a biofuel with high octane number and energy density that has the potential to replace gasoline directly. DMF is produced by hydrogenation of HMF and subsequent hydrogenolysis.

HMF is very useful also for the production of levulinic acid (LA) by acid rehydration reaction. LA is an important molecule that can be further upgraded in many sectors of industry such as fuel additives, polymer and resin (van Putten et al. 2013).

Furfural can be converted, by hydrogenation, to potential fuel components, such as furfuryl alcohol, 2-methylfuran (MF) and 2-methyltetrahydrofuran (MTHF), and to C4-C5 valuable chemicals, such as valerolactone, pentanediols, cyclopentanone, dicarboxylic acids, butanediol and butyrolactone, by oxidation, hydrogenolysis and decarboxylation processes (Li et al. 2016).

Other chemical compounds, in the form of acids and aldehydes, such as glycerol, sorbitol, xylitol, propanediol, lactic and succinic acid, acetoin or acetic acid, can be produced from lignocellulose biomass-derived sugars (Putro et al. 2016).

The value-added chemicals derived from lignin, via depolymerization or thermal degradation (e.g. oxidation, liquefaction, hydrolysis, hydrocracking, solvolysis and pyrolysis) are phenolic compounds, classified in p-hydroxyl, vanillyl, syringyl and cinnamyl, and aromatic monomers such as benzene, toluene, xylene and hydroxybenzoic acids (Thevenot et al. 2010; Kang et al. 2013; Ma et al. 2015).

5.7 Polymer Materials from Lignocellulose

Lignocellulose biomass can be also used in the preparation of polymer composites materials, using lignin as reinforcement in polymer matrix for making: thermoplastic material, thermosetting polymer composites, and rubber composites (Thakur et al. 2014).

Thermoplastic materials are polymeric materials that can be cooled and heated reversibly without affecting their inherent properties (Wang et al. 2016); several thermoplastic compounds was prepared using lignin as reinforcement, such as: lignin reinforced polystyrene (PS) composites (Barzegari et al. 2012), polydimethylsiloxane-α, ω-diol (PDMS) polymeric matrix-based composites (Thakur et al. 2014), poly(ethylene terephthalate) (PET) matrix-based composites reinforced with lignin (Canetti et al. 2009).

Thermosetting Polymer Composites are polymers that are cured into a solid form and cannot be returned to their original uncured form. Several thermosetting polymer matrix-based composites was prepared using lignin as the reinforcing material, such as: lignin-reinforced epoxy composites (Yin et al. 2012) and lignin-reinforced phenol formaldehyde (PF) polymer composites (Jagur-Grodzinski 2006). Different polymer composite systems were prepared by using rubber as the matrix and lignin as reinforcement, such as: lignin-reinforced styrene-butadiene rubber (SBR)/lignin-LDH (layered double hydroxide) composites (Frigerio et al. 2014), and polymer nanocomposites (Jiang et al. 2013).

Lignin has also been reported to be used as potential reinforcement in foam-based polymer composites, and as a compatibilizer in polymer composites (Thakur et al. 2014).

Finally, the lignin is a promising reinforcement in polymer composites, being biodegradable, CO_2 neutral, abundantly available as industrial waste, low in cost, and environmentally friendly, and having antioxidant, antimicrobial, and stabilizer properties.

5.8 Environmental Impact of Lignocellulose Feedstock Biorefineries

LCF biorefinery should be evaluated for the entire value chain of bio-based products by taking into account environmental, social and economic impacts. For biorefineries, the value chain is classified according to following charcteristics: (i) feedstocks, including production and distribution activities; (ii) conversion processes; (iii) platforms (e.g. intermediate materials used for synthesis of more processed materials and chemicals); and (iv) products obtained after conversion processes from platforms. In particular, LCF biorefieneries may play a major role in reduction of environmental impacts: in tackling climate change by reducing the demand on fossil fuel energy and providing sustainable energy, chemicals and materials. Then, LCF biorefineries are supposed to contribute to a reduction in greenhouse gas. However, biobased products and fuels may also be associated with environmental disadvantages due to, e.g. land use change/intensity or eutrophication of water. These effects also have an impact on biodiversity and ecosystem services. The environmental analysis can be done by life cycle assessment (LCA) methodology which takes into account all the input and output flows occurring along the production chain, from raw material acquisition, to production, use, and end-of-life (Cherubini 2010). This methodology is standardized in the ISO 14040 series by the International Organization of Standardization (ISO) (Mussatto 2016). From various literature data on the environmental impacts of LCF biorefineries, it can be concluded that LCF biorefinery system could be an effective option to mitigate climate change, reduce dependence on fossil fuels and improve cleaner production chains based on local and renewable resources, revitalizing rural areas (Cherubini 2010; Wertz and Bédué 2013; Valdivia et al. 2016; Cheali et al. 2015). The supply of biomass with sustainable practices is a key point to ensure a renewable energy supply to biorefineries. Howevever, an careful environmental evaluation of LCF biorefinery should include several impact categories, for example: the potential consequences due to the competition for food and biomass resources; the impact on use and quality of water; the effects on land use change and soil carbon stocks and fertility of land; the net greenhouse gas balance; impacts on biodiversity and ecosystem services; potential toxicological risks and energy efficiency (De Jong and Jungmeier 2015). Therefore, the determining of all environmental impacts is complex and a certain degree of uncertainty is always present in the final results.

5.9 Conversion Processes

Various conversion processes of lignocelluloses biomass to biofuel are being summarized in following sections giving stress on the engineering of the lignocelluloses materials and mechanism.

5.9.1 First Generation

5.9.1.1 Transesterification

This reaction is used to produce biodiesel or vegetable oil based fatty acid methyl esters (FAME). The product of the reaction is glycerol which is a high value product derived from the oil (Kulkarni et al. 2006; Narwal and Gupta 2013). Transesterification is a reversible reaction and proceeds essentially by mixing the reactant in which the catalyst is a liquid acid or liquid base (called homogeneous catalysis), however in the cases of high free fatty acids (FFA) this process fails that is why solid catalyst is recommended. The reason is that the solid catalysts can simultaneously catalyze the Transesterification of triglycerides and FFA present in biomass to methyl esters (Kulkarni et al. 2006).

5.9.1.2 Ethanol Conversion Process

A wide variety of carbohydrates containing raw materials have been used for production of ethanol by fermentation process. The fermentation process refers to the metabolic conversion of organic substrate by the activity of enzymes secreted by micro-organisms. There are two basic kind of fermentation has been conceptualized, (a) aerobic and (b) anaerobic depending upon oxygen needed in the process or not. There are many micro-organisms capable of providing fermentative changes to both sugars and starches.

5.9.2 Second Generation Biofuel

There are two basic routes for conversion of biomass to liquid biofuels viz. thermo chemical processing and biochemical processing which is described in Fig. 5.6.

- Biochemical—in which enzymes and other micro-organisms are used to convert cellulose and hemicellulose components of the feedstock to sugars prior to their fermentation to produce ethanol;
- Thermochemical—(also known as biomass-to-liquids, BTL), where pyrolysis/gasification technologies produce a synthesis gas ($CO + H_2$) from which a wide range of long carbon chain biofuels, such as synthetic diesel, aviation fuel, or ethanol, can be reformed, based on the Fischer–Tropsch conversion

The clear advantage of thermo-chemical processing is that, it can essentially convert all the organic components of the biomass compared with biochemical processing which focuses mostly on the polysaccharides (Kyung Lee et al. 2015).

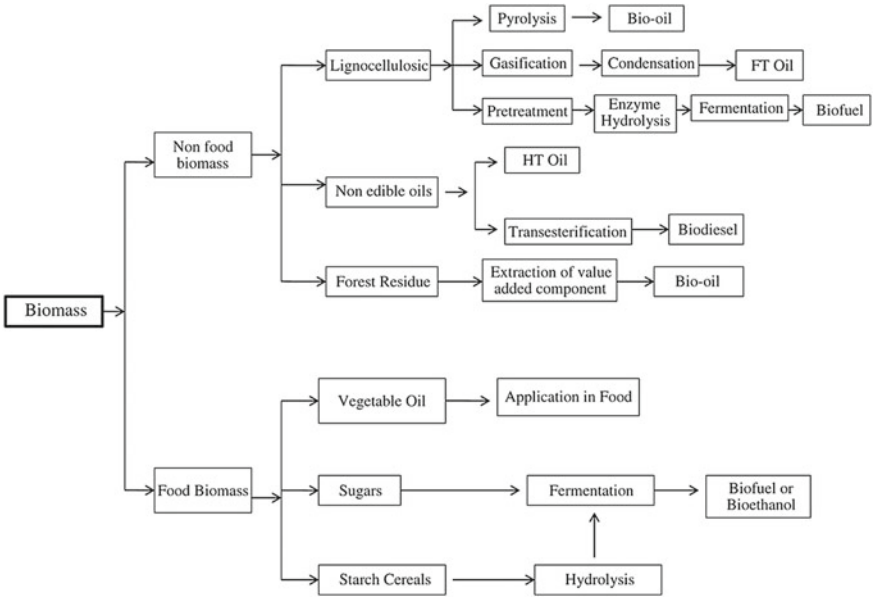


Fig. 5.6 Conversion of biomass to 2nd generation fuels (Chakraborty et al. 2012)

5.9.2.1 Bioethanol from Lignocellulosic Biomass

The ethanol that is produced from lignocelluloses biomass is called bioethanol, which is environmental friendly and renewable (Johnston 2008). It can be used directly in modified spark engines or can be blended with petrol. Ethanol also improves fuel combustion in vehicles hence reduction of emissions. In comparison to petrol ethanol contains only a trace amount of sulphur, so mixing ethanol with petrol helps reduce the sulphur content of fuel, simultaneouly lowering the emission of sulphur oxide which is the major component of acid rain. Sugar and starch can also be fermented to alcohol. This is in-fact the least complex method used in producing ethanol (Kuhad et al. 2011; Chakraborty et al. 2012). With plant biomass it's a different story altogether. Plant biomass consists of cellulose microfibers embedded in hemicelluloses, pectin and lignin. The amount of each component varies among different plant species and parts. Following steps are involved in production of ethanol.

- pretreatment of substrates,
- Saccharification process to release the fermentable sugars from polysaccharides, fermentation of released sugars
- finally distillation step to separate ethanol.

Pretreatment is designed to facilitate in the separation of cellulose, hemicellulose and lignin, so that complex carbohydrate molecules constituting the cellulose and hemicellulose can be broken down by enzyme-catalysed hydrolysis into their constituent simple sugars. The complex structure of cellulose makes it difficult to depoly-

merise into simple sugars, but once the polymer structure has been broken down, the sugar molecules are simply fermented to ethanol using fermentative microorganisms (Elshaghabee et al. 2016).

Hemicellulose consists of 5-carbon sugars, which although are easily broken down into its constituent sugars such as xylose and pentose, the fermentation process is much more difficult, and requires efficient microorganisms that are able to ferment 5-carbon sugars to ethanol.

Lignin consists of phenols, and for practical purposes is not fermentable, although it can be recovered and utilized as a fuel, providing process heat and electricity for the alcohol (ethanol, butanol) production facility.

The hydrolysis is usually, catalyzed by cellulase enzymes and the fermentation are carried out by yeast or bacteria. The factors that affect the hydrolysis of cellulose include porosity, i.e., accessible surface area of the waste materials, cellulose fiber crystallinity and lignin and hemicellulose content (Kang et al. 2014). The presence of lignin and hemicellulose makes the access of cellulase enzymes to cellulose difficult. The lignin and hemicellulose removal, reduction of cellulose crystallinity and increase of porosity in pretreatment processes can significantly improve the hydrolysis. The cellulose crystallinity can be reduced by a combination of chipping, grinding and milling (Santos et al. 2011). Steam explosion is the most commonly used method for pretreatment of plant biomass (Kang et al. 2014).

Lignin biodegradation could be catalyzed by the peroxidase enzyme with the presence of H_2O_2. Microorganisms such as brown, white and soft rot fungi are used in biological pretreatment processes to degrade lignin and hemicellulose (Cragg et al. 2015). Brown rots mainly attack cellulose, while white and soft rots attack both cellulose and lignin. The white rot fungus Phanerochaete chrysosporium produces lignin-degrading enzymes, lignin peroxidases and manganese-dependent peroxidases, during secondary metabolism in response to carbon or nitrogen limitation (Cragg et al. 2015). Other enzymes including polyphenol oxidases, laccases, H_2O_2 producing enzymes and quinine-reducing enzymes can also degrade lignin. The advantages of biological pretreatment include low energy requirement and mild environmental conditions, but the hydrolysis rate is very low (Santos et al. 2011).

Furfural is an important inhibitor of ethanol production from hemicellulose hydrolysate even at low concentrations. Various bacteria and yeast have been reported to partially transform furfural to either furfural alcohol or furoic acid, or a combination of both (Moysés et al. 2016). A few microbial species such as Neurospora, Monilia, Paecilomyces and Fusarium have been reported to hold the ability to ferment cellulose directly to ethanol by simultaneous saccharification and fermentation (SSF) (Singh et al. 2017). Consolidated bioprocessing (CBP) featuring cellulase production, cellulose hydrolysis and fermentation in one step, is an alternative approach with outstanding potential (Byadgi and Kalburgi 2016). The recombinant strain of E. Coli with the genes from Z. mobilis for the conversion of pyruvate into ethanol has been reported by Olson et al. (2015). A key challenge to commercializing production of fuels and chemicals from cellulosic biomass is higher processing costs (Manochio et al. 2017; Techaparin et al. 2017). Biological conversion opens such

low costs production path as it has the potential to achieve a higher yield and the modern tools of biotechnology can improve key process steps.

A range of residual substrates such as sugarcane bagasse, sugarcane molasses and starch has been found suitable for the bioconversion of available carbohydrates in these substrates to produce ethanol (Techaparin et al. 2017; Suryaningsih 2014). A variety of mesophilic and thermophilic microorganisms were employed to optimize the fermentation process, which could be practically viable in different climatic conditions, particularly to reduce the cost of temperature maintenance in large fermenters operating in warmer countries in summer months (Wu et al. 2016).

Sukumaran et al. have recently reported on bioethanol production from the saccharification of wheat bran, a ligno-cellulosic waste (Sukumaran et al. 2009). The cost of cellulase enzymes is a major factor in the enzymatic saccharification of agricultural biomass, which contains lignin. Production cost of cellulases and hence ultimately the cost of ethanol production may be brought down by multifaceted approaches. One important approach is the use of cheaper lignocellulosic substrates for the biosynthesis of the enzyme, and second strategy is the use of cost efficient fermentation process such as solid state or solid substrate fermentation at much cheaper cost.

Whilst bioethanol production has been greatly improved by development of new technologies but there are still challenges that need further improvements in the developed technology to bring forward to commercial scale. These challenges include maintaining a stable performance of the genetically engineered microorganisms and developing more efficient pretreatment technologies for the lignocellulosic biomass and integrating the optimal components into economic ethanol production systems.

5.9.3 Third Generation Biofuel

The conversion technologies for utilizing microalgae biomass can be separated into two basic categories of thermochemical and biochemical conversion (similar to terrestrial biomass). Thermochemical conversion covers the thermal decomposition of organic components to fuel products, such as direct combustion, gasification, thermochemical liquefaction and pyrolysis. The biological process of energy conversion of biomass into other fuels includes anaerobic digestion, alcoholic fermentation and photo biological hydrogen production (Slade and Bauen 2013).

5.10 Advancement in Different Fuels

As mentioned earlier the field of biofuels has seen meteoric change both in the techniques used and the quantities of biofuel produced. Use of food crops was supplemented by agricultural waste and residue and it was hypothesized that microscopic species can be used for further improvement. Transgenic has been used to improve the biofuel crops (Grant 2009). Efficient biotechnical methods to modify the struc-

ture of different algal species coupled with photonic techniques has been explored to give high yields. Different designs of bioreactors have been explored which aims at higher growth rate of algae. One such example is found in one of the recent work by Milano et al. 2016 but not only this there had been a thrust on developing different technologies to use biofuel in different forms. Bio-fuel cells both enzyme and microbe based to convert biofuels to electricity has been created and improved. One such advancement in the bio fuel cells is creation of organelle based biofuel cell (Marbelia et al. 2014) which uses mitochondria immobilized on paper instead of complete cells. This kind of fuel cell is more efficient than enzymatic fuel cell and has the efficiency of microbial fuel cell. These and many such other developments have propagated the hope that these biofuels can be used competitively with petroleum based products as well as production of hydrogen in recent days (Sharma 2017).

5.11 Progress in Processing of Lignocellulosics to Biofuels

5.11.1 Pre-treatment

Due to the nonfermentable nature of lignin, biomass is pretreated to separate cellulose, hemicellulose and lignin. Pretreatment is the major step in the successful production of valuable products from lignocellulosic biomass. A suitable pretreatment of biomass is necessary to ensure good yields of sugars from the polysaccharides. Pretreatment disrupts the plant cell wall and improves enzymatic access to the polysaccharides as raw and untreated biomass is usually resistant to enzymatic degradation. A number of biomass pretreatment technologies are available today (Nanda et al. 2014; Putro et al. 2016; Menon and Rao 2012), such as **physical** (comminution by chipping, grinding and milling to reduce biomass particle size; ozonolysis; gamma rays; pulsed electrical field; electron beam; ultrasound and microwave digestion), **chemical** (use of acids, bases and organic solvent in biomass hydrolysis), **thermophysical** (liquid hot water, steam explosion, supercritical water), **thermochemical** (wet oxidation, ammonia recycle percolation, ammonia fiber explosion, supercritical CO_2) and **biological** (enzymatic hydrolysis).

As regard chemical pre-treatments, hydrolysis of lignocellulosic biomass result in undesirable components found in biomass hydrolysates that are inhibitory to fermentation include sugar degradation products (e.g. hydroxymethyl furfural or HMF and levulinic acid), hemicellulose degradation products (e.g. acetic acid, ferulic acid, glucuronic acid and p-coumaric acid) and lignin breakdown products (e.g. syringaldehyde and syringic acid). New pretreatment methods were proposed to be highly efficient and effective for downstream biocatalytic hydrolysis of various lignocellulosic biomass materials, which can accelerate bioethanol commercialization, such as the hydrogen peroxide–acetic acid pretreatment (Wi et al. 2015). Recent advances included acidic treatments to deconstruct biomass in combination with organic solvents in a biphasic system, in order to increase the concentrations of products and

the efficiency of downstream processing options (Wettstein et al. 2012). It would be highly desirable if these organic solvents could be produced from biomass, thereby eliminating the need to transport solvents derived from petroleum to the biomass refining site. Recently, ionic liquids are gaining interest in biomass hydrolysis and being attractive alternatives to volatile and unstable organic solvents due to their high thermal stability and nearly absolute nonvolatility (Vancov et al. 2012). Nevertheless, there are several core issues that stand in the way of commercialization, including the relative high cost of the ionic liquids, a lack of knowledge in terms of process considerations for a biorefinery based on these solvents, and scarce information on the co-products of this pre-treatment technology (Klein-Marcuschamer et al. 2011).

Thermophysical and thermochemocal pretreatments often result in the generation of inhibitory byproducts such as furfural, HMF and acetic acid. They have adverse effects on enzymatic hydrolysis and fermentation, consequently several post-treatment steps such as detoxification, neutralization and nutrient supplementation to the hydrolysate medium could curb the inhibitory effects (Nanda et al. 2014).

As regard biological pre-treatments, lignocellulose polysaccharides are hydrolyzed to provide the mono-saccharides used by microbial biocatalysts in fermentation processes.

Synergistic interaction between different enzymes have been investigated in order to design optimal combinations and ratios of enzymes for different lignocellulosic substrates subjected to various pretreatments (Van Dyk and Pletschke 2012). Bioconversion using enzyme synergy has generally opted for two approaches, individual enzyme combinations or combinations of commercial mixtures. Based on the substrate analysis and identification of sugars, enzymes are selected for hydrolysis of bonds relating to those sugars. Enzymes required for glucose and xylose release are considered the main enzymes, while accessory enzymes are added should those sugars be present. These enzymes are then evaluated for optimal yield and synergy. Once enzyme ratios are optimized, further accessory enzymes can be evaluated for total release of all sugars (see Fig. 5.7a). Finally, commercial mixtures must be selected and characterized to identify the presence of relevant enzyme activities. Ratios of commercial mixtures are optimized based on yield of glucose and xylose. Enzyme activities that are not present in the commercial mixtures must then be added in the form of additional enzymes and evaluated for improved hydrolysis (Fig. 5.7b).

5.11.2 Cellulose and Hemicelluloses Conversion

After biomass pretreatment, cellulose and hemicellulose fractions of the lignocellulosic biomass are converted to various biofuels, while the residue fraction (lignine) is converted via combustion. Hydrotermal, thermochemical, biochemical and chemocatalytic processes are typically studied to produce biofuels from lignocellulosic biomass. Along with bio-oil, ethanol, butanol and syngas, various value-added co-products including biochar, organic acids, solvents, phenols, aromatic compounds, etc. are also obtained. The most used platform molecules include: (a) levulinic acid

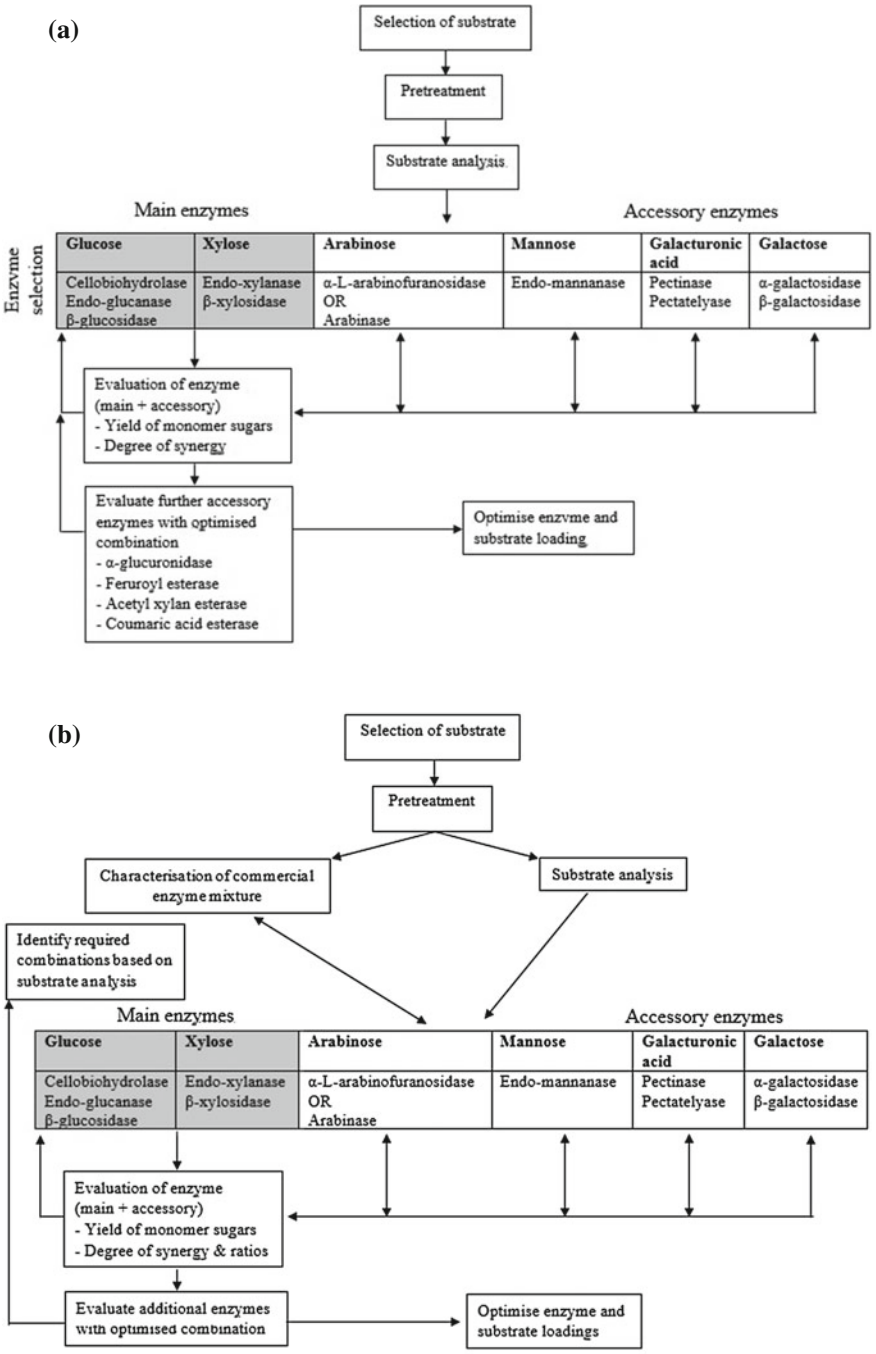


Fig. 5.7 **a** Model for developing optimal combinations with individual enzymes and **b** optimal synergistic combinations with commercial mixtures of enzymes (Van Dyk and Pletschke 2012)

that can be transformed to produce either fuels or additivies for fuels; (b) furan derivatives that can also be transformed into fuels and fuel additives; (c) polyols to produce liquid fuels as well as oxygenated additives; (d) fatty acids for producing diesel and lubricants (Climent et al. 2014).

In **hydrothermal** processes, supercritical water acts as a medium in the biomass conversion to fermentable sugars and H_2-rich syngas. This technology has been found to be promising for the production of H_2 from biomass over last few years, but it has a few limitations for industrial applications (Nanda 2012). **Thermochemical** processes do not require enzymes or microorganisms, they are applicable over a wide range of feedstocks, and they are generally compatible with conventional petroleum processing technologies. However, pre-treatment of the biomass and physical feeding into thermal processing units are challenging. The thermochemical conversion of biomass includes gasification, pyrolysis and liquefaction.

Gasification produces syngas and tar (condensable high molecular weight hydrocarbons produced by incomplete biomass gasification). Syngas is converted to biofuels by using chemical catalysts known as FT process or by using microbial catalysts known as syngas fermentation (Munasinghe and Khanal 2011). The syngas fermentation into ethanol and other bioproducts is considered to be more attractive due to several inherent merits over the biochemical approach and the FT process. In gasification, challenges include minimization of tar formation, syngas cleanup, development of effective catalysts, and integration with Fischer–Tropsch (FT) process. The direct integration of biomass gasification and FT synthesis requires an intermediate gas-cleaning system, because the gaseous stream delivered from the gasifier typically contains a number of contaminants that need to be removed before the FT unit, which is highly sensitive to impurities (Serrano-Ruiz and Dumesic 2011). The utilization of pure oxygen atmosphere, small particle sizes (lower than 1 mm diameter), and a combination of high temperatures, high pressures and low residence times favors the production of syngas versus producer gas (a mixture of CO, H_2, CO_2, CH_4, and N_2 used for heat and electricity production) (Serrano-Ruiz and Dumesic 2011).

Pyrolysis produce bio-oil, gas and char and major challenges include cleanup of the bio-oil and sufficient stabilization of it for practical delivery and use in a petroleum refinery (Hoekman 2009). A new controlled conversion of lignocellulose biomass to bio-jet and diesel fuels by catalytic pyrolysis of biomass into low carbon hydrocarbons coupled with alkylation of aromatics was recently proposed (Zhang et al. 2015).

Finally, liquefaction gives bio-oil and gas as products. Bio-oil results in a complex mixture of volatile organic acids, alcohols, aldehydes, ethers, esters, ketones, and non volatile components. This oil could be upgraded catalytically to yield an organic distillate product which is rich in hydrocarbons and useful chemicals (Naik et al. 2010).

The **biochemical** conversion involves biomass hydrolysis with dilute acids and enzymes to produce monomeric sugars followed by microbial fermentation of the sugars to fuel ethanol and butanol (Balat 2011). Recent studies have aimed to better characterize and understand the mechanisms of cellulase/hemicellulase reactions to design high performance cellulosomes/hemicellulosomes (Gao et al. 2013).

Recent articles reported the identification and characterization of novel xylanases (GH10-XA) and α-glucuronidase (GH67-GA) from *Alicyclobacillus* and *Caldicellulosiruptor* (GH67-GC) (Cobucci-Ponzano et al. 2015). Several authors focused on the current status and advances in cellulase and hemicellulase improvement (Dumon et al. 2012, Behera and Ray 2016, Gao et al. 2011). Recent developments include engineered strains for consolidated bioprocessing for cost-effective production: hydrolytic strains with a recombinant biofuel pathway and engineering of a natural ethanologenic strain by inserting cellulolytic and/or hemicellulolytic potentialities (Amore and Faraco 2012). Although the actual consolidated bioprocessing yields are lower than those of wild fermenting microorganisms on lignocellulose hydrolysates, the concept is promising. The biomass hydrolysates containing monomeric sugars (glucose and xylose) were fermented using *Saccharomyces cerevisiae* and *Clostridium beijerinckii* for ethanol and butanol production, respectively (Nanda et al. 2014). New strains and process intensification are being investigated, including the use of a pervaporation system in order to remove the produced alcohol continuously and increase the yield (Amelio et al. 2016).

Solid-state fermentation technology is expanding with increasing importance for the production of high value-added products, by involving the growth of microorganisms on moist solid substrates in the absence of free flowing water. It has gained considerable attention due to several advantages over submerged fermentation (Behera and Ray 2016). Other authors investigated the rapid bioconversion of lignocellulosic sugars into ethanol using high cell density fermentations with cell recycle by using nine different engineered microbial strains: the results showed that acceptable performance is largely correlated to the specific xylose consumption rate (Sarks et al. 2014).

Bioconversion of lignocellulose by microbial fermentation is typically preceded by an acidic thermochemical pretreatment step designed to facilitate enzymatic hydrolysis of cellulose. Substances formed during the pretreatment of the lignocellulosic feedstock inhibit enzymatic hydrolysis as well as microbial fermentation steps. Conditioning of slurries and hydrolysates can be used to alleviate inhibition problems connected with hydrolytic enzymes and the yeast *Saccharomyces cerevisiae*. Novel developments in the area include chemical in situ detoxification by using reducing agents, and methods that improve the performance of both enzymatic and microbial biocatalysts, such as fermentation technology and microbial resistance to inhibitors (Amelio et al. 2016).

Since lignocellulose conversions carried out at <50 °C have several limitations, thermophilic bacteria and thermostable enzymes were also investigated to overcome the limitations of existing lignocellulosic biomass conversion processes to biofuels (Amelio et al. 2016; Bhalla et al. 2013).

Alternatively, an integrated system including anaerobic digestion and aerobic fungal fermentation was investigated to convert corn stover, animal manure and food wastes into microbial lipids for biodiesel and methane production (Zhong et al. 2015). This novel self-sustaining advanced lignocellulosic biofuel production is based on a combined hydrolysis treating synergistically solid digestate and corn stover (see

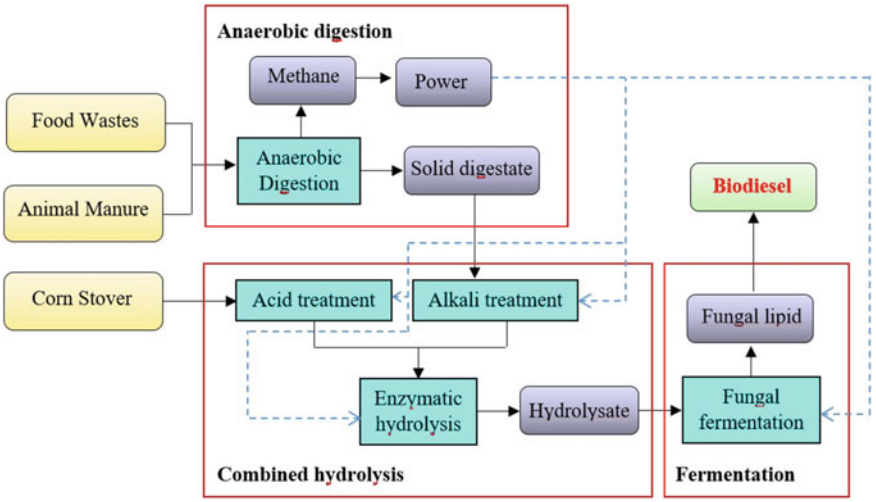


Fig. 5.8 Flowchart of a self-sustaining advanced lignocellulosic biofuel production (Zhong et al. 2015)

Fig. 5.8). Some authors also proposed to link anaerobic digestion and pyrolysis in order to convert lignocellulosic biomass more efficiently (Fabbri and Torri 2016).

In metabolic engineering, significant progress has been made using physical and chemical mutagens to increase production of lignocellulolytic enzymes (Behera and Ray 2016; Chandel and Singh 2011). A wide range of microorganisms are being engineered reflecting the effectiveness of today's gene technology. Successful metabolic engineering strategies are being applied with emphasis on xylose catabolism, inhibitor tolerance, synthetic microbial consortium, and cellulosic oligomer assimilation (Chen and Dou 2016).

In the **chemical or chemo-catalytic** approach, the cellulosic biomass undergoes catalytic hydrolysis, using acids either in aqueous solution or heterogeneous phase. Continued research is necessary to address the use and separation of mineral acids, increase the concentration of product streams, and improve product separations. Mineral acids can be eliminated by the identification of solid acid catalysts easily recoverable from the reaction mixture and recyclable for biomass deconstruction and for upgrading the resulting sugars. Alternatively, effective management and recycle of the mineral acid must be achieved to reduce costs and environmental impact.

Direct transformation of lignocellulosic biomass into 5-hydroxymethylfurfural (HMF)—emerging platform for the next generation plastics and biofuels (Wang et al. 2014)—was carried out using single or combined metal chloride catalysts in DMA–LiCl solvent under microwave-assisted heating or using Sn-Mont catalyst in a tetrahydrofuran (THF)/H_2O–NaCl biphasic system under mild conditions (Wang et al. 2014).

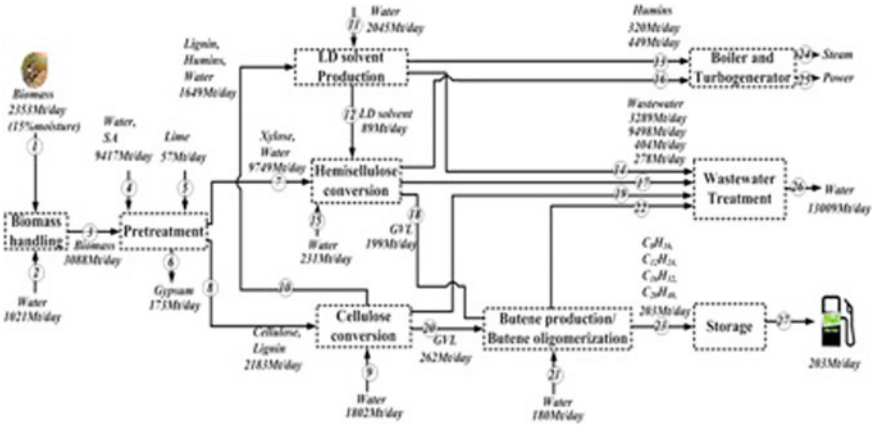


Fig. 5.9 Integrated catalytic process for monophasic conversion of cellulose to butene oligomers (using SBP solvent), biphasic conversion of hemicellulose to butene oligomers (using LD solvent), and monophasic conversion of lignin to LD solvent (Kim and Han 2016)

A novel controllable transformation of lignin into C8–C15 cycloparaffins and aromatics in the jet and diesel fuel range by catalytic depolymerization of lignin using ionic liquid was demonstrated. Ionic liquids may play a role in catalysis, in addition to their role in facilitating the dissolution of cellulose (Zhang and Zhao 2010). However, ionic liquids were not suitable for large scale applications due to their high cost and deactivation by small amounts of water, and their separation from the reaction mixture was still a problem.

An integrated process (see Fig. 5.9) based on a new alkylphenols-based biomass conversion technology was recently developed by Kim and Han (2016) as an economically competitive alternative to current biofuel production approaches. This catalytic production strategy involves separate conversion of hemicellulose and cellulose using 2-sec-butylphenol (SBP) and lignin-derived (LD) alkylphenol solvents, in order to produce liquid hydrocarbon fuels (butane oligomers). Firstly, row biomass is fractionated by dilute sulfuric acid (SA)-catalyzed pretreatment into cellulose and hemicellulose-derived xylose. These two fractions are then converted separately to levulinic acid (LA) using SBP and LD alkylphenol solvents, respectively. Finally, LA is upgraded catalytically to butene oligomers via c-valerolactone (GVL) and butene intermediates.

The proposed strategy has a high biomass-to-fuels yield (34.8 mol%) at low solids concentrations using large volumes of solvents, which are mostly recovered (99%). Energy integration reduced the total heating requirements by 72%.

Hemicellulose and cellulose can be also simultaneously converted in a single reactor, thus eliminating pre-treatments steps to fractionate biomass and simplifying product separation. This process uses gammavalerolactone (GVL) as a solvent, that is also one of the reaction products, over different catalysts (Climent et al. 2014).

Commercial processes for the conversion of biomass to fuels are now based mainly on the production of bioethanol, biodiesel and renewable fuels from gasification and pyrolysis of biomass and hydroprocessing of triglycerides, but there are few commercial processes based on the catalytic approaches. A catalytic process was developed by Avantium (Netherlands) in order to produce furan derivatives such as ethoxymethylfurfural as alternatives to petroleum-derived hydrocarbons. In 2010 Shell and Virent announced the first biogasoline demonstration plant based on Virent's Bioforming® process converting aqueous carbohydrate solutions into mixtures of hydrocarbons by combining aqueous phase reforming using heterogeneous catalysts (Virent modified ZSM-5 zeolite) (Climent et al. 2014).

5.12 Conclusions and Future Trends

Biofuels are a promising short term alternative to petroleum-derived fuels and can be derived from renewable carbon sources to mitigate greenhouse gas emissions. The applicability of biomass as a renewable resource for transportation fuels has been demonstrated by the successful integration of first generation bioethanol and biodiesel into the current infrastructure. However, first generation technologies have drawbacks related to their consequent ethic question food *vs* fuel. A more sustainable biofuels strategy would utilize widely available biomass feedstocks to the largest extent possible, drawing upon non-edible lignocellulosic biomass. A successful lignocellulosic biorefinery can be realized through a combination of different technologies and biomass processing strategies for the flexible production of varied fuel and chemical products.

There are many challenges which need to be addressed to make the syngas fermentation commercially viable in producing biofuels and other value-added products. The yields of the products from syngas fermentation are usually low; hence new recombinant microorganisms with high yields of ethanol are essential for industrial scale fermentation of syngas. Genetic manipulation of microorganisms to amplify solvent production over acetic acid can be considered as a possible option (Nanda et al. 2014).

Bioconversion using enzyme synergy is generally based on two useful approaches, individual enzyme combinations or combinations of commercial mixtures. The use of individual enzymes can lead to a greater understanding of synergy and cooperation between enzymes to degrade a complex substrate, but there is no commercial availability of pure enzymes (mostly the lesser known accessory enzymes) to study interactions between enzymes, as well as there is no characterization of the available enzymes in terms of activity on complex substrates, stability and inhibition in the bioreactor environment. New enzymes and protein engineering are necessary to improve characteristics of enzymes and to provide suitable enzymes for the future.

Instead, the use of commercial enzymes may be a quicker route to commercialization, but it is important that production of these mixtures should be optimised for different substrates with different pretreatments.

In order to perform the biocatalytic conversion of lignocellulose at industrial scale, further aspects need to be addressed. At first, the biocatalysts have to be improved so that higher yields and productivities can be achieved. Moreover, product recovery and the recycling of water as well as biocatalysts have to be considered, since both aspects are essential for industrial processes. Ultimately, the evaluation and model-based synthesis of the complete process chain needs to be performed, since pretreatment, hydrolysis, fermentation, product recovery as well as recycling steps are strongly associated and need to be harmonized (Jäger and Büchs 2012). Further research should be done with respect to hemicellulases and their contribution to lignocellulose degradation, particularly the role of enzymes such as pectinases, mannanases and other accessory enzymes. The role of other proteins and non-hydrolytic enzymes to achieve and enhance complete degradation of lignocellulose requires further investigation (Van Dyk and Pletschke 2012).

New trends in engineering synthetic microbial consortia and direct use of cellulosic or hemicellulosic oligomers are promising potential future directions for research and development. Strain improvement for enhanced cellulases biosynthesis using mutagenesis, metabolic engineering and genomics approaches, should be used for the lignocellulosic bioconversion processes. Recombinant DNA technology and protein engineering are also being used as a powerful modern approach for efficient lignocellulosic bioconversion by improving various aspects of lignocellulolytic enzymes such as production, specific activity, pH and temperature stability, or by producing novel proteins/enzymes with altered properties (Kumar et al. 2008).

Moreover, degrading the recalcitrant part of the lignocellulosic biomass (chitin for example) remains a challenge. Further challenges concern the combination of pretreatment and hydrolysis at high solids loadings to make energetic molecule production economically viable. However, efforts need to be continued to overcome biological bottlenecks and transfer limitations, crucial steps to optimize processes with high solids lignocellulosic materials (Alfenore and Molina-Jouve 2016).

In order to compete with the cost of petroleum fuels, the cost of biofuel processing should be kept as low as possible using energy efficient technologies and using less water. Producing as many co-products as possible in a biorefinery will help to reduce the cost of biofuel production. It is important that a biorefinery should be established in an appropriate location that has good water resources, access to feedstocks, and energy that is needed to process the feedstock. Several studies are in progress to enhance carbohydrate release from lignocellulose by combinations of physical and physicochemical pretreatments; combine pretreatments and hydrolysis to improve yields at high solids loadings; maximize and accelerate the conversion of sugar monomers into the final products by improving enzyme activities for separate hydrolysis and fermentation processes; improve the fermentation performances by ensuring nutritional complementation of both liquid and gaseous media, optimizing mass transfer with new configurations of bioreactor, and developing engineered strains to better understand the metabolic pathways involved in biofuel synthesis to develop over-producing engineered strains with increased inhibitor resistance; develop a cleanup system to remove inhibitors present in both the substrate (liquid or gas) and the fermented broth for purifying energetic molecules.

In conclusion, the success of a biorefinery concept depends on the development of energetically efficient processes to convert lignocelluloses biomass directly into biofuels. For this reason, the research should focus on:

- in-depth understanding of the mechanism of conversion of lignocellulosic biomass by heterogeneous catalysis and of the interrelationship among the feedstock, the catalyst, the reaction conditions and the product distribution;
- design and preparation of multifunctional catalysts for highly active and selective conversion of lignocellulosic biomass;
- development in biological and genetic fields;
- development of strategies for the production of flexible chemical platform molecules, such as levulinic acid and g-valerolactone;
- applications for the production of speciality chemicals and hydrocarbon fuels;
- technologies for biomass deconstruction, such as fast pyrolysis;
- methods for the synergistic coupling of hydrolytic and thermochemical processes into an integrated biorefinery;
- strategies for lignin utilization.

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Chapter 6
Beyond Ethanol: Contribution of Various Bioproducts to Enhance the Viability of Biorefineries

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Abstract Environmental pollution by the extensive use of fossil fuels and petroleum-based products is a current worldwide concern. In this context, the development of valuable products from renewable sources is an interesting and environmentally friendly alternative. Lignocellulosic biomass is a renewable low cost feedstock that presents in its composition high quantity of cellulose, carbohydrate extensively studied to produce cellulosic ethanol. However, considering the high cost of 2G ethanol process, the coupled production of other products can help the economic viability in a context of a biorefinery producing bioenergy, biopolymers, biopharmaceutical, nutrients, pigments, surfactants, biochemical, and others, from different fractions of biomass. Products with high economic value such as vitamins B7, B12, C and E, riboflavin, xylitol and lactic acid can be obtained by biotechnological route from sugars released after hydrolysis of cellulose and hemicellulose fraction present in biomass. Thus, an integrated industry that can direct production considering market fluctuation could be thought, taking advantage of biotechnological routes. In this chapter, biorefinery concept is briefly discussed and some bioproducts that can contribute with economic viability of current biorefineries are presented. Some interesting possibilities were discussed, including different compounds with a variety of applications as substitute of traditional products or representing new and innovative ones.

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6.1 Introduction

Lignocellulosic biomass is a low cost and readily collectable feedstock mainly composed of the carbohydrate fractions of cellulose and hemicellulose and non-carbohydrate phenyl propane fractions of lignin along with other minor components such as extractives and ash. These lignocelluloses include agro-industrial residues as sugarcane bagasse and crop stubble, wood and forest residues, and landfill waste and correspond to the most abundant feedstocks that can be used to obtain high value sustainable by-products. The aforementioned components of the biomass can be separated maximizing their valorization. E.g., currently the cellulose fractions, after pretreatment, are being enzymatically hydrolyzed to release monomeric sugars which are used for ethanol production. Besides, after an acid or enzymatic hydrolysis of hemicellulose, the released sugars, as xylose, can be used to produce 5-hydroxymethylfurfural (HMF), xylitol and other value added products. The remaining Lignin fractions can be used for electricity generation and also for the production of aromatic compounds and commodity chemicals. Typically the transformation of lignocellulosic components into valuable products needs an extensive processing collectively known as biorefining. These biorefinery products are usually biodegradable, biocompatible and eco-friendly. Also, these products can help to reduce the dependence of petroleum based products in a sustained way. Considering the complexity of the production process, several compounds such as biopharmaceutical, vitamins, nutrients and others can be commercialized in low volumes yet at high prices. Additionally, the demand of market of these products due to biocompatibility and biodegradability properties are increasing since last years. In this way, the production of these compounds coupled with existent bioenergy industry is an interesting option to improve the economic viability of biorefineries. In this chapter, the current situation of production of cellulosic ethanol and other biomolecules in the context of biorefinery is presented and discussed.

6.2 Biorefinery Concept: Integration of Different Bioproducts

The core idea of a biorefinery is rooted from a knowledge-based economy known as bioeconomy. The American National Renewable Energy Laboratory (NREL) defined biorefinery as “a facility that integrates biomass conversion processes and equipment to produce fuels, power and chemicals from biomass” (Parada et al. 2017). This definition is considering a possibility to actually provide a substitute for the petrochemical

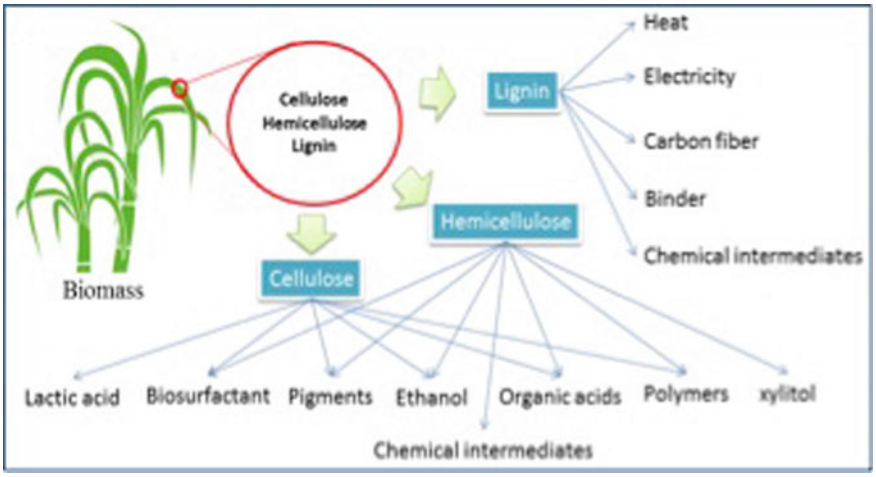

Fig. 6.1 Conversion of lignocellulosic biomass into valuable products in the concept of an integrated biorefinery

industry to yield a multitude of sustainable fuels and chemicals. The evolution of biorefineries, based on the heterogeneity of feedstocks, has seen a gradual shift from starch based refineries (SBR) to non-food based lignocellulosic refineries (LCR), which utilizes waste biomass including forest residues, whole crops, woody biomass, pulp and paper residue etc.

Lignocelluloses are the most abundant and sustainable feedstocks. They are diverse in their nature and can be exploited for the production of various bioproducts. Their three major fractions namely cellulose, hemicellulose and lignin can be completely utilized in the context of a biorefinery, allowing to establish cellulose-, hemicellulose- and lignin-based biorefineries (Manandhar and Shah 2017; Hu et al. 2017; Mihiretu et al. 2017).

The classical approach thus far adapted for the lignocellulosic biorefinery comprise of some steps such as pretreatment, hydrolysis (chemical/enzymatic) and ultimately fermentation. Among from these steps, pretreatment is envisaged as a prerequisite to disintegrate the inherent structural recalcitrant of the raw biomass matrix (Terán-Hilares et al. 2016; Ahmed et al. 2016). There are a lot of pretreatment options and, in some alternatives, hemicellulose can be hydrolyzed or lignin removed. Pretreated biomass is subjected to hydrolysis of the cellulose and hemicellulose (if no hydrolyzed in pretreatment step) fractions for the release of mostly C6 and C5 sugar fractions, respectively (Sun and Cheng 2002; Terán Hilares et al. 2017b). The remaining left over residue is rich in lignin, which has high energy density and is a great source for electricity generation along with production of green chemicals such as adhesives and binders.

In Fig. 6.1 is shown a schematic representation of different products which can be obtained from lignocellulosic biomass in an integrated biorefinery context.

6.3 Current Scenario of Cellulosic Ethanol in Brazil and New Opportunities to High Value Biomolecules Production

Currently, there are two commercial scale plants for 2G ethanol production (GranBio and Raizen) in Brazil. According to Ministry of Mines and Energy of Brazil (MME 2015), the national projection for cellulosic ethanol production in Brazil for 2024 is up to 429 million L, considering the production of seven installed and in construction plants.

The company RAIZEN started operation in 2014 with an initial investment of R$237 million in research, development and structure. The nominal capacity projected by the company was 40 million L of 2G-ethanol production per year (Raizen 2014). According to State Department of Energy and Mining of the Sao Paulo State (2017), the projected production of second generation ethanol during 2017/18 will be about 16 million L, value lower than 50% of nominal capacity of the company. That value is higher than obtained in 2016/17 which was near to 8 million liters.

The Bioflex 1, the industrial unit of GranBio, also started the production of second generation ethanol from straw and sugarcane bagasse in 2014. This company was constructed to produce 82 million L per year. However, in 2015, the company produced only 4 million L and in 2017 it was projected to achieve 50% of real capacity (Novacana 2016).

In both Brazilian 2G ethanol companies, the technological bottlenecks are the transport of bagasse along the process in the plants and the heterogeneity of the pretreatment process. Besides, other hurdles the current economic situation of Brazil is also a major blow for these companies. The Brazilian Development Bank (BNDES) is responsible for 40–50% of financing of Brazilian sucro-energetic industry. In the case of GranBio Company, BNDES has 15% of the business with a contribution of $190 million (Novacana 2017a). However, the presence of BNDES in sucro-energetic sector has been falling year after year with successive negative records. Now, with the data for the first half of 2017 released, the situation has worsened further with reduction in 26% compared to period of 2016 (Novacana 2017b).

One option to turn more favorable the economic viability of 2G ethanol production would be the extension of its production in the context of biorefineries with a larger pool of interesting bioproducts obtained from biomass in integrated industrial plants. Thus, a high volume low price product (ethanol) would be produced with different kinds of low volume high price products. In the Fig. 6.2, a representative graph of price versus volume of different molecules which could be obtained from lignocellulosic biomass is shown. For example, considering two products shown in the extremes of the Fig. 6.2, the current commercial price of ethanol in USA (November 16, 2017) is about $1.39 per Gallon (Businessinsider.com 2017) compared to the Riboflavin high purity price (November 16, 2017) of $60–$100 per kg (alibaba.com 2017).

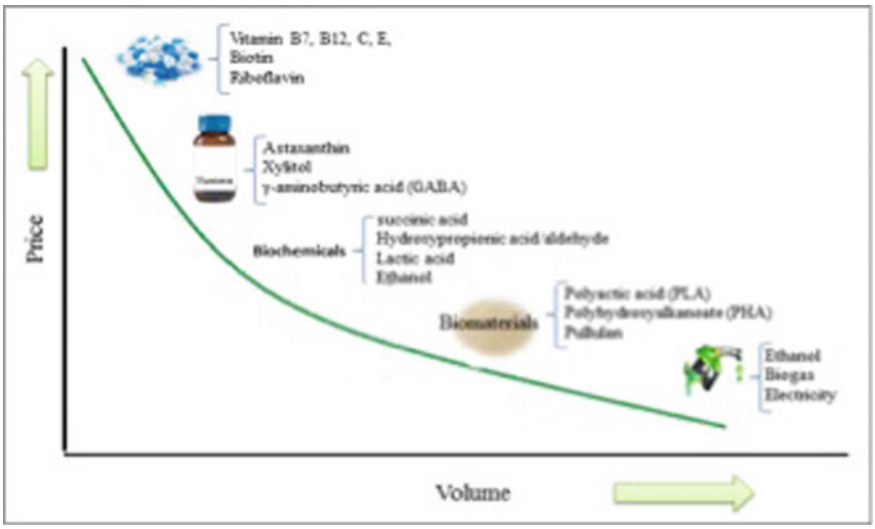

Fig. 6.2 Schematic representation of volume versus economical value of different products in context of biorefinery

6.4 Lignocellulosic Sources and Biotechnological Production of High-Value Bioproducts

Sugars as hexoses and pentoses released after hydrolysis process of biomass carbohydrate fractions (cellulose and hemicellulose) can also be used for production of different molecules besides ethanol, these including e.g. polymers, xylitol, pigments, organic acids and others (Sindhu et al. 2016; Terán Hilares et al. 2017b). In this section, some examples of bio-molecules such as polymers, biosurfactants, lactic acid, xylitol and pigments obtained from different lignocelluloses have been briefly discussed.

6.4.1 Polymers

Polymers mainly derived from petroleum resources present properties of non-biocompatibility and non-biodegradability, limiting thus their application in different areas ranging from medicine, food additives, biosensors and others (Mogoşanu and Grumezescu 2014). In recent years, alternative biopolymers have got attention, mainly due to the aspects of biocompatibility and biodegradability; these properties turn possible their applications as medical material, packaging, cosmetics, food additives and others (Raj and Mumjitha 2015).

Biopolymers can be obtained from different sources such as animal (e.g. chitosan and collagen), plants (e.g. starch and gums) and microorganisms (e.g. polyhydroxyalkanoates (PHAs), xanthan and pullulan) or can be chemically produced from monomers obtained from biological sources [e.g. poly(lactic acid)] (Koutinas et al. 2014). Microbial production way is an attractive alternative mainly due to non-dependence of environmental conditions, presenting higher yield of production and possibility of use of different lignocellulosic feedstocks as a carbon sources (Vijayendra and Shamala 2014). In Table 6.1 are listed different lignocellulosic biomass used for this purpose.

Polyhydroxyalkanoates (PHAs) correspond to microbial biopolymers which are synthesized by diverse bacteria under nutrient unbalanced conditions using different substrates as sugars (glucose and sucrose), alkanes and fatty acids (Ramya et al. 2017). These biopolymers can be used for different applications as medical (drug delivery), pharmaceutical (microcapsules in therapy or as materials for cell and tablet packaging), packaging (bottles, laminated foils, fishnets, flowerpots, sanitary goods) and others. This product is commercialized as natural white granules by the company Dalian Great Fortune Chemical Co., Ltd (alibaba.com 2017) at $3000–4000/kg of product. PHA also is commercialized at approx. $670/kg of product (prices consulted in October 31, 2017) (Sigma-Aldrich, Inc. 2017).

The homopolymer poly-3-hydroxybutyrate (P3HB) and the copolymer poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P3HB-co-3HV) are the most extensively studied PHA compounds (Silva et al. 2004; González-García et al. 2011). Considering the expensive cost of carbon sources (e.g. pure carbohydrates) usually used for PHA and PH3B production, inexpensive raw materials have been proposed, including agro-industrial and forest residues and by-products, such as sugarcane bagasse, wheat straw, rice straw, sweetgrass and others. For example, in the work of Cesário et al. (2014), P3HB was produced by *Burkholderia sacchari* DSM 17165 using commercial sugars (glucose and xylose) and enzymatic hydrolysate of wheat straw. In that work, using commercial sugars, 0.7 g PH3B/g cell dry weight (CDW) with a yield of polymer on sugars ($Y_{P/S}$) of 0.18 g/g were achieved; moreover, using wheat straw hydrolysate, 0.6 g P3HB/g CDW and 0.19 g/g of yield were achieved.

Other interesting biopolymer corresponds to pullulan produced by *Aureobasidium pullulans*, a black-yeast-like fungus. It consists of maltotriose repeating units further connected by α-(1 → 6) linkages. This particular linkage confers it a considerable solubility in water compared to other polysaccharides (Wu et al. 2015). However, *A. pullulans* strains are also associated with the formation of a pigment (melanin) actually unwanted in pullulan production process, requiring additional decolorization steps, thus making it an expensive choice (Wu et al. 2009; Ravella et al. 2010). Another key aspect in pullulan production corresponds to the necessity of replacement the expensive carbon sources (liquefied starch, sucrose and pure carbohydrates) by low cost ones. Several alternative carbon sources rice hull hydrolysate (Wang et al. 2014) and sugarcane bagasse (SCB) hemicellulosic hydrolysate (Chen et al. 2014), have been used to produce this biopolymer. In the Fig. 6.3 is shown a schematic representation of biotechnological production of pullulan by *Aureobasidium pullulans* from carbohydrate fraction of lignocellulosic biomass.

Table 6.1 Different biomaterials (biopolymers) which can be produced coupled to bioenergy industry

Lignocellulosic biomass	Microorganism	Product	Yield	References
Wheat straw hydrolysate	*Burkholderia sacchari*	Poly(3- hydroxybutyrate) (P(3HB))	60% g P(3HB)/g CDW*; 0.19 g/g	Cesário et al. (2014)
Spent coffee grounds hydrolysate	*Bacillus megaterium* *Burkholderia cepacia*	Copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate Poly(3- hydroxybutyrate) (P(3HB))	56% g P(HB-co-HV)/g CDW; 0.04 g/g 51% g PHB/g CDW; 0.24 g/g	Obruca et al. (2014)
Cellulose	*Saccharophagus degradans*	Homopolymer of poly(3-hydroxybutyrate)	14.6% g PHA/g CDW	Sawant et al. (2017)
Rice bran hydrolysate	Recombinant *Escherichia coli*	Poly(3-hydroxybutyrate)	90.1% g PHB/g CDW	Oh et al. (2015)
Perennial ryegrass hydrolysate	*Pseudomonas strains*	Medium chain length polyhydroxyalkanoate (mcl-PHA)	6–17% g mcl-PHA/g CDW	Davis et al. (2013)
Sugarcane bagasse hydrolysate	*Halogeometricum Borinquense*	Poly(3-hydroxybutyrate-co-3-hydroxyvalerate)	50% g P(3HB-co-3HV)/g CDW	Salgaonkar and Bragança (2017)
Sugarcane bagasse hydrolysate	*Burkholderia cepacia* *B. sacchari*	Poly(3-hydroxybutyrate)	62% g P2HB/g CDW; 0.39 g/g 53% g P2HB/g CDW; 0.29 g/g	Silva et al. (2004)
Sugarcane bagasse hydrolysate	*Aureobasidium pullulans*	Pullulan	20 g/L	Terán Hilares et al. (2017b)
Sugarcane hemicellulosic hydrolysate	*Aureobasidium pullulans*	Pullulan	12.65 g/L	Chen et al. (2014)
Wheat straw hydrolysate	*Xanthomonas campestris*	Xanthan gum	20.7 g/L, 0.62 g of xanthan/g sugar (glucose and xylose)	Zhang (2015)
Cassava bagasse hydrolysate	*Xanthomonas campestris*	Xanthan gum	14 g/L	Woiciechowski et al. (2004)
Rice straw hydrolysate	*Xanthomonas campestris*	Xanthan gum	10.41 g of xanthan/100 g raw material	Jazini et al. (2017)

**CDW* Cell Dry Weight

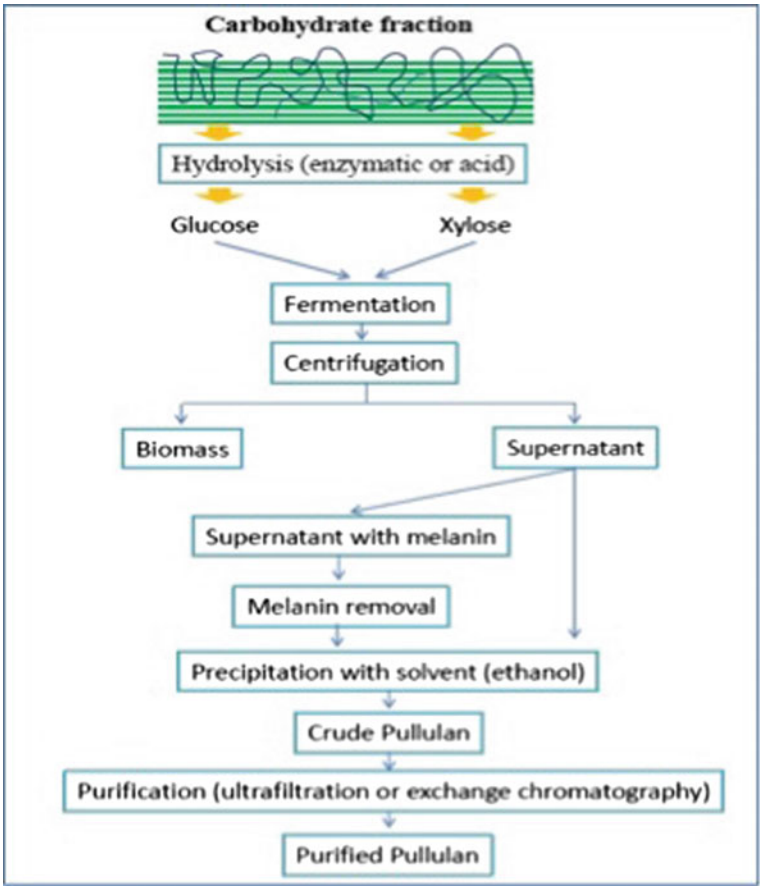

Fig. 6.3 Biotechnological production of pullulans from carbohydrate fraction of lignocellulosic biomass

In a recently study reported by Terán Hilares et al. (2017b), different strategies were evaluated in order to simplify the pullulan production process using low cost carbon source. In that study, light-emitting diodes (LEDs) of different wavelengths were used to assist the fermentation process aiming to produce low-melanin containing pullulan by the wild strain of *A. pullulans* LB83 with different carbon sources. Under white light using glucose-based medium, 11.75 g/L of pullulan with high melanin content (45.70 UA_{540nm}/g of pullulan) was obtained. The process was improved by assisting the fermentation by blue LED light, resulting in 15.77 g/L of pullulan with reduced content of melanin (4.46 UA_{540nm}/g of pullulan). By using SCB hydrolysate as carbon source, similar concentration of pullulan (about 20 g/L) was achieved using white and blue LED lights, with lower melanin contents in last option.

Moreover, the production of pullulan can be coupled with bioenergy production via a process intensification approach. This product, depending on the degree of

purity, can be sold in the market at high prices. For example, high quality food and cosmetic grade pullulan powder is commercialized by Zhengzhou zhenhua medicine science and technology service co., Ltd at $32–43/kg of product (alibaba.com 2017). Other product corresponds to high quality pharmaceutical grade sweetener pullulan also with 99% of purity is commercialized by Suzhou Ruiying-Runze Trading Co., Ltd at $72–82/kg of product (alibaba.com 2017). Additionally, this product is also commercialized with extrapure quality (Sigma-Aldrich, Inc. 2017) and Sisco Research Laboratories Pvt. Ltd (Maharashtra, India) at $1969/25 mg of product and $174.28/25 g of product, respectively.

6.5 Biosurfactants

Biosurfactants (BSs) naturally produced by microorganisms present advantages compared to synthetic surfactants derived from petroleum or oleo-chemical sources, mainly due to their low or non-toxicity, biodegradability, biocompatibility, surface activity comparable to the synthetic surfactants, and several other properties (Lee et al. 2008; Samad et al. 2017).

BSs can be extracted from natural sources or obtained through chemically mediated processes (called "first generation biosurfactants"). However, microbial BS's (the so-called "second generation biosurfactants") have attracted great attention in research works (Madsen et al. 2015).

Microbial BSs are classified according to their chemical structure (Table 6.2) and include glycolipids, lipopeptides and lipoproteins, phospholipids and fatty acids, polymeric surfactants, and particulate surfactants (Desai and Banat 1997; Campos et al. 2013; Shah et al. 2016).

Among BSs, Sophorolipids (SLs) can be considered the most promising to be produced from lignocellulosic hydrolysates. Actually, although there are other important

Table 6.2 Classification of microbial biosurfactantes and types of these

Biosurfactantes	Main types
Glycolipids	Rhamnolipids, Sophorolipids, Mannosylerytrhitol lipids, Cellobiose lipids or Ustilagic acid, Xylolipids, Lipids of oligosaccharides, Complexes carbohydrate-lipids, Polyol lipids
Fatty acids, neutral lipids and phospholipids	Fatty acids, Neutral lipids, Phospholipids
Polymerics	Emulsan, Biodispersan, Liposan, Carbohydrate-lipid-protein, Mannoproteins
Lipopeptides and lipoproteins	Surfactin, Iturin, Serrawetin, Subtilysine, Cerelipin, Gramicidin, Viscosin, Peptide-lipids (lysine lipid and ornithine lipid)
Particulates	Membranes vesicles and *whole cells*.

BSs classes, as rhamnolipids, SLs are advantageous because they can be produced by non-pathogenic yeast strains as *Candida apicola*, *Rhodotorula bogoriensis*, *Wickerhamiella domericqiae* and others (Van Bogaert et al. 2011). SLs are composed by two components: a sophorose head (a dimeric sugar residue) and a hydroxylated fatty acid (Roelants et al. 2016).

Some current challenges in SLs production correspond to the use of low cost substrates and the optimization of fermentation parameters. Different economical carbon sources can be used for SLs production; however, glucose and oleic acid are the most common substrates. In this way, considering the massive availability and low cost of lignocellulosic biomass, different studies were reported using cellulosic hydrolysates for this purpose (Moldes et al. 2007; Samad et al. 2017).

In the study of Moldes et al. (2007), detoxified hemicellulosic hydrolysate of corn cob was used for intracellular biosurfactant production by *Lactobacillus pentosus*. In this work, 4.7 g/L of intracellular biosurfactant, 0.53 g of intracellular biosurfactant per g of biomass ($Y_{BS/BM}$) and 0.53 g of intracellular biosurfactant per g of sugar consumed ($Y_{BS/S}$), were achieved. Corn stover was also used as a substrate for SLs production by *Candida bombicola*, as reported by Samad et al. (2017). In that study, initial experiment was carried out in 250-mL Erlenmeyer flask with 50 mL of medium. About 11.6 g/L of SL (yield of 0.12 g of SL per g of carbon source) was produced in 14 days, using as carbon source the enzymatic hydrolysate of corn stover (65 g/L of initial total sugars) supplemented with 10 g/L of soybean oil. Additionally, sophorolipid was produced in 3-L of bioreactor using same hydrolysate and supplemented with yellow grease (10 g/L of initial concentration). Under these experimental conditions, 52.1 g/L (0.35 g/g of sugar plus yellow grease) was achieved.

In another report, Liu et al. (2016) related the use of enzymatic hydrolysate from rice straw pretreated via SO_3 micro-thermal explosion for SLs production by *Wickerhamiella domercqiae* var. *sophorolipid* CGMCC 1576. In that work, using 60 g/L of glucose as hydrophilic carbon source and 60 ml/L of hydrophobic carbon source, 53.7 g/L of SLs were produced after 168 h of fermentation at 30 °C of process temperature.

BSs are considered sustainable products with physico-chemical and biological properties similar or superior to synthetic surfactants. Due to its highlighted advantages the BSs market has expanded quickly due to the variety of applications of these compounds. The main companies producing BSs in the world are TeeGene Biotech (UK), AGAE Technologies LLC (USA), Jeneil Biosurfactant Co. LLC (USA), Paradigm Biomedical Inc. (USA), Rhamnolipids Companies Inc. (USA), Fraunhofer IGB (Germany), Cognis Cares Chemicals (China, Germany, USA), Saraya Co. Ltd. (Japan), Ecover Belgium (Belgium), Groupe Soliance (France), MG Intobio Co. Ltd. (South Korea), Synthezyme LLC (USA), Allied Carbon Solutions (ACS) Ltd. (Japan), Henkel (Germany), Lion Corporation (USA), Lipo Chemicals (USA), Kaneka Co. (Japan), Ecochem Ltd. (Canada) and Evonik (Germany) (Randhawa and Rahman 2014). The industrial sectors that show the greatest interest in BSs are petrochemical, pharmaceutical, cosmetics, food and agrochemical industries.

Recent market analysis shows that main consumers of BSs are European and North American countries, responsible for 75–80% of the global consumption of these

biomolecules, followed by developing countries and the Asia-Pacific. According to data from Transparency Market Research (2012) and Grand View Research (2014), an average global production of 344,000 ton of BSs and approximately 1.7 billion dollars has been recorded between 2011 and 2013. By 2020, an important increase in production is expected, reaching 462,000 tons and 2.2 billion dollars.

Despite the market expansion, BSs production is still considered low, since the costs, when compared to the synthetic surfactants, can be equal or superior, due to the substrates used. In view of these problems, the use of industrial by-products in bioprocesses can be an economically viable alternative.

6.5.1 Lactic Acid

Lactic acid [2-hydroxypropanoic acid, CH_3–CH(OH)–COOH] is a natural organic acid with several applications in food, polymers, pharmaceuticals, cosmetics, and chemical industries, which is produced by different strains of *Lactobacillus*. It can be produced in two optical isomers: L-(+)-lactic acid produced by *L. casei*, *L. paracasei*, and *L. rhamnosus*; D-(-)-lactic acid produced by *L. delbrueckii*, *L. coryniformis*, *L. jensenii*, and *L. vitulinus*; DL- lactic acid produced by *L. pentosus, L. plantarum, L. brevis, L. sake*, and *L. acidophilus* (Mack 2004; Abdel-Rahman and Sonomoto 2016). L-Lactic acid is used for the synthesis of poly L-lactic acid (PLLA), a biodegradable and thermosetting polymer with several applications in orthopedic fixation, packaging and dental applications; on the other hand, D-Lactic acid is used for the production of poly D-lactic acid (PDLA) (John et al. 2007).

Lactic acid can be produced by chemical synthesis or microbial fermentation. Some advantages of the microbial way correspond to the utilization of renewable carbohydrate biomass (Table 6.3), less thermal conditions, and the production of high optical purity lactic acid by selecting an appropriate strain (Kuo et al. 2015; Abdel-Rahman and Sonomoto 2016). For fermentation process, different carbon sources have been used; for example, in the work of Kuo et al. (2015), nondetoxified wood hydrolysate was employed for optically pure L-lactic acid production by using a newly isolated and D-lactate dehydrogenase gene-deficient *L. paracasei* strain. In that work, 99 g/L of L-lactic acid with respective yield of 0.96 g/g and productivity of 2.25 g/L h, was produced in 120 h of fermentation.

In the report of Adsul et al. (2007), enzymatic hydrolysate of cellulosic fraction of SCB was used for production of L-(+)-lactic acid by *L. delbrueckii* mutant Uc-3 in a simultaneous saccharification and fermentation (SSF) process. In that study, 67 g/L of lactic acid, with respective productivity of 0.93 g/L h and yield of 0.83 g/g, was produced from 80 g/L of cellulose. Additionally, the conversion of cellobiose into lactic acid was observed in a homo-fermentative way. Oonkhanond et al. (2017) also reported the use of SCB hydrolysate for lactic acid production by *L. casei* in a 3-L bioreactor. Those authors observed production of 21.3 g/L after 120 h with a productivity of 0.63 g/L h.

Lactic acid can also be used for the production of different polymers as poly-lactones such as poly-lactic acid (PLA), poly-glycolic acid (PGA), and poly-

Table 6.3 Lactic acid production by different microorganisms using different lignocellulosic materials as carbon source

Lignocellulosic biomass	Microorganism	Product	Volumetric production/yield	References
Cellulosic date palm wastes	*Lactobacillus delbrueckii* subsp. *Lactis*	L-(+)- Lactic acid	27.8 mg/mL	Alrumman (2016)
Sugarcane bagasse hydrolysate	*Escherichia coli*	D-(−)-Lactic acid	0.95 $g_{D\text{-}LA}$/g of sugar consumed	Utrilla et al. (2016)
Sophora flavescens residues	*Lactobacillus casei*	D-(-)-Lactic acid	55.1 g/L, 0.835 $g_{D\text{-}LA}$/g of sugar consumed	Wang et al. (2016)
Waste wood chips hydrolysate	*Lactobacillus paracasei*	Lactic acid, 95.1% of OP-LA	52.61 $g_{D\text{-}LA}$/L	Kuo et al. (2015)
Brewer's spent grain hydrolysate	*Lactobacillus fermentum*	Racemic mixture of L-(+)- and D-(−)-LA	0.44 g of total LA/g of sugar consumed	Pejin et al. (2015)
Brewer's spent grain hydrolysate	*Lactobacillus rhamnosus*	L-(+)-LA, 95–98% of OP-LA	0.95 $g_{L\text{-}LA}$/g of sugar consumed	Pejin et al. (2015)
Corn stover hydrolysate	*Bacillus coagulans*	L-(+)-Lactic acid, optical purity of 99.5%	92 g/L, 0.91 $g_{L\text{-}LA}$/g of sugar consumed	Ma et al. (2016)
Coffee pulp hydrolysate	*Bacillus coagulans*	L-(+)-lactic acid; 99.5% of OP-LA	0.78 $g_{L\text{-}LA}$/g of sugar consumed; 45.3 $g_{L\text{-}LA}$/L	Pleissner et al. (2016)
SCB hydrolysate (cellulose fraction)	*L. delbrueckii*	L-(+)-lactic acid	0.83 g/g; 67 g/L of lactic acid	Adsul et al. (2007)
SCB hydrolysate	*L. casei*	L-(+)-lactic acid	21.3 g/L; 0.63 g/L/h	Oonkhanond et al. (2017)

caprolactone (PCL) by chemical transformations. These compounds are commonly used in medical, packaging, agricultural products and textile industry, mainly due to the additional properties of thermoplastic processability and eco-friendly nature (Lee et al. 2016; Zeidan et al. 2017). PLA correspond the most promising polymers with several applications in medical area as reported for orthopedic regenerative engineering in the work of Narayanan et al. (2016), or in the review paper of Lasprilla et al. (2012) and Gentile et al. (2014). PLA can be obtained by different polymerization process as polycondensation, ring opening polymerization and by direct methods like azeotopic dehydration and enzymatic polymerization (Lim et al. 2008; Castro-Aguirre et al. 2016).

Currently, some prices correspond to $2000–$4000 per ton of PLA (www.alibaba.com, price consulted in November 14, 2017). Lactic acid and PLA market size was

valued, in 2016, at USD 2.08 billion and 1.29 billion, respectively. Values projected to 2025 indicate the global demand is expected to reach USD 9.8 billion and USD 6.5 billion, respectively, for lactic acid and PLA, according to reported by Grand View Research, Inc. (Grandviewresearch.com 2017).

Considering the value and the market of the lactic acid, the implementation of the production of this important molecule integrated with bioenergy production is promising, mainly contributing towards the economic viability of biorefineries. Additionally, the abundant availability of lignocellulosic feedstocks such as sugarcane bagasse (in the case of Brazil) seemingly becomes quite attractive for the biotechnological production of lactic acid.

6.5.2 Xylitol

Xylose is one of the major sugars obtained during the hydrolysis process of hemicellulosic fraction of the biomass. It is considered the second most abundant monomeric sugar in the biosphere and is used in the conversion process to xylitol, either industrially or microbiologically (Albuquerque et al. 2015).

Xylitol is a polyalcohol, considered an important input that can be used in different sectors, such as cosmetics, food and pharmaceutical industry. It is interesting considering its non- and anticariogenic properties, with application in odontological industry. Because it has a sweet taste and its metabolism is independent of insulin, xylitol can be used as a sweetener, with indication in some works for use by people with diabetes (Pal et al. 2013). With a number of other applications, xylitol is one of the main products that can be generated together with ethanol in processes integrated in a biorefinery (Hernández-Pérez et al. 2016).

Industrially xylitol is synthesized from the chemical reduction of xylose by catalytic hydrogenation of D-xylose, but due to the high costs and low yields of the process, in addition to several chemical purification stages of xylose before reaction and of the produced xylitol, several studies have been conducted to obtain via biotechnological (Fig. 6.4) with the use of specific microorganisms (Chen et al. 2010; Wei et al. 2010).

Among these microorganisms we can highlight the yeasts of the genus *Candida*, where xylitol can be considered an intermediary metabolite of these microorganisms. To further reduce processing costs and, in addition, in search of solutions for waste recycling, there are several renewable raw materials with potential use, consisting of a range of different lignocellulosic materials, such as wood bark rich in xylan, rice bran, sugarcane bagasse, among others (Zhang et al. 2014).

The hemicellulosic fraction of sugarcane bagasse can be hydrolyzed by enzymatic or by acid process. The resulted liquor has in its composition mainly xylose which is used for xylitol production after a detoxification process, necessary when acid way is used for hydrolysis. In the work of Rao et al. (2006), the detoxified hemicellulossic hydrolysate was used for xylitol production by adapted *C. tropicalis*. In that work, 0.65 g of xylitol/g of xylose after 48 h of fermentation process was achieved. Vallejos

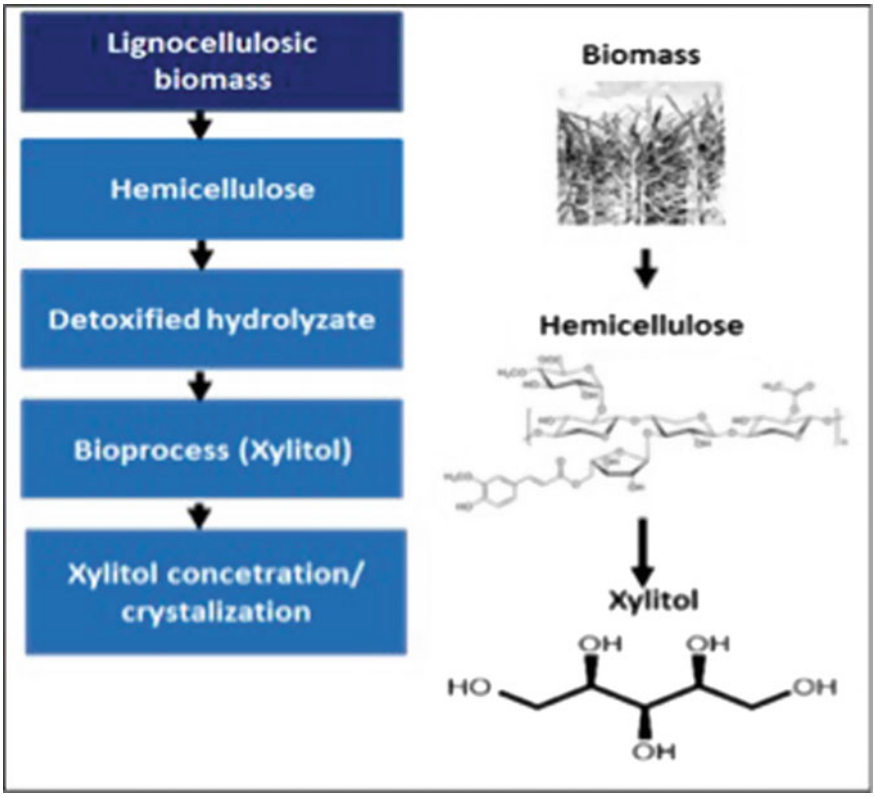

Fig. 6.4 Biotechnological process of the production of simplified xylitol

et al. (2016) reported also the xylitol production by *C. tropicalis* from hemicellulosic hydrolysate previously detoxified including treatment with $Ca(OH)_2$, IR-120 resin, activated charcoal, and IRA-67 resin. In that work, 32 g/L of xylitol production (fermentation efficiency of 46%, productivity 0.27 g/L/h) after 120 h of fermentation was achieved. In another recent work reported by Vaz de Arruda et al. (2017), the scale-up of xylitol production using sugarcane bagasse hemicellulosic hydrolysate by *Candida guilliermondii* FTI 20037 was studied. In that work, xylitol yield of 0.648 and 0.55 g/g were achieved when the fermentation were carried out in reactors with volumetric capacity of 2.4 L and 125 L, respectively.

The demand for xylitol on the planet is increasing, as consumers are more likely to consume less caloric and healthy foods. In 2013 the global consumption of xylitol was 160 thousand tons, with an estimative of 242 thousand tons in 2020. Among the industries, the manufacture of chewing gums and candies consumes about 67% of the xylitol produced, with Asia producing 50% of the total used (Rao et al. 2016).

6.5.3 Pigments

Pigments are being extensively used in food and non-food products mainly improving their organoleptic quality. In food industry, synthetic pigments (coloring agents) like amaranth, erythrosine and tartrazine are extensively used and in the last years studies have shown them as potential carcinogenic compounds (Mpountoukas et al. 2010). In this way, an increase in the consumption of safe and natural pigments seems inevitable.

Natural pigments can be extracted from vegetables, animals and microorganisms, with the last more advantageous due to its high productivity, fast growth and non-dependence of environmental conditions, besides the possibility of improvement in the cost effectiveness by using low-cost substrates (Nigam and Luke 2016; Rodriguez-Amaya 2016).

The main microbial pigments used in food industry are represented in the Fig. 6.5. In this section, β-carotene and *Monascus* pigments are briefly discussed, considering the scarcely reports about these pigments production using lignocellulosic biomass as carbon source.

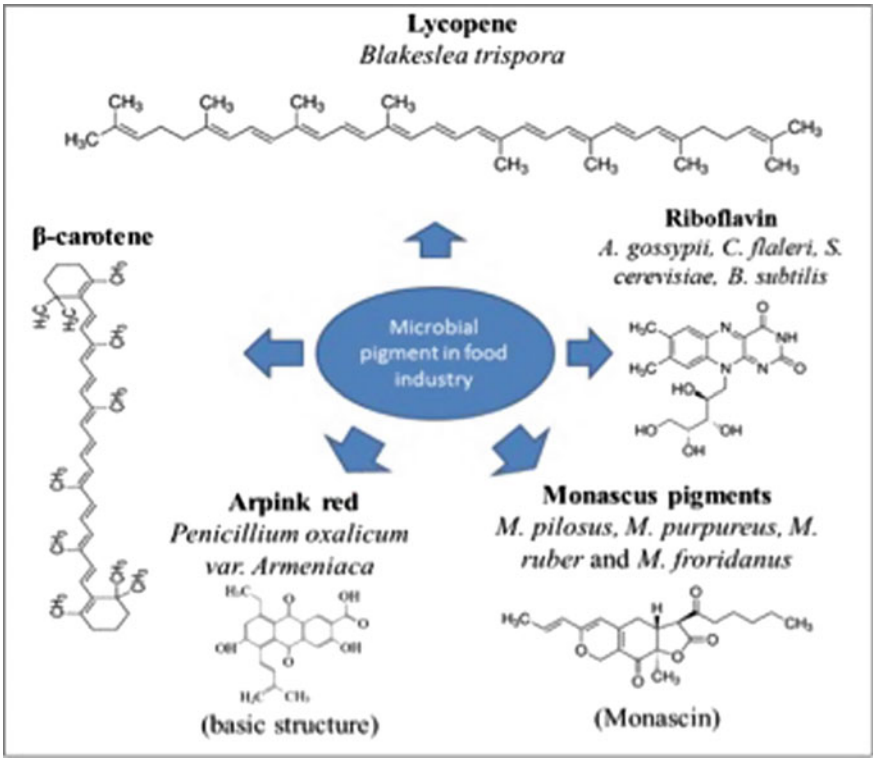

Fig. 6.5 Microbial pigments used in food industry

β-carotene (yellow to orange-red colored pigments) can be produced using different microorganisms as yeast (*Rhodotorula*), fungi (*Blakeslea trispora* and *Mucor circinelloides*) or bacterias (*Serratia*, *Micrococcus*, *Mycobacterium*, *Agrobacterium and Blakesleatrispora*) (Phan-Thi et al. 2016; Nigam and Luke 2016; Akçakaya et al. 2017). Different low cost carbon sources were reported for β-carotene production, e.g. hydrothermally pretreated wheat straw hydrolysate (Petrik et al. 2013), sugarcane bagasse hydrolysate (Goswami et al. 2015) and pith portion of the sugarcane bagasse (Abdelhafez et al. 2016). In the work of Abdelhafez et al. (2016), 0.13 mg of β-carotene/kg of biomass was produced using *Serratia marcescens* ATCC 27117 and the pith portion of the sugarcane bagasse at particle size of 0.2–2 mm as carbon source.

Monascus pigments correspond to yellow, orange, and red pigments, which depends on the used cultivation conditions (temperature, pH, dissolved oxygen), composition of medium (presence of amino acids) and fermentation mode (submerged or solid state). These pigments have several biological properties as antimicrobial, antioxidant and anticholesterol activities and are used in food, textile, cosmetic, and pharmaceutical industries (Vendruscolo et al. 2016; Koli et al. 2017). Currently, there are few reports in the literature using specifically lignocellulosic biomass for their production. For example, Velmurugan et al. (2011) reported *Monascus* pigments production by solid-state fermentation with corn cob substrate. In that work, pigments yield of 25.42 OD Units/gram of dry fermented substrate (powder of corn cob) was achieved when the fermentation process was carried out under optimized conditions: 60% (w/w) initial moisture content, incubation at 30 °C, inoculation with 4 mL of spores/gram of dry substrate, and an incubation period of 7 days. In other work, Silveira et al. (2013) reported the red pigment production by *Monascus purpureus* in submerged cultivations with sugarcane bagasse (20 g/L) as carbon source and soy protein isolate (2.5 g/L) as nitrogen source. In that work, pigment yield near to 4 UA_{500nm}/mL was achieved after 14 days of fermentation incubated at 27 °C on a rotary shaker at 125 rpm.

The hydrolysate of carbohydrate fraction of corncob was also used for *Monascus* red pigment production in submerged fermentation (Zhou et al. 2014). In that work, low citrinin production in corncob hydrolysate medium compared to conventional glucose medium was observed and the pigment production yield using the hydrolysate based medium ($25.8 \pm 0.8 UA_{500nm}$/mL) was slightly higher compared to glucose based medium ($24.0 \pm 0.9 UA_{500nm}$/mL). Additionally, glucose and xylose present in corncob hydrolysate were metabolized during the fermentation process; turning this substrate as potential carbon source for pigment production. Therefore, hydrolysate obtained from different lignocellulosic biomass can be used for pigment production, e.g. sugarcane bagasse hydrolysate, as observed in the Fig. 6.6 (photographs obtained in a laboratory at University of São Paulo/Lorena/SP-Brazil).

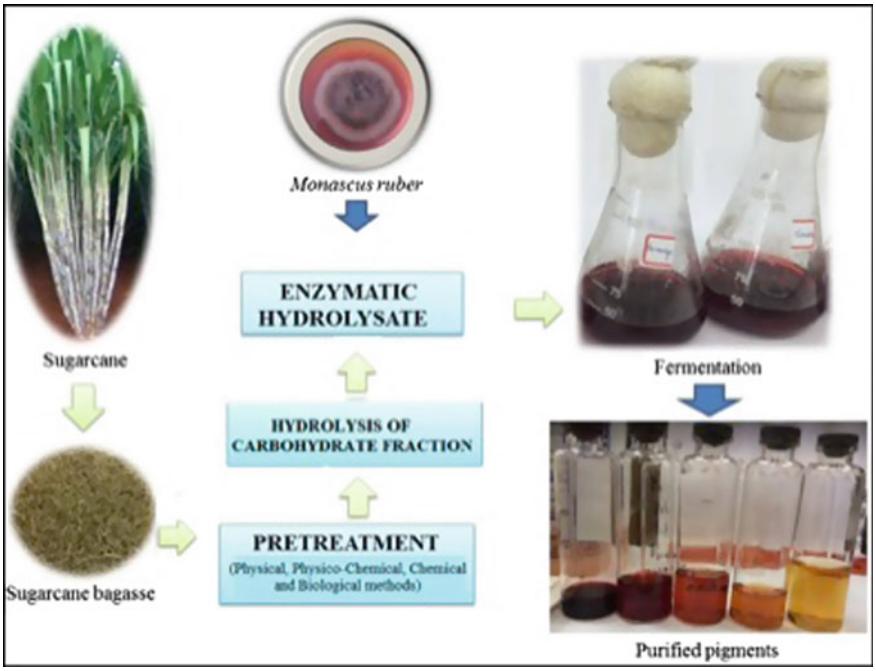

Fig. 6.6 Schematic representation of red pigment production by *Monascus ruber* using enzymatic hydrolysate of sugarcane bagasse. (Photographs obtained in LBBSIM laboratory, EEL, University of São Paulo/Lorena/SP-Brazil)

6.6 Conclusions and Future Perspectives

There are undoubtedly multiple valuable sustainable products that can be produced in a biorefinery. However, for these refineries, to enhance their economic viability, extensive utilization of lignocellulosic fractions at industrial scale is an absolute need. In this regard, some interesting possibilities were discussed, including different compounds with a variety of applications, either as substitute of petroleum based products or for some other unique and specific applications. Biotechnological inherent characteristics can also be advantageous, with a range of possibilities of process optimization by changing conditions and process alternatives.

Even considering lignocelluloses economical and available alternative raw materials to produce, by biotechnological route, polymers, pigments, biosurfactants, latic acid and others, reports in literature in this topic are curiously scarce compared to their potential applications in food, pharmaceutical and chemical industries. However, considering the mandatory necessity of substitution of petroleum based economy by a biobased one, in a near future, more studies and industrial plants are expected to be a reality. Thus, not only ethanol could be an interesting alternative as environmental friendly energy source, but also a new and varied interconnected and integrated economy based on biomass products will be a part of our daily lives.

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Chapter 7
Techno-economic Assessment of Bioethanol Production from Major Lignocellulosic Residues Under Different Process Configurations

Pornkamol Unrean

Abstract Technological and economical potentials of integrated biomass-to-ethanol conversion process is investigated using process flowsheeting simulation for an estimation of the minimal ethanol selling price (MESP). Implementing optimal process configuration with yeast consortium for efficient C_5/C_6 co-fermentation, fed-batch high-solid operation for high-ethanol-titer, on-site enzymes together with enzyme synergism for low enzyme demand and efficient saccharification could potentially lower the MESP of integrated cellulosic ethanol production process to meet economic feasibility for industrialization. Techno-economic study provides an economically viable prototype for high-ethanol-titer process via fed-batch SSF using yeast consortium and on-site enzymes production which offer better economic value for the successful commercialization of lignocellulosic ethanol production process. Such process platform is an important strategy for the development of low-cost biorefinery industry that can outperform the current starch- or sugar-based process for the production of biofuels.

Keywords Techno-economic analysis · High-solid lignocellulosic bioprocess
High-ethanol-titer fed-batch · Yeast consortium · On-site enzymes
Cellulase and hemicellulase synergism

7.1 Introduction

The finite nature of fossil fuels and the concerns about their environmental impact have propelled the world's efforts to develop and industrialize biofuels production process. Biorefineries using lignocellulosic biomass for bioconversion of transportation biofuels are among the most promising options. The production of cellulosic

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ethanol could reduce dependence on crude oil and minimize competition between crops for food and fuel production (FitzPatrick et al. 2010). The focus has, therefore, shifts toward designing sustainable lignocellulose-to-ethanol process that can be technological and economical feasible and provides energetic and environmental benefits.

Lignocellulosic feedstock is typically composed of 35–40% cellulose, 25–30% hemicellulose and 30–40% lignin. Conversion of cellulose and hemicellulose to ethanol requires cellulase and hemicellulase enzymes, respectively, for hydrolysis and yeast cells for co-fermentation of C_5/C_6 sugars. The efficiencies of enzymatic hydrolysis and co-fermentation of C_5 and C_6 sugars are critical process variables in lignocellulosic ethanol process which directly affects MESP and its economic feasibility (Chovau et al. 2013; Liu et al. 2016). Cost-effective bioconversion of lignocellulose to ethanol remains a major financial and technical challenge at industrial scale. The biomass-to-fuel ethanol process contains multiple processing steps. After pretreatment, enzymatic saccharification and fermentation is performed for hydrolyzing pretreated biomass into mixed sugars and for converting released sugars into ethanol, correspondingly (Bertilsson et al. 2009; Bezerra and Ragauskas 2016). Owing to the interdependency of each processing step, turning lignocellulosic ethanol production towards a successful industrial scale is only possible by optimizing and defining optimal integrated process option meeting techno-economic feasibility requirements (Gírio et al. 2010). Optimizing the techno-economic feasibility of lignocellulose bioconversion technologies by defining optimal process configurations is crucial for a sustainable lignocellulosic bioprocess. A slight change in input costs or process yields in each process step can have a major impact on process profitability. It is therefore essential to understand the entire integrated process and how each process step or process parameters within each step affecting the overall performance. The complexity of the lignocellulosic bioconversion process and the strong interdependent effects among process steps has made it difficult to establish the true optimum value for each parameter in each process step. The question then becomes, which lignocellulose-based processes and conversion technologies are economically viable for industrialization.

Flowsheet modeling framework could provide dynamic simulation of plant wide operation, facilitate the optimization of integrated process based on economic objective as well as determine key process requirements to improve the overall process economy (Wingren et al. 2008; Kazi et al. 2010; Morales-Rodriguez et al. 2011; Geraili et al. 2014a, b; Sassner et al. 2008). With the fully-integrated process simulation model, the implicit correlations between upstream and downstream stages of the process can be assessed such that the actual profitability of the process based on production yields, operating cost and energy consumption can be determined. A fully integrated process simulation should be composed of model steps describing (1) upstream processing (e.g. pretreatment, hydrolysis and fermentation steps), (2) downstream processing (e.g. distillation or product purification step), and (3) energy/heat integration step including lignin processing step (e.g. solids/lignin combustion for power co-generation) and waste treatment. The process integration model can assist the study of interactions and tradeoffs between process steps and the iden-

tification of optimal process conditions or possible process bottlenecks for further improvement to reduce time and cost for scale-up and large-scale production.

Herein techno-economic (TE) analysis is performed to compare different upstream process configurations for lignocellulose-to-ethanol process and to determine the cost effective process option suitable for commercialization based on minimal selling price of ethanol produced. The integrated lignocellulose-based process for ethanol conversion is investigated to design the optimal process meeting technological and economic feasibility. Specifically, the process economy is evaluated through MESP under the varying process scenarios e.g. different process configurations (SHF vs. SSF), different operating mode (batch vs. fed-batch), different solid loading, different on-site enzyme supply mode, different enzyme loading or utilization of yeast consortium. The economic impact of these process schemes on commercialization are compared to prove its techno-economic feasibility for industrialization as well as to identify process bottlenecks for maximizing process profitability. Eventually, the design of integrated lignocellulose-based process with cost-competitive MESP to the current selling price is proposed.

7.2 Integrated Process Flowsheet Simulation

A process flowsheet model has been considered a critical tool for comparison of lignocellulosic ethanol production options and for design of cost-effective process configurations with improved techno-economic and environmental characteristics. The most commonly used process integration model is relied on steady-state flowsheet simulation (e.g. Aspen Plus, SuperPro Designer). The flowsheet simulation solves mass and energy balance across each unit operation and across the entire biomass-to-product process. Examples of process scale-up and integration which incorporates unit operating steps for conversion biomass feedstock to ethanol is provided below. The integrated process is typically composed of pretreatment, yeast propagation, enzyme production, enzyme hydrolysis and fermentation, which can be performed in a separated manner (Separated Saccharification and Fermentation, SHF) or in a simultaneous manner (Simultaneous Saccharification and Fermentation, SSF). SHF process configuration is an attractive process in which both enzyme hydrolysis and fermentation can operate at their optimum. SSF process configuration has also attracted many investigators as a feasible option to reach high production efficiency by minimizing feedback inhibition effects of enzyme hydrolysis (Zhou et al. 2016). Chemical, material and utility usage, equipment sizing and operating time for processing, transferring, draining and cleaning can be determined by simulation data. With the fully-integrated process analyses the implicit correlations between each unit operation and across the entire process can be assessed to determine comparative process profitability of various process configurations based on process parameters, operating cost and energy consumption from mass and energy balance.

7.2.1 Integrated SHF Process of Lignocellulose-to-Ethanol

The integrated lignocellulose to ethanol SHF process in Fig. 7.1 begins with pretreatment (diluted-acid, DA) followed by pH adjustment and dilution to a desired water-insoluble solid (WIS) concentration. DA pretreatment with sulfuric acid as a catalyst is commonly utilized since it is considered an economically viable technology to solubilize hemicellulose as well as to increase the digestibility of cellulose in enzymatic hydrolysis (Canilha et al. 2011; Tao et al. 2012). No solid–liquid separation after the pretreatment is preferred to eliminate wastewater pretreatment. Then, enzyme hydrolysis is performed with the addition of enzymes (e.g. cellulases, hemicellulases) converting biomass into sugar monomers. Enzymatic hydrolysis of the whole pretreated slurry is carried out by cellulase and hemicellulose enzymes to produce monomeric C_5 and C_6 sugars from cellulose and hemicellulose, respectively, for fermentation (Zhou et al. 2009). Fed-batch saccharification can be performed through a pulse-feeding of pretreated biomass at desired feed profile. After hydrolysis, the addition of required nutrients and yeast cells for fermentation of sugars into ethanol is followed. *Scheffersomyces stipitis* (C_5-fermenting yeast) and *Saccharomyces cerevisiae* (C_6-fermenting yeast) are typically utilized for ethanol fermentation. A *S. stipitis* and *S. cerevisiae* single-strain or consortium is inoculated at optimized cell ratio to initiate fermentation. *S. stipitis* and *S. cerevisiae* yeasts can be produced on-site in separate aerated propagation tank to obtain satisfactory yeast cell concentration, then the culture is separated in continuous centrifuge, re-suspended and added into fermentation reactor. Liquid hydrolysates fraction and/or other substrates (e.g. molasses, glucose, xylose) can be used as culture media for yeast propagation. Dried yeast can also be used to replace yeast propagation step. If solid-liquid slurry after the enzymatic hydrolysis unit is directly used for fermentation, in practice enzyme hydrolysis and fermentation process can be carried out in one tank in a sequential manner. However, the process diagram of separated saccharification and fermentation units in Fig. 7.1a is depicted in separate tank for clarification purpose. The output stream from fermentation unit is transferred to downstream operations to recover purified ethanol solution. The downstream processing step is described in details elsewhere. The reader is referred to the optimal downstream process configuration in Wingren et al. (2008), which has conducted cost analysis and energy consumption evaluation of the integrated downstream processing steps to identify optimal process configurations for ethanol recovery with the lowest energy demand, resulting in a lower ethanol production cost than traditional distillation process. The remaining solid and liquid wastes are separated out for waste treatment and re-utilization.

7.2.2 Integrated SSF Process of Lignocellulose-to-Ethanol

Figure 7.2 shows an integrated lignocellulose to ethanol process composing basic steps of pretreatment, yeast propagation, enzymatic hydrolysis and fermentation, downstream process and utilities system. Briefly, milled biomass feedstock is steam-

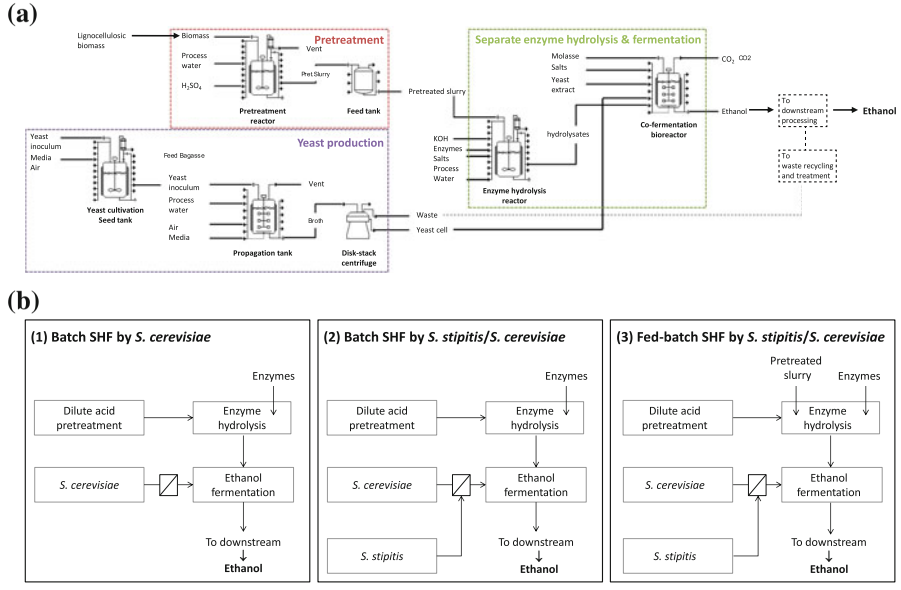

Fig. 7.1 **a** Process diagram for separate saccharification and fermentation of lignocellulosic biomass to ethanol using *S. stipitis*/*S. cerevisiae* consortium and commercial saccharification enzymes. The process configuration includes diluted-acid pretreatment (in red dashed square), yeast propagation (in purple dashed square), enzyme hydrolysis and co-fermentation (in green dashed square) steps for the conversion of biomass to ethanol. Process conditions including material inputs, yields, rates, titers, processing times and unit operations required in pretreatment, yeast propagation, enzyme hydrolysis and fermentation processes for material and energy balance simulation are based on experimental data in Unrean et al. (2016). Downstream processing for ethanol recovery is described in Wingren et al. (2008). **b** Process configurations under techno-economic evaluation. Three process scenarios for the production of lignocellulosic ethanol are (1) *S. cerevisiae* in batch separate hydrolysis and fermentation (SHF), (2) *S. stipitis*/*S. cerevisiae* yeast consortium in batch SHF, (3) *S. stipitis*/*S. cerevisiae* yeast consortium in fed-batch SHF

pretreated with diluted acid. The pretreated biomass can be neutralized using base (e.g. NaOH, KOH) and diluted to a specified water-insoluble solids (WIS) concentration prior to use in simultaneous saccharification and fermentation (SSF) process. Nutrients, yeast cells suspension and cellulase and hemicellulose enzymes is then added at optimized dosage to convert pretreated biomass to ethanol in SSF. Yeast cell suspension is from on-site yeast propagation or purchased dried yeast. A single-strain or yeast consortium can be used as appropriate. Fed-batch process can be performed through a pulse-fed of pretreated biomass slurry, yeast cells or enzymes at desired feed profiles. In SSF process, waste can be minimized if the whole slurry of pretreated biomass is used with no solid-liquid separation after the pretreatment. If dried yeast is used, no wastewater pretreatment is required in the yeast cultivation step. Output stream from SSF unit is transferred to downstream operations to recover ethanol solution. Downstream process for ethanol purification is described in Wingren et al. (2005). The remaining solid and liquid waste is separated out after downstream processing for waste treatment and re-utilization for electricity and biogas production.

7.2.3 Techno-Economic Analysis of Lignocellulosic Ethanol Production Process

The process integration models in Figs. 7.1 and 7.2 are combined with economic model for techno-economic (TE) assessment which calculates production cost and analyze economic feasibility of the integrated process. The economic model estimates capital and operating costs given the mass and energy balance from the flowsheet model. The analysis provides profitability assessment for comparative analysis of various process configurations for the production of cellulosic ethanol which must be taken into consideration for decision making process. The purpose of the fully integrated process simulation and the economic evaluation is to evaluate process techno-economic feasibility under different configurations as well as to explore other process alternatives for further economic improvement. This is by no mean to determine an exact ethanol production cost. The exact ethanol selling price could be determined using the fully integrated process simulation that is extend beyond upstream and downstream steps by incorporating all unit operations for processing biomass feedstock prior to pretreatment (e.g. pre-processing, transportation and storage), waste treatment, waste re-utilization after end-product recovery including energy and heat integration of lignin processing step (e.g. combustion of solids/lignin waste for electricity generation).

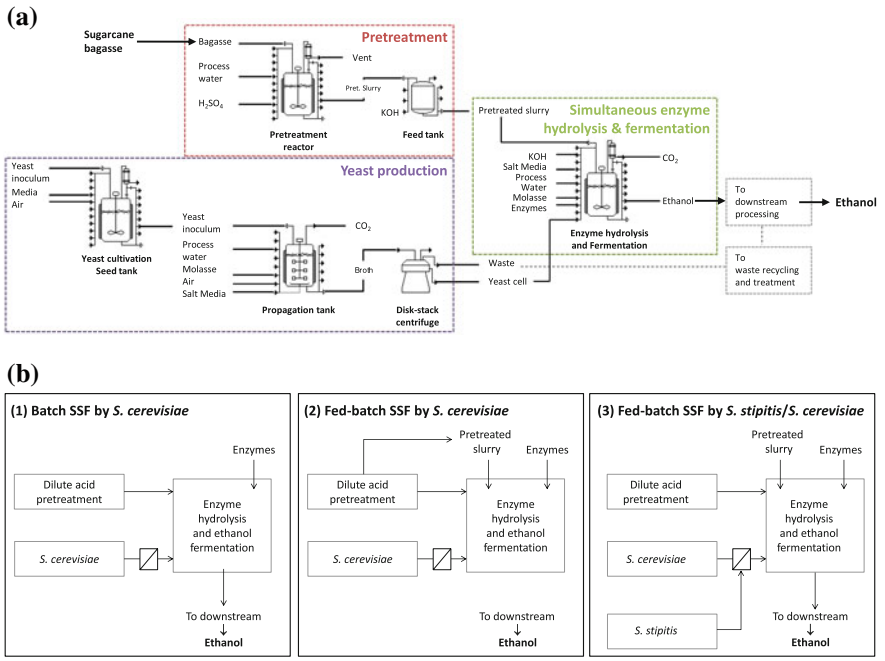

Fig. 7.2 **a** Process diagram for simultaneous saccharification and fermentation of lignocellulose to ethanol using dried yeast, *S. cerevisiae*, and commercial saccharification enzymes. The process configuration includes diluted-acid pretreatment (in red dashed square), yeast suspension (in purple dashed square), enzyme hydrolysis and fermentation (in green dashed square) steps for the conversion of biomass to ethanol. Process parameters including material inputs, yields, rates, titers, processing times and unit operations required in each step for material and energy balance simulation are obtained from previous experimental studies (Unrean et al. 2016; Buaban et al. 2010; Puseenam et al. 2015). Downstream processing for ethanol recovery is described in Wingren et al. (2008). **b** Process configurations under techno-economic evaluation. Three process scenarios for the production of lignocellulosic ethanol are (1) *S. cerevisiae* in batch simultaneous hydrolysis and fermentation (SSF), (2) *S. cerevisiae* in fed-batch SSF, (3) *S. stipitis*/*S. cerevisiae* yeast consortium in fed-batch SSF

7.3 Comparative Process Design of Lignocellulose-to-Ethanol

The effectiveness of enzyme saccharification and fermentation conditions contributes significantly to the economics of overall process. To select an optimal process configuration for sustainable and economical lignocellulosic ethanol bioprocess, six possible integrated process scenarios are simulated and compared for their techno-economics: *S. cerevisiae* in batch SHF, *S. stipitis*/*S. cerevisiae* yeast consortium in batch SHF, *S. stipitis*/*S. cerevisiae* yeast consortium in fed-batch SHF, *S. cerevisiae* in batch SSF, *S. cerevisiae* in fed-batch SSF, and *S. stipitis*/*S. cerevisiae* yeast consortium in fed-batch SSF. The process scenarios for SHF and SSF under study are

shown in Figs. 7.1b and 7.2b. These different process configurations are analyzed through comparative techno-economic analysis. The evaluation is mainly focused on the process conditions in enzyme hydrolysis and fermentation that have been optimized in experimental studies to examine how each of these conditions affects the production capacity and profitability of the process based on minimal ethanol selling price (MESP). The MESP is used as an indicator to show the economic impacts of different process designs and conditions.

7.3.1 Techno-Economic Assessment of SHF Process

Optimized SHF process using *S. stipitis*/*S. cerevisiae* consortium as shown in Fig. 7.1a is examined its potentials for industrialization and economic feasibility through process integration and techno-economic analysis. For TE analysis, demo-scale SHF process is simulated using SuperPro Designer software (Intelligen Inc., USA) to determine material and energy flow for all streams in the integrated process. Economic evaluation from mass and energy balances consists of estimating raw materials, operating, cleaning and labor costs associated with the ethanol production process (Table 7.1). Downstream processing cost estimation is from the previously reported value of required energy for ethanol purification, 10.2 MJ/L ethanol (Wingren et al. 2008). In the SHF process, on-site cultivated yeast cells suspension is implemented. The process performance and cost estimation via MESP of all the process steps in SHF at different configurations is summarized in Table 7.2. The MESP is defined as total production cost divided by liter of ethanol produced referring to the ethanol price where production cost and income of selling ethanol are equal (Humbird et al. 2011). Based on comparative process techno-economic, using *S. stipitis*/*S. cerevisiae* consortium could reduce the minimal selling price by 6% compared to when single-strain of *S. cerevisiae* is used in batch process, hence increasing profit margin. With fed-batch strategy, the MESP is decreased further by 18% compared to batch process. Comparison between batch and fed-batch process reveals that increasing solid loading from 10% WIS (in batch) to 22% WIS (in fed-batch) in SHF could increase the production capacity by 29%. The similar trend is also observed in SSF process configuration (Table 7.2) as well as in several previous studies (Wingren et al. 2003, 2008; Humbird et al. 2011; Gao et al. 2014). Thus, the process options with single-strain or with batch process should not be considered for practical applications because of their high cost.

An overview of material and utility requirements of the fed-batch SHF process using *S. stipitis*/*S. cerevisiae* per 1 dry ton sugarcane bagasse is summarized in Fig. 7.3. The estimated MESP of fed-batch yeast consortium process at 2.83 USD/gal is competitive with the current selling price of starch-based ethanol process (2.88 USD/gal, 2015 average selling price, www.thaiethanol.com). The fed-batch SHF using *S. cerevisiae*/*S. stipitis* consortium culture yields the lowest MESP due to the high ethanol titer reached by increasing solid content using fed-batch and the efficient utilization of mixed sugars by yeast consortium which have a significant

Table 7.1 Raw materials and utilities cost used in the techno-economic evaluation of the integrated lignocellulose-to-ethanol process

	Cost	Unit[a]	Sources
Raw materials			
Sulfuric acid	0.03	USD/kg	Kazi et al. (2010)
$MgSO_4$	0.50	USD/kg	Wingren et al. (2008)
Molasses	0.11	USD/kg	Sassner et al. (2008)
Cellulases	11.42	USD/kg	Geraili et al. (2014a, b)
$(NH_4)_2SO_4$	0.78	USD/kg	Unrean et al. (2016)
KOH	0.24	USD/kg	Unrean et al. (2016)
KH_2PO_4	0.53	USD/kg	Unrean et al. (2016)
Yeast extract	13.44	USD/kg	Unrean et al. (2016)
Utilities and labor			
Electricity	7.84	USD/GJ	Wingren et al. (2008)
Process water	0.17	USD/m^3	Wingren et al. (2008)
Chilled water	0.36	USD/m^3	Wingren et al. (2008)
Cooling water	0.28	USD/m^3	Wingren et al. (2008)
Cleaning agent	0.46	USD/kg	Wingren et al. (2008)
Steam	11.48	USD/ton	Unrean et al. (2016)
Labor	8.40	USD/d	Unrean et al. (2016)
Byproducts income			
CO_2	3.36	USD/ton	Wingren et al. (2008)

[a]The values reported in literatures are converted to USD using following exchange rate of 0.028 USD/Baht (the 2015 rate average, www.exchange-rates.org)

positive effect on process economy. Thus, the fed-batch yeast consortium process platform could potentially be suitable to meet the economic demand of large-scale ethanol production process, thereby replacing the starch-based ethanol production process. Techno-economic assessment to further improve process economy of fed-batch yeast consortium process platform under different process configuration (SHF vs. SSF) is performed in the next section.

7.3.2 Techno-Economic Assessment of SSF Process

Integrated SSF process containing various interdependent steps of pretreatment, yeast propagation, enzymatic hydrolysis and fermentation and downstream processing in a demonstration ethanol plant shown in Fig. 7.2 is evaluated for material and energy balance, and then used in techno-economic evaluations of the SSF process. The

Table 7.2 Techno-economic evaluation of different lignocellulosic ethanol process configurations using *S. stipitis* and *S. cerevisiae* (adapted from Unrean et al. 2016; Khajeeram and Unrean 2017)

SHF process configuration using yeast single- or co-culture[a]	Batch SHF *S. cerevisiae*	Batch SHF *S. stipitis*/*S. cerevisiae*	Fed-batch SHF *S. stipitis*/*S. cerevisiae*
Ethanol titer (g/L)	42.10 ± 0.25	46.68 ± 0.09	60.09 ± 3.92
Ethanol yield (kg-etoh/ton-bagasse)	173.1	193.7	250.0
Production capacity (L-etoh/year)[b]	26,988	30,190	38,969
Minimal ETOH selling price (USD/gal)[c]	3.82	3.46	2.83
SSF process configuration using yeast single- or co-culture[d]	Batch SSF *S. cerevisiae*	Fed-batch SSF *S. cerevisiae*	Fed-batch SSF *S. stipitis*/*S. cerevisiae*
Ethanol titer (g/L)	42.17 ± 0.65	65.43 ± 3.86	70.70 ± 1.61
Ethanol yield (kg-etoh/ton-bagasse)	173.5	267.3	288.6
Production capacity (L-etoh/year)[b]	27,041	41,664	44,997
Minimal ETOH selling price (USD/gal)[c]	3.69	2.91	2.58

[a]Process conditions of SHF using *S. cerevisiae* or *S. stipitis*/*S. cerevisiae* are according to experimental data reported in Unrean et al. (2016). Batch and fed-batch sugarcane bagasse-to-ethanol SHF processes are performed as separated enzymatic hydrolysis at 10% WIS (for batch) and 22% WIS (for fed-batch) followed by fermentation using yeast consortium

[b]Production capacity is based on demonstration scale operating at 3 dry tons sugarcane bagasse per batch and 41 batches annually

[c]Ethanol selling price is estimated based on total production cost per total ethanol produced

[d]Process conditions of SSF using *S. cerevisiae* or *S. stipitis*/*S. cerevisiae* are according to experimental data reported in Unrean et al. (2016). Batch and fed-batch sugarcane bagasse-to-ethanol SSF process is operated at accumulated WIS concentration of 10 and 22% respectively

optimized batch and fed-batch SSF process implemented using lignocellulose, purchased cellulase and hemicellulase enzymes and *S. cerevisiae* yeast cell is evaluated for its techno-economic feasibility using SuperPro Designer software (Intelligen Inc., USA). Process performance for batch and fed-batch SSF process is summarized in (Table 7.2). Demo-scale SSF process model is analyzed to determine material flow rates, composition and energy flow for all streams in the integrated process. Techno-economic evaluation from mass and energy balances consisted of estimating the raw materials, operating, cleaning and labor costs associated with the production process.

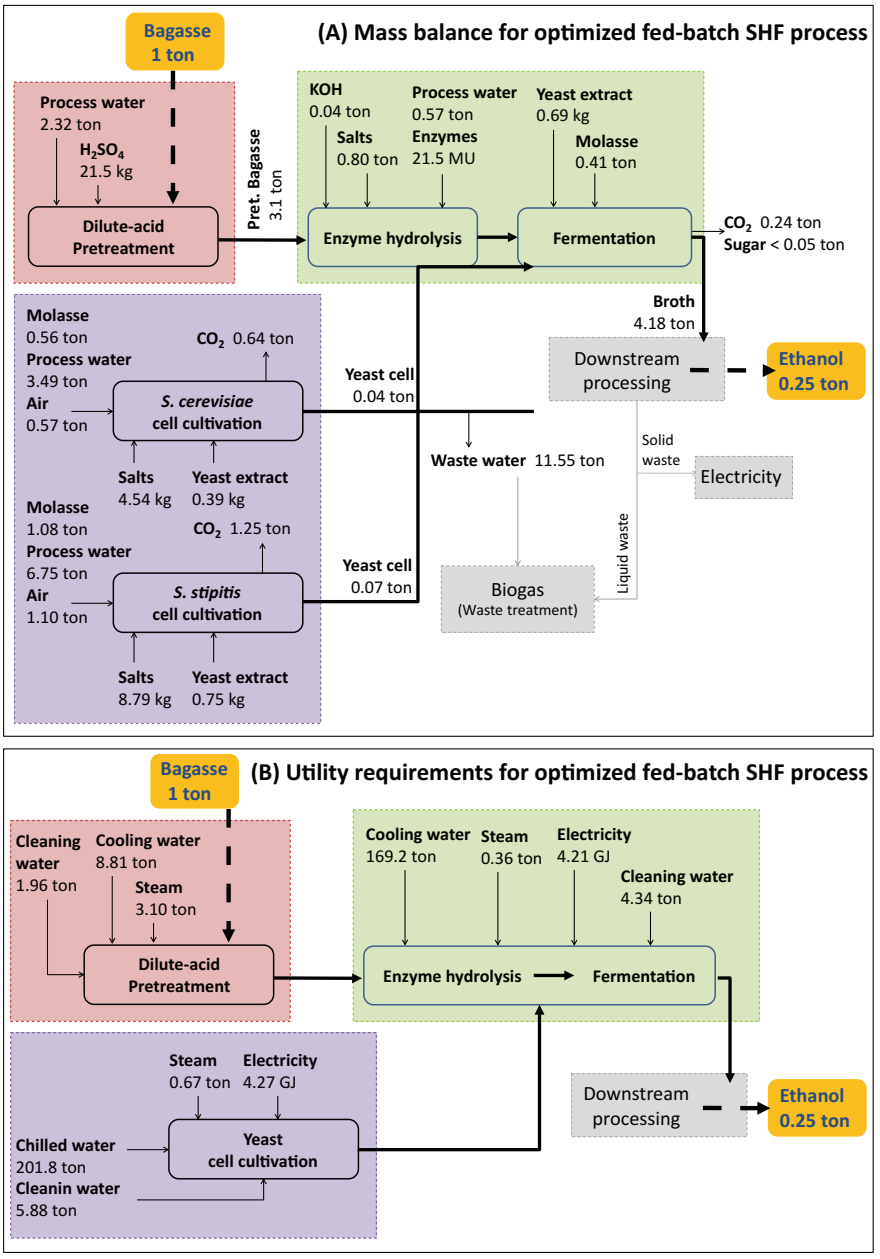

Fig. 7.3 Summary of mass and utility requirements for the conversion of sugarcane bagasse to ethanol in the optimized fed-batch SHF by *S. stipitis*/*S. cerevisiae* consortium. All values are based on 1 dry ton sugarcane bagasse

The cost of raw materials and utilities together with the byproduct income used in this evaluation is summarized in Table 7.1.

Using process flowsheet simulation, the SSF process is economically evaluated through minimal ethanol selling price (MESP) to assess its cost competitiveness with starch-based processes. The cost analysis of all the process steps for MESP determination estimated based on raw materials and utilities costs from the economic data is summarized in Table 7.2. TE analysis reveals that SSF process in fed-batch mode for high ethanol titer could reduce MESP by up to 15-20% hence increasing profit margin compared to batch process (Unrean et al. 2016; Wingren et al. 2003, 2008; Humbird et al. 2011), suggesting a positive effect of fed-batch SSF on the process economy. The fed-batch SSF process using *S. stipitis/S. cerevisiae* demonstrates economic viability with the MESP of 2.58 USD/gal, 8.8% lower cost compared to the SHF process (at 2.83 USD/gal) under the same condition. Techno-economic comparison of different sugarcane-to-ethanol process configurations in Table 7.2 reveals that utilization of yeast consortium is preferred than single-yeast culture for lignocellulosic ethanol production. In addition, SSF configuration offered better process economy than SHF configuration in both batch and fed-batch mode. Hence, the fed-batch SSF with yeast consortium platform is determined as the most effective process configuration for low-cost lignocellulosic ethanol process.

However, the ethanol cost under fed-batch yeast consortium is still higher than the ethanol selling price at 1.76 USD/gal from sugar-based process (2016 average selling price, www.tradingeconomics.com/commodity/ethanol). Further improvement to lower production cost would allow the SSF process platform to meet economic demand of large-scale ethanol production process. An overview of material and utility requirements of the fed-batch SSF process using yeast consortium per 1 dry ton sugarcane bagasse is summarized in Table 7.3. The fed-batch SSF process using yeast consortium is chosen for process sensitivity examination for improved economic viability.

7.4 Process Bottleneck Identification Through Cost Distribution Analysis

Main costs in fed-batch *S. stipitis*/*S. cerevisiae* yeast consortium process contributing to raw materials and utilities are analyzed. Cost distribution analysis for identifying various process bottlenecks that decrease the efficiency of fed-batch yeast consortium process economic is demonstrated in Fig. 7.4. In Fig. 7.4a, the cost of enzymes constituted 45% of the raw material costs, to which alkaline used for detoxification is the second contributor of 22% as has also been observed in several previous reports (Wingren et al. 2003; Zhuang et al. 2004a, b). Since the enzyme cost is the main concern for the ethanol production from lignocellulose, the on-site enzyme production or reducing enzyme usage in the process seems most reasonable to offer economic advantages. The production of enzymes on-site could be done using fungus cell or

Table 7.3 Raw material and utility requirements for the conversion of 1 dry ton sugarcane bagasse to ethanol by *S. stipitis*/*S. cerevisiae* in optimized fed-batch SSF process (adapted from Unrean et al. 2016)

Raw materials	SSF fed-batch Per 1 dry ton bagasse	
Process water	13.13	ton
H_2SO_4	0.021	ton
KOH	0.041	ton
Molasses	2.05	ton
Purchased enzymes	0.18	ton
Mineral salts	0.014	ton
Yeast extract	0.012	ton
Utility requirements		
Cooling water	228.98	ton
Chilled water	201.78	ton
Steam	3.42	ton
Electricity	8.79	GJ
Cleaning water	12.18	ton
Products		
Ethanol	0.29	ton
CO_2[a]	2.13	ton
Waste water	11.55	ton
Ethanol yield[b]	288 kg-etoh/ton-bagasse	

[a]CO_2 is sale as byproduct income
[b]The value is estimated from upstream process only thereby excluding the loss during downstream steps

genetic engineered yeasts (Kovacs et al. 2009; Puseenam et al. 2015). The advantage of the production of enzymes on-site includes eliminating transportation and the need to add stabilizers to reduce enzyme degradation during storage. However, multiple steps for on-site enzyme production would add to the overall process cost including operating cost, cost of cell removal, enzyme concentrating and purifying steps. Hence, techno-economic feasibility analysis is required to demonstrate what yield and production cost tradeoffs occur when enzymes are produced on-site. Increasing enzymes activity using enzyme enhancer or recycling enzymes for reuse are options for reducing enzyme loading. Minimizing enzyme irreversible binding to lignin is also essential for enzymatic hydrolysis efficiency which could be achieved through addition of surfactants (Li et al. 2016).

The cost of alkali for detoxification step also contributes significantly to the cost of cellulosic ethanol. Thus, from process economics perspective, improvement in cell robustness against the inhibitors present in pretreated biomass slurry is a prerequisite to reduce cost on detoxification step. Development of robust yeast cell factory could

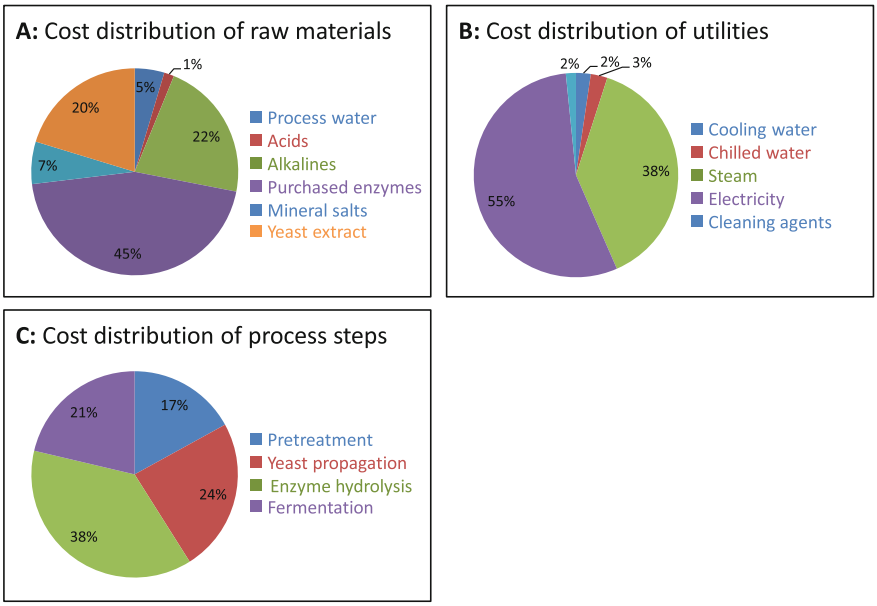

Fig. 7.4 Cost distribution of fed-batch SSF using *S. stipitis*/*S. cerevisiae* consortium based on raw materials **a**, utilities **b** and processing steps **c**

be accomplished through metabolic engineering or adaptive evolution approaches as being demonstrated in several studies (Wallace-Salinas and Gorwa-Grauslund 2013; Cheng et al. 2015; Unrean and Franzen 2015). Engineering robust yeast cell that could grow and propagate in high-solid SSF could also reduce the amount of yeast addition into the process since major limitation of high-solid operation is high concentration of inhibitors which hamper yeast cell growth and fermentation (Unrean et al. 2016). The robust yeast strains would also make it possible to run enzymatic hydrolysis and fermentation at higher solid loading since one of major limitations of high-solid operation is high concentration of inhibitors inhibiting yeast cell growth and fermentation.

For every one ton of dry bagasse being processed, 2.13 tons of carbon dioxide is generated (Table 7.3). Therefore, the co-product credit would have a significant effect on the overall process economy. Upgrading the CO_2 byproduct into valued-added chemicals through catalytic or biological conversion process would increase income and profit margin of the current integrated process. In addition, based on material flow balance, 13.13 ton of process water is required for upstream processing of 1 ton of raw sugarcane bagasse. To reduce the plant makeup water and further improve process economic, major part of the process water required could be replaced with steam condensates without affecting ethanol production yield.

The cost of electricity and steam energy covers more than 90% of the overall utility cost (Fig. 7.4b), thereby reducing production cost could be done by lowering utility

cost. Several alternatives for lower utility cost are recycling steam condensates for increasing energy efficiency, utilizing lignin and other solid wastes as energy source, and integrating biogas production from liquid waste into the process for electricity and steam generation. Integrating the wastewater treatment would also reduce the utility cost for steam and electricity, the two major cost of the process (Fig. 7.4) making the sugarcane bagasse-to-ethanol process more cost effective and energy efficient in the economic outcome. Liquid waste from the downstream steps could be used for biogas production in anaerobic digestion which can be used for steam generation. The solid waste containing the yeast could also be utilized as cattle feed. Furthermore, the solid, lignin-rich waste obtained from the downstream process is a co-product that can be dried and re-used as a solid fuel for electricity generation, underlining the importance of lignin recovery. Investigating potentials for increasing income from by-products and solid-liquid wastes generated during the production is necessary for meeting process economic requirements.

It should be noted that the cost analysis has not yet considered the cost due to mixing energy consumption when the process is operated at high solid condition (25% solid or more). Experiments have shown that the ratio of the mixing energy consumption to the thermal energy production in the ethanol is increased exponentially with the increasing solids loading from 15 to 30% solid but is decreased quickly with the increasing enzyme dosage from 7 to 30 FPU/WIS (Zhang et al. 2010). Thus, a balance for achieving the optimal energy cost between the increased mixing energy cost, the increased cost of enzyme and the reduced distillation energy cost needs to be thoroughly evaluated and account for during process integration and TE analysis of high-solid loading lignocellulose-based process.

Figure 7.4c points to enzyme hydrolysis as the most expensive processing steps within the conversion of sugarcane bagasse to ethanol process due to high enzyme cost. Comparing among process steps, yeast propagation process was the second largest cost distribution. Therefore, the production of yeast from molasses or other waste products is necessary for cost saving. On-site yeast production is also more economical compared to purchasing dried yeast for use in the process (Table 7.2). Further reduction of yeast propagation cost should be implementing high-cell-density fermentation to maximize yeast production while minimize operating cost. Engineering robust cell that could grow and propagate in high solid lignocellulose-based process is another process option to reduce the amount of yeast addition into the process.

7.5 Process Sensitivity Analysis

The effectiveness of enzyme saccharification and fermentation conditions contributes significantly to the economics of the overall process. Techno-economic analysis can be used for studying the effects of process parameters such as ethanol titer, solid substrate concentration, enzyme dosage, and yeast cell loading on the overall energy demand and production cost of lignocellulose-based process (Sassner et al. 2008). Through cost distribution analysis, process parameters, e.g. enzymes hydrolysis effi-

ciency, utilization of the pentose fraction and operation at high dry matter content for high ethanol titer, are keys for improving overall process economy of lignocellulosic ethanol. Hence, techno-economic process simulation is made to investigate process sensitivity.

7.5.1 Economic Impact of Yeast Consortium Utilization on MESP

Carbohydrates content of lignocellulosic feedstock consisted of a mixture of C_6 sugars e.g. glucose (from cellulose) and C_5 sugars e.g. xylose (from hemicellulose) (Canilha et al. 2011; Sluiter et al. 2008; Unrean et al. 2016). The utilization of both C_5/C_6 sugars is required for economical lignocellulosic ethanol process. Implementation of yeast consortium containing two yeasts, *S. stipitis* (C_5-fermenting yeast) and *S. cerevisiae* (C_6-fermenting yeast) for improved ethanol titer and productivity has been previously described (Suriyachai et al. 2013). Earlier reports have applied fed-batch strategy using yeast consortium approach to achieve high cumulative solid loading of lignocellulose-based process thus improving its working capability (Unrean et al. 2016). Co-fermentation of C_5 and C_6 sugars which could be accomplished by yeast consortium of *S. stipitis* and *S. cerevisiae* is essential to achieve high ethanol yield for economic advantages. Hence, Techno-economic feasibility analysis to evaluate the yield and production cost tradeoffs for the use of yeast consortium is performed. The economic impact of yeast consortium for C_5/C_6 co-fermentation on MESP is studied using the techno-economic model of integrated SSF process (Fig. 7.2b). Comparing between process configurations (Table 7.2), the impact of yeast consortium on MESP revealed up to 11.6% reduction of MESP when the yeast consortium technology was utilized in fed-batch SSF compared to the use of single-strain. This trend is consistent with previous study showing up to 30% production cost reduction if both C_6 and C_5 sugars are converted to ethanol compared to only C_6 sugar conversion (Liu et al. 2016). The result, therefore, validates the yeast consortium technology for economical biomass-to-ethanol conversion.

7.5.2 Economic Impact of Ethanol Titer on MESP

High-substrate loading process can be accomplished through fed-batch under optimal feeding of pretreated biomass, enzymes and/or yeast cells to maintain the low level of viscosity and sufficient mixing as demonstrated in recent studies (Gupta et al. 2012; Koppram et al. 2014). The development of an integrated biomass-to-ethanol process at high-solid operation for high ethanol titer has several economic advantages over conventional batch process including lower operating and labor costs (Hoyer et al. 2009; Zheng et al. 2009). The high-ethanol-titer process could maximize the overall

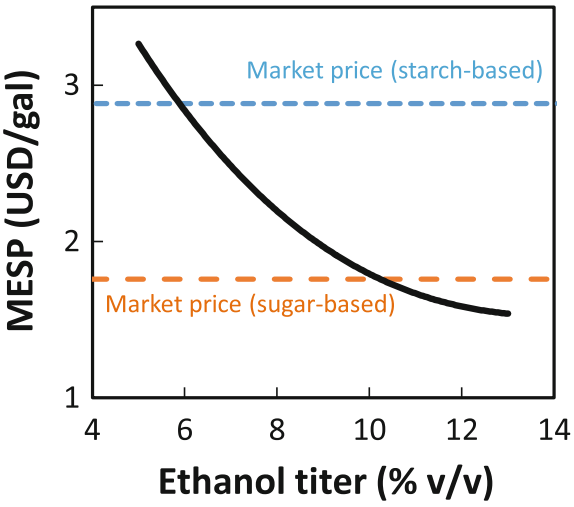

Fig. 7.5 Process sensitivity analysis examining final ethanol titer on the minimum ethanol selling price (MESP) for fed-batch *S. stipitis*/*S. cerevisiae* consortium process (adapted from Unrean et al. 2016). Dashed line represents current ethanol selling price from starch-based process (2015 average selling price, www.thaiethanol.com) in blue and sugar-based process (2016 average selling price, www.tradingeconomics.com/commodity/ethanol) in red for comparison purpose

process economy by lowering water consumption and greatly reducing (>80%) the energy demand and cost for downstream processing steps (Wingren et al. 2003; Soderstrom et al. 2005; Zheng et al. 2009). Previous study has shown that increasing the solid substrate concentration from 7% to 15% could reduce the energy demand and operating cost by as much as 50% (Sassner et al. 2008). Operation at high-solid load also requires less energy and smaller equipment for a given throughput (Koppram et al. 2014). Process sensitivity analyses the relative effect of ethanol titer on overall process economy in term of MESP to provide information on potential cost reduction when varying ethanol titer in upstream processing (Fig. 7.5). Increasing ethanol titer from 6 to 12%, while all other parameters are kept constant, would reduce the MESP significantly up to 44% (Fig. 7.5). Reducing the production cost and MESP can be explained by the lower energy demand in downstream processing at higher titer of ethanol. As been studied previously, doubling the ethanol titer from 2.5 to 5% could reduce the energy required in distillation by 33% (Sassner et al. 2008).

The result indicates that the ethanol titer is one of key factors controlling over the overall production cost. Higher ethanol titer could be accomplished either by improving ethanol yield in the yeast cell through genetic or evolutionary engineering or by increasing concentration of solid loading in enzyme hydrolysis followed by fermentation. One option of engineering yeast with improved ethanol yield could be based on the utilization synthetic biology or systems metabolic engineering to redirect more fluxes towards ethanol synthesis (Trinh et al. 2008). It is also expected

that the ethanol yield may be decreased when the solid concentration is increased due to increasing toxicity and stress caused by high viscosity of high-solid content (Koppram et al. 2014; Kristensen et al. 2009). Thus, having an efficient and robust yeast cell is desirable and essential trait for economical lignocellulose-based process. Additionally, as the concentration of solid-content increases, a specialized reactor for high-solid operation may be necessary. This has been demonstrated in several experimental studies (He et al. 2014; Palmqvist et al. 2011, 2015; Zhang et al. 2010) showing stirred tank reactor with helical impellers could provide sufficient mixing at solid load as high as 30% (w/v).

7.5.3 Economic Impact of Enzyme Hydrolysis Efficiency on MESP

Making cellulosic ethanol process economically viable also requires high saccharification efficiency at high sugar yield and low enzyme load for achieving a competitive MESP. Enhancement of the hydrolytic capacity of cellulases to increase sugar yield could be accomplished through a supplementation of hemicellulases due to the synergistic effect of enzymes mixture (Tabka et al. 2006; Kumar and Wyman 2009; Buaban et al. 2010; Zhang and Viikari 2014). The presence of hemicellulases such as xylanase removes hemicellulose hence improving the accessibility and the digestibility of cellulases to the cellulose leading to higher sugar production and lower overall process cost (Chandel and Singh 2011). Additionally, the use of on-site or near-site enzyme production is proposed as a promising way to the significant reduction of enzyme cost up to 30–70% owing to its simplified purification and logistics (Merino and Cherry 2007; Takimura et al. 2013; Szakacs et al. 2006; Hong et al. 2013). Due to the cost benefit of on-site enzymes, on-site hemicellulase production was investigated together with the integrated fed-batch SSF process in techno-economic process simulation (Fig. 7.6). A commercial cellulase preparation is employed due to its present low-cost (Aden et al. 2002; Wingren et al. 2005; Sassner et al. 2008; Zhou et al. 2009) and the effect of varying dosage of cellulase and on-site hemicellulase enzyme mixtures on the MESP is investigated.

Sensitivity analyses on the use of on-site hemicellulase and cellulase enzyme mixtures in SSF, are performed with the main focus on the effect of cellulases dosage and the supplementation of hemicellulases to cellulases enzyme on SSF process economy. Two integrated process configurations differed in enzyme supply mode (Fig. 7.6) are investigated in the techno-economic model of SSF process: SSF-Co: *S. stipitis*/*S. cerevisiae* in fed-batch SSF using commercial cellulases, and SSF-Co-Enz: *S. stipitis*/*S. cerevisiae* in fed-batch SSF using commercial cellulases and on-site hemicellulase enzymes production. In SSF-Co, effect of cellulases enzyme dosage on MESP is examined. In SSF-Co-Enz, hemicellulase enzymes are prepared on-site and added to the SSF process to examine economic effect when cellulases and on-site

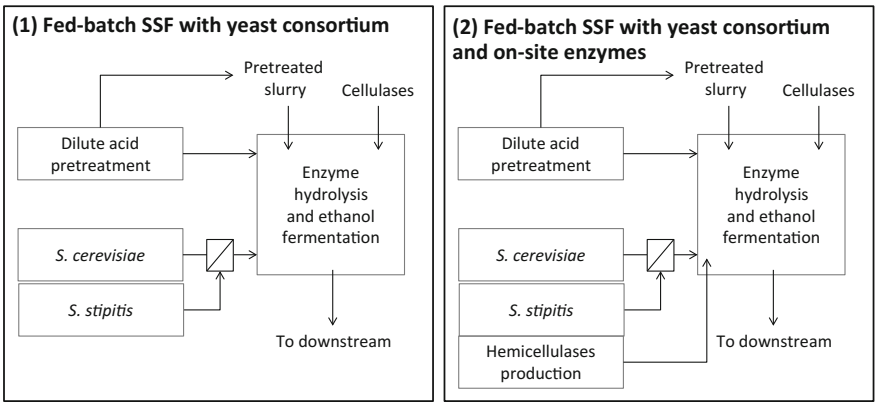

Fig. 7.6 Process scenarios for the conversion of lignocellulose to ethanol under techno-economic evaluation: (1) SSF-Co: Fed-batch SSF by *S. stipitis*/*S. cerevisiae* using commercial cellulases, and (2) SSF-Co-Enz: Fed-batch SSF by *S. stipitis*/*S. cerevisiae* using commercial cellulases and on-site hemicellulase enzymes production. SSF-Co is the reference case with the use of purchased cellulase enzymes and no on-site hemicellulase enzymes production, while SSF-Co-Enz is the reference case with the addition of purchased cellulases together with on-site produced hemicellulase enzymes supplementation. SSF process conditions and performances are described in previous studies (Unrean et al. 2016; Khajeeram and Unrean 2017). The production of hemicellulase enzymes and hydrolysis yield of cellulases supplemented with hemicellulases are as previously described (Buaban et al. 2010; Puseenam et al. 2015)

hemicellulases are used together compared to other scenarios with only cellulases enzyme.

7.5.3.1 Effect of Cellulase Enzyme Dosage on MESP

Enzyme cost is one of major cost distribution in the lignocellulosic ethanol production process (Fig. 7.4). The MESP could be further reduced to make the cellulose ethanol market competitive if enzyme loading can be further reduced and the sugar yield from enzyme saccharification process for ethanol fermentation can be further improved (Georgieva et al. 2008). Based on experimental SSF studies using yeast co-culture with different cellulase loading reported in Khajeeram and Unrean (2017) (Table 7.4), MESP is calculated.

Figure 7.7 shows the relative impact of cellulase enzyme dosage on the process economy in term MESP in SSF using *S. cerevisiae*/*S. stipitis* yeast consortium. Reducing cellulase enzymes load from 20 FPU/WIS (equivalent to 25 mg-protein/WIS) to 2.5 FPU/WIS (equivalent to 5 mg-protein/WIS) increases MESP by 31%, yielding a 1.9-fold higher price compared with the current selling price of ethanol at 1.76 USD/gal. Reducing cellulases load from 20 FPU/WIS to 2.5 FPU/WIS yields an increased MESP from 2.58 USD/gal to 3.39 USD/gal. Although at low cellulases loading the cost of purchased enzyme was reduce naturally (Zheng et al. 2009),

Table 7.4 Lignocellulosic ethanol production performance in SSF process by *S. stipitis*/*S. cerevisiae* yeast consortium under different cellulases loading operations (adapted from Khajeeram and Unrean 2017)

Process conditions[a]	Fold change in ethanol production[b]
SSF process with 25 FPU/WIS cellulases dosage	1.00
SSF process with 15 FPU/WIS cellulases dosage	0.92
SSF process with 10 FPU/WIS cellulases dosage	0.86
SSF process with 5 FPU/WIS cellulases dosage	0.78
SSF process with 2.5 FPU/WIS cellulases dosage	0.70

[a]Fed-batch SSF process for ethanol production from sugarcane bagasse is operated with yeast cell dosage of 0.02 g/g-WIS and solid loading of 22% WIS as depicted in process diagram in Fig. 7.6. Commercial cellulases is used

[b]Fold change is calculated as ethanol titer obtained in SSF process under investigation compared to base SSF process of *S. stipitis*/*S. cerevisiae* with 25 FPU/WIS purchased cellulases dosage under the same condition

releasing sugar yield of enzyme hydrolysis is also decreased when lower cellulases load is applied resulting in lower final titer of ethanol fermentation (Table 7.4). Due to lower ethanol production efficiency at low enzyme dosage, the decrease in cellulase dosage causes an increase in MESP. The results indicates that the performance of enzyme hydrolysis had higher impact on MESP than the cost of enzyme usage and the balance between enzyme hydrolysis efficiency through enzyme dosage and ethanol production efficiency is critical for achieving cost-effective lignocellulosic ethanol production process. The result is another confirmation that optimizing enzyme load while maintaining high enzymatic activity for high ethanol fermentation is essential for economic feasibility of the biomass-to-ethanol process.

7.5.3.2 Effect of Cellulase-Hemicellulase Enzyme Synergism on MESP

Since enzyme hydrolysis is the main contributors to the overall costs of producing ethanol from biomass, on-site or near-site enzyme production is desirable in order to further reduce the MESP to be competitive with the current selling price. Increasing enzymes activity using enzyme synergism is also one of the options for reducing enzyme loading and enhancing efficiency of enzyme hydrolysis. The synergistic action of the cellulases/xylanase mix has been reported to increase > 50% superior saccharification yield and ethanol production compared to the cellulases enzymes alone (Zhuang et al. 2004a, b; Kumar and Wyman 2009; Buaban et al. 2010; Lever et al. 2010). Thus, the production economics of lignocellulosic ethanol is largely dependent on the usage of cellulase and hemicellulase enzymes (Chandel and Singh 2011). On-site enzyme production can significantly decrease the overall MESP up to 40% compared to the use of purchased enzymes (Barta et al. 2010; Liu et al. 2016),

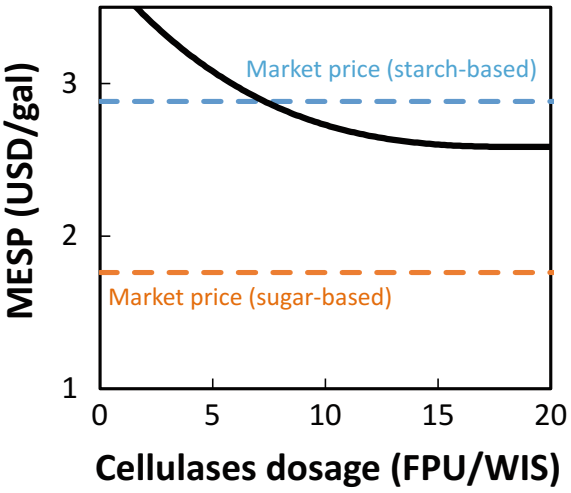

Fig. 7.7 Process sensitivity analysis examining impacts of cellulase enzymes dosage on the minimum ethanol selling price (MESP) for fed-batch SSF by *S. stipitis*/*S. cerevisiae* consortium using purchased cellulases. The condition and performance of SSF yeast consortium process using commercial cellulase enzymes was as described in previous study (Unrean et al. 2016). Dashed line represents current ethanol selling price from casava-based process (2015 average selling price, www.thaiethanol.com) in blue and sugar-based process (2016 average selling price, www.tradingeconomics.com/commodity/ethanol) in red for comparison purpose

providing a promising alternative approach for economical cellulosic ethanol production. The obvious advantages of on-site enzyme production include no additional cost on enzyme concentrating and purifying steps, storage and transportation cost, thereby providing a significant cost advantages to the process (Kovacs et al. 2009; Zhu et al. 2014; Lever et al. 2010). Thus, we examine economic impact of on-site hemicellulases (e.g. xylanase) supplementation on MESP.

Figure 7.8 shows the effect of cellulases and hemicellulase supplementation on MESP in SSF-Co-Enz scenario as depicted in Fig. 7.6. The cost analysis is evaluated based on SSF experiments using *S. cerevisiae*/*S. stipitis* yeast consortium and purchased cellulases mixed with varying load of crude xylanase (Table 7.5). The production of crude xylanase is simulated based on the performance of recombinant strain of *S. stipitis* expressing xylanase as previously reported Puseenam et al. (2015). As shown in Fig. 7.8, increasing xylanase dosage from 5 to 20 IU/WIS lowers the MESP by 24% under 10 FPU/WIS cellulase dosage in SSF process by *S. cerevisiae*/*S. stipitis* yeast consortium. Adding 20 IU/WIS xylanase produced on-site to SSF process with 10 FPU/WIS purchased cellulase dosage led to a 39.2% reduction in MESP compared to SSF process with only purchased cellulases. Xylanase hydrolyzes xylan in the pretreated bagasse into xylose allowing more accessibility of cellulose by cellulases yielding increased glucose release. Higher C_5 (xylose) and

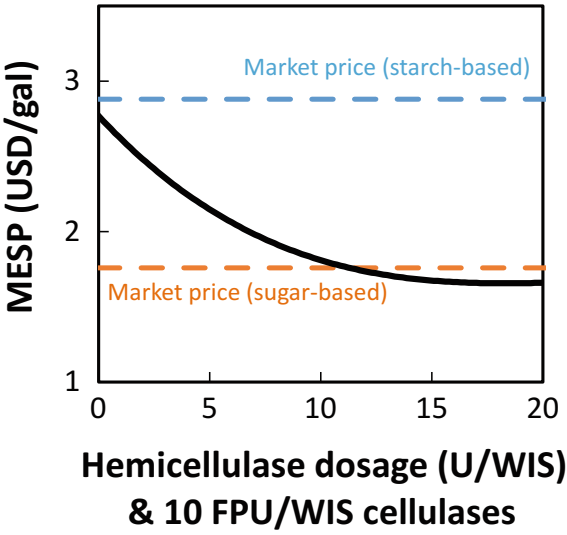

Fig. 7.8 Process sensitivity analysis examining impacts of cellulase/on-site hemicellulase enzymes loading on the minimum ethanol selling price (MESP) for fed-batch SSF by *S. stipitis*/*S. cerevisiae* consortium. The condition and performance of SSF yeast consortium process using commercial cellulase enzymes is described in previous studies (Unrean et al. 2016). The production of hemicellulase enzymes and hydrolysis yield of cellulases supplemented with hemicellulases are as previously described (Buaban et al. 2010; Puseenam et al. 2015). Dashed line represents current ethanol selling price from starch-based process (2015 average selling price, www.thaiethanol.com) in blue and sugar-based process (2016 average selling price, www.tradingeconomics.com/commodity/ethanol) in red for comparison purpose

C_6 (glucose) sugars released during hydrolysis in cellulases/hemicellulases mixture is then converted to ethanol by the yeast consortium resulting in higher ethanol yield and titer. Thus, this result demonstrates the impact of enzyme synergism on lignocellulosic process economy. Varying ratio of cellulases and xylanase enzyme load also affects the economics of biomass-to-ethanol production process. Thus, a proper combination of cellulase and hemicellulase enzyme mixtures is one of important factors for the biomass-to-ethanol process economy. Process sensitivity results emphasize that the dosage ratio between cellulases and hemicellulases mixture is necessary for achieving the cost-effective biomass-to-ethanol process.

7.6 Cost-Effective Lignocellulosic Ethanol Process Using on-Site Yeast Consortium and Enzyme Production

Based on techno-economic analysis of different process schemes, key features that must be reached for economic feasibility of lignocellulose-to-ethanol process are (1)

Table 7.5 Lignocellulosic ethanol production performance in SSF process by *S. stipitis*/*S. cerevisiae* yeast consortium under different hemicellulases supplementation with cellulase enzymes (adapted from Khajeeram and Unrean 2017)

Process conditions[a]	Fold change in ethanol production[b]
SSF process with 10 FPU/WIS cellulases dosage	1.00
SSF process with 10 FPU/WIS cellulases & 5 IU/WIS xylanase dosage	1.25
SSF process with 10 FPU/WIS cellulases & 10 IU/WIS xylanase dosage	1.25
SSF process with 10 FPU/WIS cellulases & 15 IU/WIS xylanase dosage	1.33
SSF process with 10 FPU/WIS cellulases & 20 IU/WIS xylanase dosage	1.33

[a]Fed-batch SSF process for ethanol production is operated with yeast cell dosage of 0.02 g/g-WIS and solid loading of 22% WIS as depicted in process diagram in Fig. 7.6. Commercial cellulases is used

[b]Fold change is calculated as ethanol titer obtained in SSF process under investigation compared to base SSF process of *S. stipitis*/*S. cerevisiae* with 10 FPU/WIS purchased cellulases dosage under the same condition

high sugar yield of enzyme hydrolysis with optimized cellulase and hemicellulase enzyme mixtures, (2) reduction of enzyme cost through on-site production, (3) efficient conversion of both C_5 and C_6 sugars to ethanol using yeast consortium and (4) enhanced ethanol titer through fed-batch, high-solid SSF process. Comparing all process schemes under investigation, the lowest MESP achieved is 1.66 USD/gal, obtained from the high-solid SSF process scheme with *S. cerevisiae*/*S. stipitis* yeast consortium using 10 FPU/WIS purchased cellulases supplemented with 20 IU/WIS on-site crude xylanase. This MESP was a 29% lower compared to that obtained from the reference case using *S. cerevisiae* and purchased cellulases. Under this condition, the ethanol cost is below the current market price of 1.76 USD/gal, thus the commercialization feasibility exists. The estimated MESP under the best process configuration will generate up to 6% increase in profit margin based on the current selling price of fuel ethanol reported. The proposed process scheme for low-cost ethanol production process from sugarcane bagasse is shown in Fig. 7.9.

Overall, the addition of optimized cellulase and xylanase enzyme loading which is produced on-site together with optimal yeast consortium of *S. stipitis* and *S. cerevisiae* should permit a highly efficient and low-cost simultaneous enzyme hydrolysis and fermentation for the production of ethanol from biomass. A proposed process with fed-batch, high-solid SSF using yeast consortium and cellulases/on-site hemicellulase mixture still requires the validation of industrial-scale operations for the future cellulosic ethanol industry. Potential economic benefit with higher activity enzymes (e.g. through supplementation of enzyme enhancer, addition of surfactants

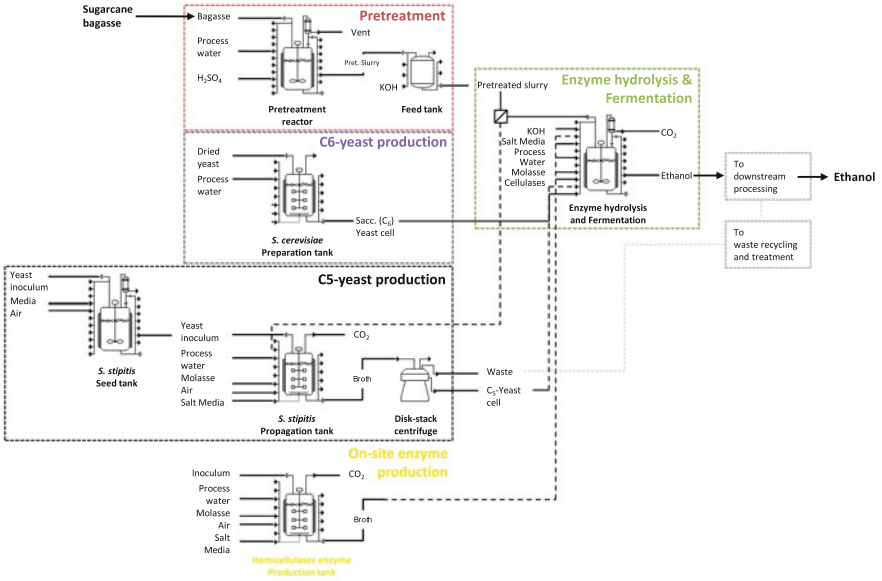

Fig. 7.9 Proposed process diagram of fed-batch, high-solid SSF by *S. stipitis*/*S. cerevisiae* cell consortium and on-site hemicellulase enzyme production for low-cost ethanol production from biomass. The process includes diluted-acid pretreatment (in red dashed square), yeast propagation for *S. cerevisiae* (in purple dashed square) and *S. stipitis* (in black dashed square), on-site hemicellulase production (in yellow dashed square), and simultaneous enzyme hydrolysis and co-fermentation (in green dashed square) steps

to minimize enzyme irreversible binding to lignin (Li et al. 2016) and more robust yeast cell is not yet considered in the MESP, and should be addressed in the future.

7.7 Process Uncertainty

Process operating conditions may be varied due to different equipment configurations during scale-up or large scale production. In addition, these experimental data contain uncertainty from measurement. This uncertainty can pass into the mass and energy balance in process integration model and combine with uncertainty in the capital cost of the process equipment in the economic model leading to uncertainty in the total production cost. The uncertainty from various sources such as change in market or operational parameters should be included into the future process integration model for evaluation of the impact of uncertainties affecting the overall production cost in order to improve a degree of realism of the simulated large-scale production process (Morales-Rodriguez et al. 2011; Kazi et al. 2010; Vicari et al. 2012; Zhang et al. 2013). Several studies have developed a modelling framework for evaluation

of the impact of measurement uncertainties on the uncertainty in production cost. The study by Vicari et al. (2012) has estimated the uncertainty of minimal ethanol selling price based on the uncertainties in input process parameters such as feedstock compositions and product yield from each step in integrated process using Monte Carlo simulations. Sensitivity analysis is another type of analysis that can be used to determine key parameters affecting the overall process (Morales-Rodriguez et al. 2011). Such uncertainty effect and sensitivity analysis should be account for during process selection and decision making of integrated process.

7.8 Concluding

Techno-economic of the integrated biomass-to-ethanol conversion process is analyzed based on process flowsheet simulation to provide insights into the effect of operational conditions on process economics and possible process design of an economical-viable biomass-to-ethanol process. A mass and energy balance based on process flowsheet simulation of the integrated process is implemented to estimate minimal ethanol selling price (MESP) of the integrated process. Overall, techno-economic analysis has identified the process configuration, the substrate loading, the efficiency from enzyme hydrolysis step and the efficiency for conversion of sugars to ethanol in fermentation as key parameters with the significant impact on the overall production cost and are the major driver for calculation of minimal ethanol selling price. The process cost analysis pointed to a potential ethanol production cost reduction via lowering enzyme demand of SSF through (1) on-site production and (2) enzyme synergism for high saccharification, as well as increasing ethanol titer during fermentation by (3) increasing solid content through fed-batch and (4) implementing yeast consortium for C_5/C_6 co-fermentation. These process strategy would improve techno-economic characteristics of the biomass-to-ethanol conversion process for large-scale production, providing economic feasible for industrialization. Among process scenarios considered, fed-batch SSF with yeast consortium and on-site enzymes production is the most cost-effective process option. The techno-economic modelling approach could provide basis for cost comparisons among different processes and for designing the most promising process integration for lignocellulosic ethanol production process. Incorporation of process uncertainty and sensitivity should be included into the process integration simulation model to improve a degree of realism of the simulated process.

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Chapter 8
Lignocellulolytic Enzymes from Thermophiles

Vikas Sharma and D. Vasanth

Abstract Thermophilic microorganisms are considered as the important source for the production of novel enzymes for various industrial applications including degradation of lignocellulosic biomass. Bioprocessing of lignocellulosic biomass has gained significant attention for the synthesis of bio-based products by focusing on its three major components, i.e. cellulose, hemicellulose and lignin. Thermophiles (optimally grown at 60 ± 80 °C) obtained from hot springs are of great interest for providing novel thermostable enzymes that can catalyze under harsh conditions comparable to those existing in various industrial processes. Metagenomic studies helps in identifying lignocellulolytic enzymes with novel properties from the culturable and unculturable micro-organisms. In this chapter, the biotechnological significance of thermostable lignocelluloses degrading enzymes will be briefly discussed particularly cellulases, xylanases and laccases.

Keywords Extremophiles · Lignocellulosic biomass · Hotsprings Thermophiles · Cellulases · Xylanases · Laccases

8.1 Introduction

Microbial life is not limited to specific environments, some microbial communities can also withstand extreme pH, temperature, pressure and salinity conditions (Van Den Burg 2003). Such organisms are known as extremophiles. Extremophiles are further classified into different categories which include thermophiles, acidophiles, alkalophiles, psychrophiles, and barophiles (piezophiles) and others. These organisms transformed themselves to survive in immoderate places for example hot springs, sulfataric fields and deep-sea hydrothermal vents etc. Therefore, the enzymes obtained from these extremophiles can function under such conditions where mesophilic organisms cannot even survive (Demirjian et al. 2001). To date, few microbial

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communities have been explored (Van Den Burg 2003). The discovery of extremophiles and novel enzymes produced by them can contribute in the development of industrial processes (Demirjian et al. 2001). In a particular environment, nearly 10% of the organisms are cultivable, so metagenomics can play an important role in revealing these organisms which leads to the exploration of microbial diversity. Now it is possible to create gene expression libraries of microorganisms from extreme sources. The screening of these libraries with fast and precise detection technologies can discover numerous new extremozymes. Till now more than 3000 different enzymes have been explored and most of them are being used for biotechnological and industrial applications, still they are not sufficient to fulfill industry demands (Van Den Burg 2003). The main reason is that most of the enzymes are not capable to survive in extreme conditions of industrial processes. Thus, enzymes produced by the extremophiles have a great potential to be used in new bioprocessing techniques that are more specific, faster and ecofriendly.

8.2 Importance of Lignocellulosic Biomass

There is a requirement of utilizing renewable, economic and easily available biomass for the production of wide varieties of products and lignocellulose is the best suitable option existing (Turner et al. 2007). Lignocellulosic biomass contains three types of biopolymers i.e. lignin (25–30%), cellulose (35–50%) and hemicellulose (25–30%) (Wongwilaiwalin et al. 2010). Cellulose is the most opulent organic molecule present on earth and an essential component of all plant material, whereas hemicelluloses is the polysaccharides present in the plant cell wall (Turner et al. 2007). While lignin is a complex compound made up of complicated phenylpropane units that are nonlinearly and arbitrarily linked with each other. But the transformation of lignocellulosic biomass to fermentable sugars is a major task to utilize renewable resources. So many approaches including thermal, biochemical and chemical have been anticipated but none have proven to be adequate. There is a need for possibilities with new conversion technologies that are unaffected with the variation in feedstock and can face vigorous process-operating conditions (Blumer-Schuette et al. 2008) (Fig. 8.1).

8.3 Role of Thermophiles in Degradation of Lignocellulosic Biomass

In past two decades, thermophiles and thermostable enzymes have gain much importance, but the study on thermophilic mocroorganisms and their proteins started in the early 1960's by the revolutionary work of Brock and his colleagues (Turner et al. 2007). Thermostable enzymes are suitable for extreme processes, as high temperature

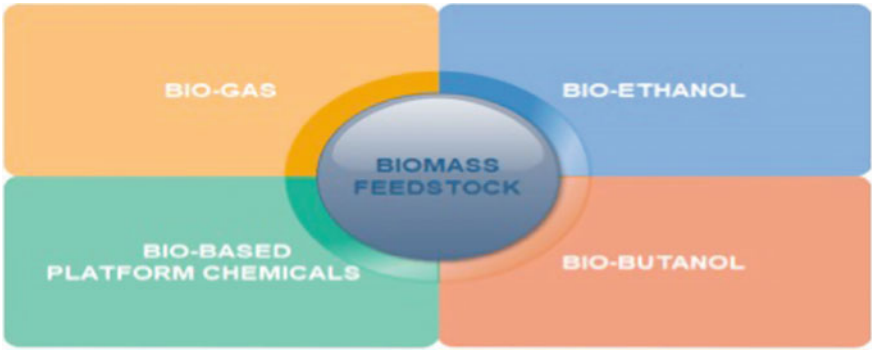

Fig. 8.1 Primary industrial biotechnology renewable product sectors supported by enzymatic release of lignocellulosic carbohydrates from biomass feedstock

often stimulates better enzyme penetration and cell-wall degradation of raw materials. Thermostable enzymes are produced by both the thermophilic and mesophilic organisms, but thermophiles are the more potential sources for such enzymes (Viikari et al. 2007). Extreme ecosystems such as hotsprings are of great interest as a source of novel extremophilic species, enzymes, metabolic functions for survival and biotechnological products (Saxena et al. 2017).

8.4 Sample Collection from Geothermal Areas

The images from Fig. 8.2a–d showcases collection of samples in the form of water, soil, rock mattings and pebbles from different sites of Tattapani hot water spring, India. These samples were placed in sterilized bottles and kept in an icebox immediately, then brought to the laboratory and stored at 4 °C in refrigerator till further processing. The temperature and pH must be measured at the time of sampling.

Thermostable enzymes obtained from thermophiles have numerous advantages over mesophiles in the degradation of lignocellulosic biomass e.g. (Viikari et al. 2007; Bhalla et al. 2013)

- the higher solvability of reactants and products, that result in higher reaction velocities thus reducing the quantity of enzyme required
- small hydrolysis period
- chances of contamination is less therefore, better productivity
- promotes restoration of evaporative compounds e.g. ethanol
- reduce the cost of power for cooling after thermal pretreatments.

The hydrolysis of lignocellulosic biomass with thermo-alkaliphilic and thermo-acidophilic enzymes could elude the neutralization phase during pretreatment (Bhalla et al. 2013). Many microbes that produced at extreme temperatures are capable of

Fig. 8.2 Sample collections from geothermal areas

utilizing a variable polysaccharides related to the transformation of lignocellulosic biomass to bioenergy (Blumer-Schuette et al. 2008). The microbial community that produces thermostable cellulases, xylanases and laccases are the most acknowledged micro-orgnisms involved in the bioprocessing of huge quantity of lignocellulosic material.

8.5 Thermostable Cellulases Obtained from Thermophilic Microbes

Cellulases (EC 3.2.1.4) are enzymes that catalyze the hydrolysis of β-1, 4 glucosyl linkages exist in the insoluble linear glucose homopolymer cellulose (Wilson 2009). Most commonly these enzymes are used in the degradation of lignocellulosic biomass and conversion of this biomass into fermentable sugar elements that can further used for the generation of other valuable products (Cerda et al. 2017). Recently, thermophilic bioprocessing techniques for bioconversion of cellulosic biomass have gained much attention, as these processes work at high tem-

perature (Rastogi et al. 2010). Considering the applications of cellulases, constant and functional thermostable cellulases would be more beneficial as compared to thermolabile enzymes in terms of time, cost reduction, and getting the appropriate product with desired yields/productivities (Franzén et al. 2017). Advancement in the proteomics, genomics, and fermentation strategies can contribute in searching more effective and unique thermostable cellulases obtained from thermophilic microorganisms of extreme environments. Most thermostable cellulases are isolated from either bacterial or fungal sources. Thermophilic bacteria are the most commonly reported source of cellulases. They have the capacity to directly ferment cellulose to ethanol and organic acids (Margaritis et al. 1986).

Thermophiles that can produce thermostable cellulase have been isolated from various hot springs around the world including Egypt, India, Thailand, Pakistan, China, Turkey and Sweden etc. These sources have harsh environmental conditions similar to those in industrial processes, so enzymes isolated from these microorganisms would be more feasible than other sources (Table 8.1).

8.6 Thermostable Xylanses Obtained from Thermophilic Microbes

Xylanases (EC 3.2.1.8) are enzymes that catalyze the hydrolysis of 1,4-β-D xylosidic linkages in xylan, therefore mainly responsible for the degradation of hemicelluloses component of the lignocellulosic biomass (Bhalla et al. 2013). Xylan requires various enzymes for its complete hydrolysis because of its complex structure, which are collectively termed as xylanases (Ellis and Magnuson 2012). Bacteria and fungi are major producers of thermostable xylanases. Thermostable xylanases produced by thermophilic bacterial strains are normally preferred for hydrolysis of lignocellulosic biomass over fungal xylanases because of their stability and better activity at elevated temperature (Viikari et al. 2007; Bhalla et al. 2015). Most of these processes require extreme conditions or extreme pre-treatment, which create a bottleneck for xylanase in industrial applications (Zhang et al. 2012). Study of extremophiles with metagenomics can further improves the understanding of xylanses to enhance its role in bioprocessing of lignocellulosic biomass (Walia et al. 2017).

Several xylanases has been produced from the thermophilic microbes isolated from geothermal areas around the world including hot springs of Argentina, China, Thailand, Japan, India, USA, Taiwan, Italy etc. Other sources of isolation of thermophilic micro-organisms are biogas reactor, local farm, mushroom compost, poultry compost, pulp samples, cow dung etc. (Table 8.1).

Table 8.1 Lignocellulolytic enzymes isolated from thermophiles

Name of the isolated organism	Extreme environment location	Temperature (°C)	MW(KDa)/pH	References
Thermostable cellulases from thermophilic microbes				
Bacillus sonorensis HSC7	Gorooh hot spring, Egypt	70	pH 4.0	Azadian et al. (2017)
Clostridium sp. DBT-IOC-C19	Puga thermal hot spring—Himalayan hot springs, India	55		Singh et al. (2017)
Bacillus subtilis J12	Hot spring soil and water sample (Thailand)	60	pH 6	Kuancha et al. (2017)
Bacillus amyloliquefaciens AK9	Hot spring of TattaPani, Pakistan	60	MW 47, pH 7	Irfan et al. (2017)
Geobacillus sp. HTA426	Hot spring of China	60	MW 40	Potprommanee et al. (2017)
Anoxybacillus kaynarcensis	Water and sludge samples from hot springs of Turkey	65		Baltaci et al. (2017)
*Bacillus licheniformis*WBS1, *Bacillus* sp. WBS3	Hot spring, India	60	pH 8 for rice and 9 for wheat	Acharya and Chaudhary (2011)
Thermoanaerobacter tengcongensis MB4	Tengcong hot springs (Yunnan, China)	75–80	MW 42.5, pH 6.0–6.5	Liang et al. (2011)
Bacillus subtilis DR	Hot spring water sample collected in YangLing, Shannxi province, China	50	MW 55, pH 6.5	Li et al. (2008)
Rhodothermus marinus	Alkaline submarine hot springs (Sweden)	95	MW 49, pH 7.0	Hreggvidsson et al. (1996)
Thermostable xylanases from thermophilic microbes				
Paenibacillus dendritiformis LT-4 and *Bacillus licheniformis* LT-5	Sediments, water, and biofilms (geothermal areas Argentina)	55	–	Cavello et al. (2017)

(continued)

Table 8.1 (continued)

Name of the isolated organism	Extreme environment location	Temperature (°C)	MW(KDa)/pH	References
Bacillus subtilis J12	Hot spring Water and soil sample from Sankamphaeng in Thailand	60	pH 5.5	Kuancha et al. (2017)
Anoxybacillus flavithermus TWXYL3 (*facultative Anaerobe*)	submerged plant material in the Mickey Hot springs area of the Alvord Basin, USA	65, retained up to 85		Ellis and Magnuson (2012)
Thermoanaerobacterium saccharolyticum NTOU1	Oceanic hydrothermal vent (Taiwan)	63	MW 50.0, pH 6.4	Hung et al. (2011)
Alicyclobacillus sp. A4	Hot spring(Yunnan Province, China)	55	MW 42.5, pH 7	Bai et al. (2010)
Acidothermus cellulolyticus 11B	Acidic hot springs in Yellowstone National Park (California)	90	MW 50, pH 6.0	Barabote et al. (2010)
Geobacillussp. MT-1	Deep-sea hydrothermal field in east Pacific (China)	70	MW 36, pH 7	Wu et al. (2006)
Bacillus thermantarcticus	Antarctic geothermal soil near the crater of Mount Melbourne (Italy)	80	MW 45, pH 5.6	Lama et al. (2004)
Bacillus sp. strain SPS-0	Hot spring in Portugal (France)	75	MW 99, pH 6	Bataillon et al. (2000)
Bacillus thermoleovorans strain K-3d	Hot spring in Kobe (Japan)	70–80	MW 40, pH 7	Sunna et al. (1997)
Bacillus flavothermus strain LB3A	Alkaline Lake Bogoria, (Kenya)	70	MW 80, pH 7	Sunna et al. (1997)
Thermotogasp. strain FjSS3-B. 1	Intertidal hot spring on savusavu beach in Fiji (New Zealand)	80	MW 31, pH 5.5	Simpson et al. (1991)

(continued)

Table 8.1 (continued)

Name of the isolated organism	Extreme environment location	Temperature (°C)	MW(KDa)/pH	References
Thermostable laccases from thermophilic microbes				
Brevibacillus sp. Z1	water and sludge samples of Diyadin Hotspring in provinces of Agri in Turkey	70	93 KDa, pH 4	Bozoglu et al. (2013)
Geobacillus thermocatenulatus MS5	Manikaran thermal hot springs, in Himachal Pradesh	55–60	pH 4.0–5.0	Verma and Shirkot (2014)
Bacillus sp. strain WT	Urmia lake, ahypersaline lake in northwest of Iran	55	pH 5	Siroosi et al. (2016)
Bacillus sp. SL-1	Aran-Bidgol Saline Lake in central region of Iran	70		Safary et al. (2016)
Anoxybacillus gonensis P39	Erzurum-Ilica Spring	60	pH 5.0	Yanmis et al. (2016)

8.7 Thermostable Laccases Obtained from Thermophilic Microbes

Laccases (E.C. 1.10.3.2; oxygen oxidoreductase) are the blue multi-copper oxidases that are responsible for catalyzing the oxidation of various phenolic and non-phenolic compounds by converting oxygen molecule to water with collateral four-electron reduction (Chauhan et al. 2017). Plants, fungi and bacteria are the major sources of this enzyme but only fungal laccases are commercially available and has been extensively studied (Muthukumarasamy and Murugan 2014). Lignin peroxidase, manganese peroxidase, and laccase are the three major enzymes associated with lignolysis (lignin component of the lignocellulosic biomass). Laccase is more readily available and easier to manipulate than both lignin peroxidase (LiP) and manganese-dependent peroxidase (MnP). The benefit of using laccases instead of peroxidases is that laccases require O_2 rather than H_2O_2 (Sriharti et al. 2017). Laccases are considered as the lignin-modifying enzymes as they involved in the formation of lignin by promoting the oxidative coupling of monolignols, a family of naturally occurring phenols (Solomon et al. 1996). Thermophilic microbes are the promising sources of novel thermostable laccases. So far, very few thermohphilic micro-organisms have been explored for the production of lacasses. Moreover, thermostable laccase has

more resistance to alkalinity, acidity, chemical denaturants and withstand high substrate concentration without losing its catalytic efficiency (Hildén et al. 2009).

Thermostable laccase has been produced from the thermophilic microbes isolated from geothermal areas around the world including hot springs of India, China, Turkey, and Iran etc. Other sources of isolation of thermophilic micro-organisms are hypersaline lake, textile industry effluents, and rhizosphere of rice etc.

8.8 Role of Metagenomics in Mining Lignocelluloses Degrading Micorbes

Metagenomics is an approach that identifies enzymes with novel characteristics from the culturable and unculturable component of microbiomes. This methodology offers identification of enzyme at much lower price and time than conventional methods (Ausec et al. 2017; Garrido-Cardenas and Manzano-Agugliaro 2017). Metagenomics comprise a series of high-throughput DNA sequencing technologies and bioinformatics tools for the study which include sample processing, sequencing technology, assembly, binning, annotation, experimental design, statistical analysis, data storage, and data sharing (Thomas et al. 2012; Garrido-Cardenas and Manzano-Agugliaro 2017).

Metagenomics is a culture independent approach as it offers study of the genes originated from uncultured microbes encoding enzymes with remarkable biochemical and biophysical characteristics (Nimchua et al. 2012). Screening of functional activity and DNA data mining can be very beneficial for the identification of useful enzymes (Van Den Burg 2003) (Fig. 8.3).

Metagenomic study of microbial genes from hot springs in central India reveals thermophiles that degrade hydrocarbon and provided the information regarding the survival conditions required in extreme environments (Saxena et al. 2017). The first acidobacterial laccase-like multicopper oxidase studied through metagenomics expressed high salt and thermo-tolerance in an acidic bog soil metagenome. A gene that encods three-domain LMCO (LacM) was identified by using molecular screening of a small metagenomic library (13,500 clones) which shows resemblance to copper oxidases of *Candidatus Solibacter* (Acidobacteria) (Ausec et al. 2017). Metagenomics study of thermophilic cellulose-degrading microbial community reveals new thermo-stable cellulolytic genes (Xia et al. 2013). In a metagenomic study, lignocellulose-degrading microbial consortia with structural stability and aerotolerance were obtained from industrial sugarcane bagasse pile (BGC-1), fluid of cow rumen (CRC-1), and pulp mill activated sludge (ASC-1). BGC-1 isolated cellulolytic *Clostridium* and *Acetanaerobacterium* with ligninolytic *Ureibacillus* showed maximum degradation of agricultural waste and industrial pulp residues (Wongwilaiwalin et al. 2013). 2 cellulases and 12 xylanases were isolated from the microbes in the guts of wood-feeding higher termites when analyzed through metagenomics (Nimchua et al. 2012). Similarly, genes of sticky microbes on plant fiber incubated in cow rumen

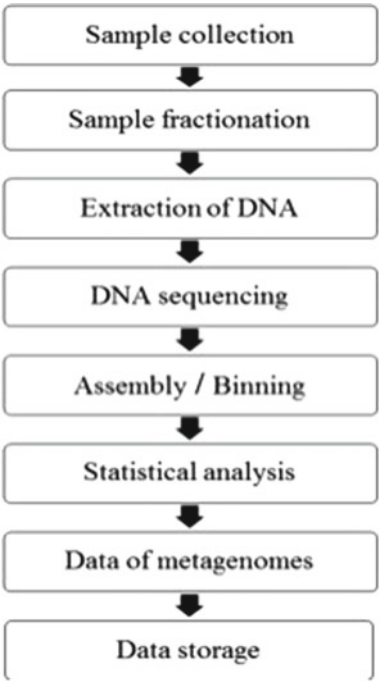

Fig. 8.3 Flow diagram of a typical metagenome study modified from Thomas et al. (2012)

were also studied through metagenomics. The study disclosed 27,755 carbohydrate-active genes out of which 57% had catalytic activity against cellulosic substrates (Hess et al. 2011). Several genes encoding cellulases, xylanases, laccases from different environments comprising termite guts, cow rumen, sugarcane bagasse pile, pulp mill activated sludge have been analyzed and identified by metagenomics studies (Hess et al. 2011; Nimchua et al. 2012; Wongwilaiwalin et al. 2013; Xia et al. 2013; Ausec et al. 2017; Saxena et al. 2017). These data sets provide information regarding genes and genomes responsible for the hydrolysis of lignocellulosic biomass. A variety of genomes from different environment have been studied but still new and suitable lignocellulose-degrading microbes are not entirely explored (Nimchua et al. 2012). So there is a need to investigate lignocellulose-degrading microbes from extreme environments through metagenomic studies that could probably provide industrial important informations applicable in bioconversion and processing.

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Chapter 9
Application of Enzymes in Sustainable Liquid Transportation Fuels Production

Nivedita Sharma and Poonam Sharma

Abstract These days the shortage of petrochemicals and environmental pollution are two major challenges, which need to be overcome by our society. As limited petroleum resources have become increasingly depleted, shortage of petroleum oil as well as rise in gasoline prices have become crucial factors in restricting the global economy. Therefore use of biofuels produced from bio-based materials serve as good alternate to petroleum based fuels as these offer various benefits to society and environment. Biofuels also offer a sustainable liquid fuel as bioethanol and biodiesel for transport sector. But different challenges have been associated with biofuel sector and one of them is need of efficient hydrolysis methods. Use of enzymes for effective hydrolysis of biomass can address the issue of hydrolysis of biomass as different enzymes can target the different components of biomass specifically. Therefore different enzymes used for hydrolysis of biomass, fermentation, limitations of enzymes are discussed in this review. Because of the different topics and challenges listed in this review and paucity of government policies to create the demand for biofuels, it may take more time for the enzymes to hit the market place than previously projected.

Keywords Hydrolysis · Fermentation · Extracellular enzymes · Enzyme producing microorganisms · Biofuel

9.1 Introduction

Modern world rely mostly on energy supply and it not only restricts the energy security of country but also associated with sustainable development. The unavoidable depletion of fossil fuels and increased level of green house gases have resulted into a quest for non-petroleum based energy sources. Oil is the basic backbone of

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transportation sector. According to International Energy Agency statistics, transport sector is responsible for approximately 60% of world's total oil usage (IEA 2008) and contributes to half of the green house gas emissions (Mielenz 2001). These days fermentation derived biofuels have already become a part of global carbon cycle (Carriquiry et al. 2011). Brazil is a world's leading player for ethanol production and according to the Brazilian federal government, 20–25% content of ethanol is blended in gasoline (Forge 2007). By building a new bioenergy sector with the help of biology, it can be beneficial to our energy security, economy and environment in many ways. In United States and Europe, during world oil crisis in the 70s, an interest arose in the use of cellulases to produce fermentable sugars from cellulosic wastes (Urbanchuk 2001; Lynd et al. 2003; Samson and Girouard 1998). The goal of this was to ensure the less dependency on oil and to reduce oil imports. At present, the need of hour is even bigger not because of increasing cost of oil, but also to reduce greenhouse gas emissions for maintaining and improving the quality of life for present and future generations.

With the help of different techniques, researchers have currently produced low cost ethanol that has drastically reduced the cost of biofuel production, boosting market growth. Different types of biomasses are being used for production of sustainable fuel with the help of biofuel enzymes- amylases, cellulase, xylanase, lipase and protease etc. Biofuels serve as alternatives to fossil fuel, is biodegradable, non-toxic and producing few emissions. Biofuels produced by different enzymes can be used in various sectors significantly in transportation.

Research for production of more effective biofuels is continuing that could reduce the risk of engine damage and could be cheaper than petroleum fuels. In different parts of world like U.S. and Europe, the enzymes cellulase and xylanase are used for biogas and bioethanol applications in power generation. Many agricultural and irrigation sector equipments are powered by biofuels, mainly by biodiesel. Besides this, biofuels produced through enzymes are used by many other industries, such as chemical, automobile, aviation, marine and research etc. According to BCC Research report, Global Markets and Technologies for Biofuel Enzymes (EGY009B), the global market for biofuel enzymes is projected to reach $652.1 million and $1.1 billion in 2015 and 2020, respectively, reflecting a healthy five-year compound annual growth rate (CAGR) of 10.4%. The arising market region is chiefly dominated by Brazil, China, Thailand, Japan, Australia and South Africa and been projected as biggest segment with revenues totaling $389.7 million through 2020. The U.S. trails the emerging market countries but still ahead from European countries due to its novel enzymes and techniques as well as government aid and support. The European region is also progressing due to a biofuel mandate by the European Union and launching of different products and plants. The increased production of biofuels has acted as key driver for biofuel market. Therefore it has been made easier to hydrolyze different plant feedstocks with varieties of enzymes. For example, Codexis Inc.'s launch of novel enzymes, as well as the use of enzyme cocktail mix for matter, has helped to make biofuel a reality and bioethanol production cost effective.

9.1.1 First Generation Biorefineries

The first generation biorefineries use corn, cassava, rye, soybean, sugar beet, sugarcane, sweet sorghum, rye and wheat as feedstocks for biofuel. In first generation feedstocks, sugary and starchy crops utilized for producing biofuel due to easy separation of their constituent reducing sugar units in water after hydrolysis and then fermented. Starch is mainly composed of two main units: amylose and amylopectin. Amylose composed of maltose's repeating units with 1–4 linked D-glucopyranose units. Amycolopectin is major constituent of most starches and composed of glucose units linked by 1–4 linkages. A wide variety of raw materials have been used for production of ethanol. These raw materials are classified under two major categories:

(a) Sugar containing crops: Sugar cane, wheat, beet root, fruits, palm juice, etc.
(b) Starch containing crops: Grain such as wheat, barely, rice, sweet sorgum, corn, etc. and root plants like potato, cassava.

9.1.2 Second Generation Biorefineries

Lignocellulosic biomass for e.g. sugarcane baggase, wheat or rice straw, corn stover, forestry, paper mill residues and municipal wastes which are abundant and renewable, have been recognized as potential low cost feedstocks for bioethanol production. Inspite of sugary crops, the utilization of lignocellulosic feedstock is difficult for ethanol production because of its complex and compact structure. Lignocellulosic feedstock contains three major parts: hemicellulose (~30% dry wt.), cellulose (~45% dry wt.), lignin (~25% dry wt.) (Carriquiry et al. 2011). Cellulose is main component of plant cell walls. It is a polymer of glucose and linked by β-(1–4)-glycosidic bonds. Hemicelluloses are branched, containing sugar residues as hexoses, pentoses and uronic acids but they also contain nonsugars like acetyl groups (Lynd et al. 2002a, b). Different raw material contained varied amount of hemicellulose depending upon source of raw material (Carriquiry et al. 2011). To cut down the cost of biofuel production, several hurdles have been encountered for converting lignocellulosic biomass to biofuels need to be overcome (Hoekman 2009; Menon and Rao 2012; Luo et al. 2010)

9.1.3 Third Generation Biorefineries

Algae have been considered as third generation biorefineries because they produce fatty acids by esterification of membrane lipids which is composed of 5–20% of their dry cell weight. Fatty acids contain medium chain, long chain and very long chain fatty acid derivatives. Some algae species contain up to 80% oil of their dry

cell weight. For e.g., an algae *Bortyococcus braunii* contain large quantities of hydrocarbons up to 80% DCW and can be explored as a raw material for biofuels (Hu et al. 2008).

9.2 Important Enzymes and Their Mechanistic Application on Different Substrates

Various kinds of enzymes are required to break different kinds of bonds for hydrolysis of biomass due to complex network of various compounds (Somerville et al. 2004). These enzymes are termed as molecular scissors and can produce specific monomer sugars from complex carbohydrates (Gao et al. 2010, 2011a, b; Banerjee et al. 2010). Every individual enzyme must be present in appropriate ratio to be most efficient. Trichoderma reseei, a filamentous fungus is used by different companies to produce biomass depolymerizing enzymes because of its efficiency to produce high quantities of enzymes up to 100 g/L (Balan et al. 2013). Various other bacterial and fungal enzymes have been introduced to perform at higher temperatures to prevent *Lactobacillus* contamination (to convert sugars to lactic acid) to the market (Acharya and Chaudhary 2012; Liszka et al. 2012). Various microbes produce enzymes to hydrolyze biomass for producing monomeric sugars for their survival. Microbes use biomass degrading enzymes in two ways in a natural environment. First one is cellulosomal enzyme system which involves a complex mixture of enzymes which are docked to cohesive and doctrine domains anchoring on the surface of the organisms (Bayer et al. 2013). Commercial companies produce free biomass degrading enzymes in large scale by using fungus or bacteria. For this microbes are fed with various agriculture crops (grains, hulls and different biomass waste). This mixture contains 40–50 different biomass degrading enzymes, mainly classified into three main categories: cellulase for degrading cellulose, hemicellulases for degrading hemicellulose, pectinases for degrading pectin (Zhang et al. 2012). Cellulase enzyme constitute about 70–85% of the mixture whereas hemicellulases and pectinase constitute remaining 15–30%.

9.2.1 Amylase

Amylase enzyme catalyzes the hydrolysis of starch into sugars. it is an important homopolysccharide and found in abundance as food and energy source in plants. It is present in seeds, leaves, bulbs and tubers. It is structurally composed of amylase and amylopectin polymers which differ in proportions depending upon its source (Anonymous 2016). In amylase appx. 103 glucose residues are linked by α-1, 4 bonds (Mayes 1996) whereas amylopectin contains 104–105 glucose residues as it is a highly branched poly-

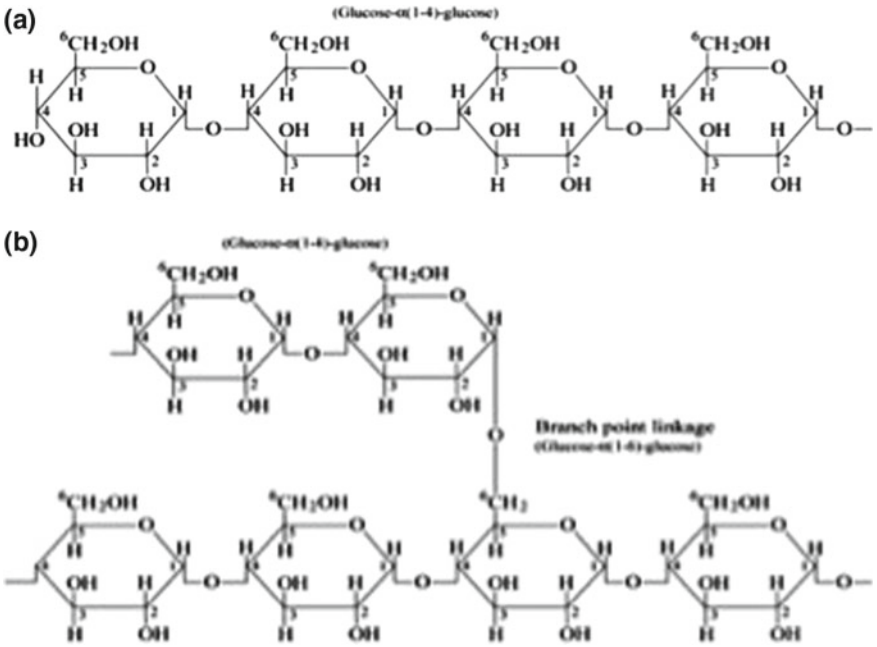


Fig. 9.1 **a** Structure of amylose. **b** Structure of amylopectin

mer comprising of α-1, 4-linked glucose chains (20–25 residues long) with α-1, 6 linkages present at branching points (Morrison and Karkalas 1990). Amylase enzymes constitute approximately 25% of the enzyme market (Anonymous 2016) (Fig. 9.1a, b).

Amylase enzymes can be divided into two types: exoamylases and endoamylases. Exoamylases act on the non-reducing end and result into short end products whereas endoamylases catalyze hydrolysis in interior of the starch molecule in a random fashion, producing linear and branched oligos of different chain lengths (Gupta et al. 2003). Pullulanase (EC 3.2.1.41) and isoamylase (EC 3.2.1.68) are the examples of debranching enzymes and are specific for 1,6 bonds in amylopectin and branched chain dextrins. According to the inability or ability to degrade also the 1,4-glucosidic bonds, pullulanases are classified into two categories (Wind 1997): pullulanase I and pullulanase II, respectively. Pullulanase type II is usually referred to as α-amylase-pullulanase or amylopullulanase.

9.2.1.1 Mode of Action of Amylase

Enzyme action can be in form of single attack or multi-chain attack action (Azhari and Lotan 1991).In single attack, the polymer molecule is completely hydrolyzed before dissociation of enzyme substrate complex whereas in multi chain attack only

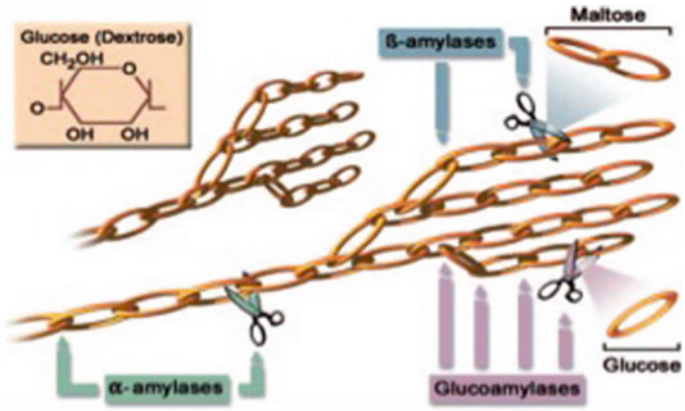


Fig. 9.2 Structure of starch and general characteristics of starch degrading enzymes

one bond is hydrolyzed. In multiple attack action, enzyme cleaves various glycosidic bonds after random hydrolytic attack before dissociation of substrate and serve as a bridge between single chain and the multi-chain action (Bijttebier et al. 2007). The multiple attack action is most accepted concept to explain the differences in action behaviour of amylases (Kramhoft et al. 2005). Despite of the fact that most of the endoamylases have very low level of multiple attack action (Bijttebier et al. 2007) (Fig. 9.2).

9.2.2 Cellulase

Cellulase catalyzes the hydrolysis of cellulose and is the enzyme of industrial importance (Jing et al. 2005). These are synthesized by fungi, bacteria and plants (Da-Silva et al. 2005). Three major types of cellulases involved in cellulose degradation are: exo-β-1,4-glucanase, endo-β-1,4-glucanase and β glucosidase (Fig. 9.3a–c).

9.2.2.1 Mode of Action of Cellulase

Cellulase is a mixture of several enzymes among which three major groups are involved in hydrolysis of cellulose are:

- Endoglucanases cut at random internal sites in the cellulose chain, liberating oligosaccharides of different lengths and subsequently new chain ends.
- Exoglucanases act in a progressive manner on the reducing or non-reducing ends of cellulose chains, releasing glucose or cellobiose as major products.
- β-Glucosidases hydrolyze soluble cellodextrins and cellobiose to glucose (Lynd et al. 2002a, b).

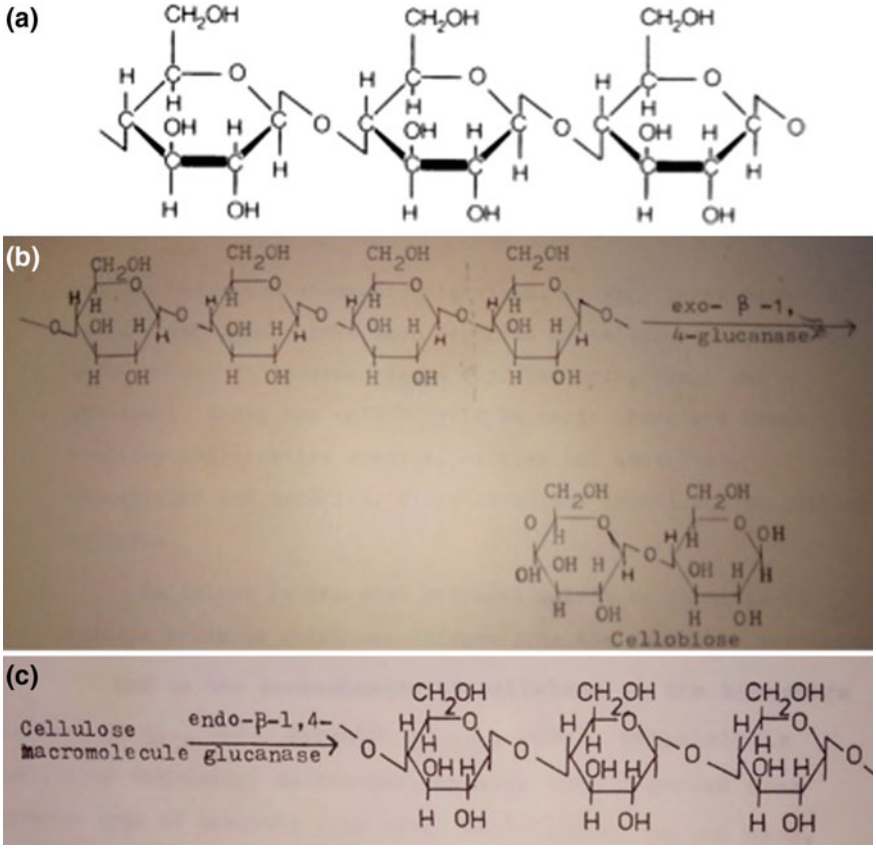


Fig. 9.3 **a**–**c** Structure of cellulose and modes of action of various components of cellulose

Most cellulases contain a modular structure including both catalytic and carbohydrate binding modules (CBMs). The CBM effects facilitate the cellulose hydrolysis by binding to cellulose surface by pulling the catalytic domain in close relation to the substrate, cellulose (Sharma and Sharma 2014). Cellulase enzyme system contains higher collection activity than activities of individual enzymes, a phenomenon called as synergism. Four types of synergism are reported: (i) endo-exo synergy (ii) exo-exo synergy (iii) synergy between exoglucanases and β-glucosidases (iv) intramolecular synergy between catalytic domains and CBMs.

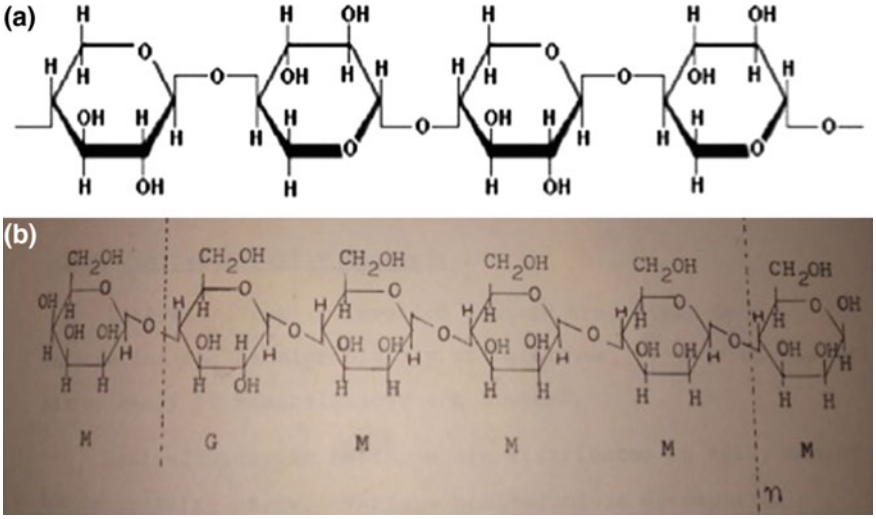


Fig. 9.4 **a** Structure of xylan. **b** Hydrolysis of xylan

9.2.3 Xylanases

The xylanolytic enzymes that carry out the xylan hydrolysis and generally composed of hydrolytic enzymes, endoxylanases (endo-1,4-β-xylanase, E.C.3.2.1.8), β-xylosidase (xylan-1,4-β-xylosidase, E.C.3.2.1.37), α-glucuronidase (α-glucosiduronase, E.C.3.2.1.139), α-arabinofuranosidase (α-Larabinofuranosidase, E.C.3.2.1.55) and acetylxylan esterase (E.C.3.1.1.72) (Belancic et al. 1995). These enzymes act in co-operation to convert xylan into constituent sugars. Among xylanases, endoxylanases are most important due to their involvement in breaking the glycosidic bonds and in releasing short xylooligosaccharides (Verma 2012).

Xylan is the second most abundant polysaccharide in nature and constitutes about one third of renewable carbon on earth. It is the main component of hemicellulose including xylan, xyloglucan (heteropolymer of D-xylose and D-glucose), glucomannan (heteropolymer of D-glucose and D-mannose), galactoglucomannan (heteropolymer of D-galactose, D-glucose and D-mannose) and arabinogalactan (heteropolymer of D-galactose and arabinose) (Kulkarni et al. 1999).

Xylan are the heteropolysaccharides consisting of a β-1,4-xylopyranosyl backbone with branches of acetyl arabinosyl and glucuronyl residues (Fig. 9.4a).

9.2.3.1 Mechanism Action of Xylanase

Xylanases being endoactive enzymes are produced in xylan medium, containing xylanase hydrolysates being the carbon source and it attacks the xylan chain in a

random manner, which cause a decrease in degree of polymerization of the substarte and produce shorter oligo, xylobiose and xylose. the working of different xylanases and hydrolysis products varies as per the source of enzyme. Xylanase, act as catalyst on the primarly xylan and related compounds to simple sugars. the primary product of this reaction is xylose. β-3-1,4-xylan are the heterogeneous group of polysaccharides, which are found in the cell wall of plants and in every plant part. The main product of hydrolysis of their characteristic backbone is β-1,4-linked D-xylosyl residues, which include β-1,4-xylanases (1,4-β-D-xylan xylanohydrolase; EC 3.2.1.8 and β-xylosidase (1,4-β-D-xylan xylohydrolase, EC 3.2.1.3.7). Xylanase attack on the xylosidic linkage, which is present on the backbone and β–xylosidases lead to the production of xylosyl residue by attacking on the endside of xylooligosaccahrides (Rani and Nand 2001) (Fig. 9.4b).

xylanase

$$\text{Xylan} + H_2O \rightarrow \text{xylose sugar}$$

9.2.4 Pectinase

Pectinase (E.C.3.2.1.15) comprise of system with complex enzymatic action, being responsible for the breakdown of the various pectin substances (Farooqahamed et al. 2003). These are the glycosidic macromolecules of higher molecular weight. Pectic substances have the protopectins, pectinic acids, pectins and pectic acids. The main chain of pectin is partially methyl esterified 1,4—D-glacturonan. Demethylated pectin is known as pectic acid (pectate) or polygalacturonic acid. This enzyme breaks the polygalacturonic acid into monogalacturonic acid by opening the glycosidic linkages. Two major sources of pectinase enzyme are plants and microorganism. Importance of the pectinase enzyme from technical as well as economic point of view, has been increasing day by day (Padmapriya et al. 2012) (Fig. 9.5a).

9.2.4.1 Mechanism of Action of Pectinase

Pectinases or pectinolytic enzymes leads to the hydrolysis of pectic substances and consist of mainly two groups; (i) pectinesterases which are able to de-esterify pectin by removal of methoxyl residues and (ii) depolymerases which readily split the main chain. The depolymerizing enzymes are divided into polygalacturonase (PG), which cleave the glycosidic bonds by hydrolysis, and into lyases, which break the glycosidic bonds by transelimination (Blanco et al. 1999). The major pectinolytic enzymes are homogalacturonan breaking enzymes.

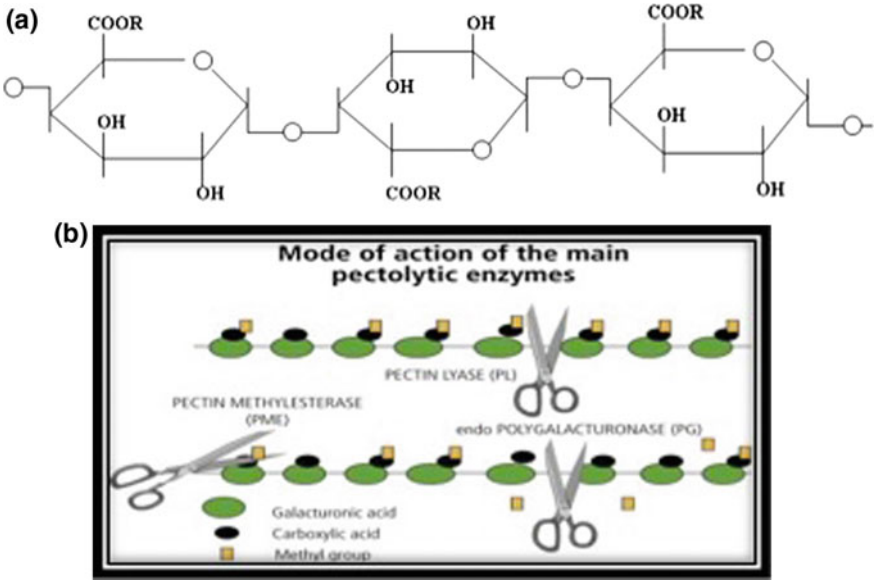


Fig. 9.5 **a** Chemical structure of pectin. **b** Mechanism action of pectinase

Protopectinases

Protopectinases solublize the protopectin, which lead to the formation of highly polymerized soluble pectin (Jayani et al. 2005). These are divided mainly into two groups; first one reacts with polygalacturonic acid region of protopectin, A type; second one reacts with the polysaccharide chains, which may connect the polygalacturonic acid chain and cell wall constituents, B type (Shevchik and Hugouvieux-Cotte-Pattat 1997).

Pectin Methyl Esterases (PME)

Pectin methyl esterase or pectinesterase (EC 3.1.1.11) leads to the de-esterification of the methoxyl group of pectin, which lead to the formation of the pectic acid and methanol. The enzyme acts mainly on the methyl ester group of galacturonate unit, which is present next to a non-esterified galacturonate unit. This act before the polygalacturonases and pectate lyases, which requires the non-esterified substrates (Kashayp et al. 2003). It is classified into carbohydrate esterase family 8 (Favela-Torres et al. 2005) (Fig. 9.5b).

Polymethylgalacturonases (PMG)
Pectin Acetyl Esterases (PAE)

Pectin acetyl esterase (EC 3.1.1.-) leads to the hydrolysis of the acetyl ester of pectin leading to the formation of pectic acid and acetate. It is classified into carbohydrate esterase families 12 and 13 (Favela-Torres et al. 2005). Polymethylgalacturonase

calalyse the hydrolytic cleavage of β-1,4-glycosidic bonds in the backbone of the pectin, mainly on highly esterified pectin which lead to the formation of 6-methyl-D-galacturonate (Sakai et al. 1993).

Polygalacturonases (PG)

Polygalacturonase catalyzes the hydrolysis process of α-1,4-glycosidic linkages in polygalacturonic acid, which lead to the production of D-galacturonate. It is classified into glycosyl-hydrolases family 28. Both groups of hydrolase enzymes (PMG and PG) can act in an endo- or exo- mode. Endo-PG (EC 3.2.1.15) and endo-PMG leads to the catalysis of hydrolytic cleavage of the substarate, whereas exo-PG (EC 3.2.1.67) and exo-PMG leads to the catalysis of hydrolytic cleavage at substrate nonreducing end producing monogalacturonate or digalacturonate. Hydrolases are produced mainly by fungi, being more active on acid or neutral medium at temperatures between 40 and 60 °C.

9.2.5 Lipase

For the production of biodiesel, lipase and phospholipase enzymes are the major source. Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are the important biocatalysts, because of possessing excellent biochemical and physiological properties. A few companies are commercializing biodiesel produced with enzymatic processes. Lipase transform the free fatty acids (FFA) and triacylglycerol into fatty acid methyl esters, which is the main product of biodiesel. The phospholipase is liable for the conversion of phospholipids to diacylglycerol, which acts as a substrate for the production of lipase. The traditional process include the use of methanol and the catalyst remove the FFAs and phospholipids, before the reaction improve the quality of biodiesel. The reason being that the enzyme can easily utilize the substrate, and the yield is higher and also, the process saves the chemical wastage. The fact is that enzymes are well known and easily available, but their cost is too high, to make their use on all feedstocks, mainly for the production of clean plant oils. One of the way to extend the life and also, to lower the cost of enzyme is to immobilize them on a solid substrate to enable multiple cycles of use.

9.2.5.1 Mechanism of Action of Lipase

Lipases are divided into three groups based on their specificity as 1,3-specific lipases, fatty acid-specific lipases and nonspecific lipases. 1,3-specific lipases release fatty acids from the 1 and 3 position of a glyceride and hydrolyse the ester bond in these positions as in case of *Aspergillus niger*, *Rhizopus oryzae* and *Mucor miehei* catalyze transesterification reactions efficiently (Ribeiro et al. 2011; Yahya et al. 1998; Freire and Castilho 2001; Choi et al. 2013; Antczak et al. 2009) (Fig. 9.6).

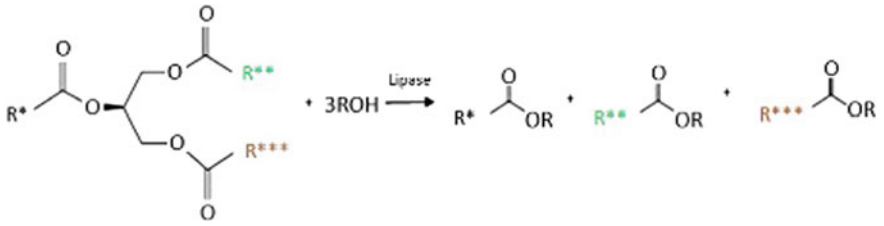


Fig. 9.6 Mode of action of lipase

9.3 Microorganisms Involved in Production of Different Enzymes

9.3.1 Microrganisms Involved in Production of Amylase

The production of amylase is done by several bacteria, fungi and genetically modified species of microbes. Major source of Amylase enzyme among bacterial species are the *Bacillus* spp., *B. amyloliquefaciens* and *B. licheniformis* which are widely used for the commercial production of enzyme. *B.cereus* and *B. subtilis* are also utilized up to some extent. *B. licheniformis*, *B. stearothermophilus* and *B. amyloliquefaciens* leads to the production of α-Amylases (Konsoula and Liakopoulou-Kyriakides 2007; Muwalia et al. 2014) and *Penicillium expansum* MT-1 produce the enzyme by solid state fermentation. *Eriobotrya japonica* Lindley (Loquat) kernels were also used for the growth of fungi as a substrate (Balkan and Ertan 2007). Other species used for the commercial production of this enzyme are *Aspergillus oryzae*, *A. niger and A. awamori* (Konsoula and Liakopoulou-Kyriakides 2007). In 2005, Sohail et al., isolated *Bacillus* sp. and *Aspergillus* sp. as the most active amylase producers showed maximum activity at slightly elevated temperature and at alkaline pH while one of the fungal enzymes retained most of its activity even at a temperature of 80 °C. In India, 71 isolates were isolated and were identified as *Klebsiella* sp., *Micrococcus* sp., *Bacillus* sp., *Staphylococcus* sp., *Enterobacter* sp., *Citrobacter* sp., *Neisseria* sp., *Pseudomonas* sp. WL-2 isolate was selected as best amylase producer and identified as *Staphylococcus aureus* and shown 24,000 U of amylase production. Enzyme showed an optimum activity at pH 6.5 and highly stable at optimum temperature at 7.5 (Table 9.1).

9.3.2 Microorganisms Involved in Production of Cellulases

Both bacteria (e.g. *Bacillus*, Bacteriodes, *Cellulomonas*, *Clostridium*, *Streptomyces*) and fungi (e.g. *Phanerochaete chrysosporium*), *Tricoderma reesei*, *Aspergillus niger*, *Gracibacillus* species, *Penicillium oxalicum* can produce cellulase (Szabo et al. 1996;

Table 9.1 Important amylase producing micro-organisms

Source	Type of enzyme	pH/stability and other stability	References
Aspergillus, *Penicillium* and *Mucor* spp.	α-Amylase	pH 8, 30 °C	Chandel et al. (2013)
Bacillus aerius GC6 *Bacillus sonorensis* GV2	α-Amylase	pH 9.0, 50 °C	Vyas and Sharma (2015)
Bacillus axarquiensis P6	α-Amylase	pH 8.0, 30 °C, 72 h	Sharma (2017)
B. amyloliquefaciens SH8	Amylase	pH 7, 30 °C	Kumar et al. (2015)

Sukumaran et al. 2009a, b; Huang et al. 2015). Many types of substrates have been used for the production of cellulase. fungi which produce the cellulase enzyme are *Sclerotium rolfsii*, *P. chrysosporium* and species of *Trichoderma*, *Aspergillus*, *Schizophyllum* and *Penicillium* (Fan et al., 1987). From the Fungus genra, *Trichoderma* and *Aspergillus* has been most extensively used for cellulase production (Sun and Cheng 2002). Mainly the fungal strains secrete the high amount of cellulase as compared to the bacterial strains, as the *Trichoderma* being the leader. Most of the commercially used cellulase are mesophilic enzymes, which are used by the filamentous fungus *Trichoderma reesei* and *Aspergillus niger* (Jeoh et al. 2007). In 2009, Odeniyi et al. isolated the *Bacillus coagulans* strain from palm fruit husk and also tested its ability to hydrolyse plan structural polysaccharides through the depolymerising activities of carboxymethylcellulase and polygalacturonase. An actinomycetes, *Streptomyces* sp. reported to produce cellulase was isolated decayed fruit waste. *Streptomyces* sp. S7 produced the cellulase enzyme on cellulose agar medium after 4 days of incubation at 28 °C. Maximum enzyme production was reported at pH 5 and temperature 40 °C in a medium having fruit waste as carbon source. Rahna and Ambili, 2011, reported *Streptomyces* sp. *S7* as a powerful cellulase producer strain. Chandrakant and Bisaria (1998) produced cellulase by using the corn syrup as a substrate and *T. reesei*; whereas in 2015 Jing et al. used hydrolyzed sugarcane bagasse residue as substrate for cellobiohydrolase production using *P. oxalicum*; Vijayaraghavan et al. (2016) reported production of carboxymethyl cellulase from cow dung by using *Bacillus halodurans* ID 18. By using the cheap source of lignocellulosic biomass substrates for enzyme production can greatly reduce the cost of production of cellulase. Wheat bran has been reported to be an effective substrate for the preparation of cellulases using *T. reesei* and *A. niger* (Sukumaran et al. 2009a, b) (Table 9.2).

Table 9.2 List of some of microorganisms producing cellulase

Microorganisms	Cellulase	References
Myceliopthora thermophila SH1	35.32 U/g	Sharma and Chand (2013)
A. niger and F. oxysporum	787.89 U/g	Kaushal et al. (2014)
B. coagulans B30, *P. mucilaginous B5 and Bacillus* sp. *B21*	6.9 IU/g (FPase) and 23.76 IU/g (CMCase)	Kaushal et al. (2014)
Alternaria brassicicola	106.93 U/ml	Deep et al. (2014)
S*erratia quinivorans* A5-2 and *Serratia quinivorans* B8		Kumar et al. (2015)
Aspergillus niger F7	2201 ± 23.91 U/g	Sharma et al. (2012)
Bacillus stratosphericus $N12_M$	2.02 IU/ml	Sharma and Chand (2013)
Bacillus axarquiensis P6	2.01 IU/ml	Sharma (2017)

9.3.3 Producing Microorganisms of Xylanase

A large number of microorganisms, including bacteria, fungi, actinomycetes and yeasts have been reported to produce xylanase (Archana and Satanarayan 1997; Lemos et al. 2000). A complete set of xylanolytic enzyme systems have been found to be of widespread among fungi and bacteria (Elegir et al. 1994; Kulkarni et al. 1999). Mesophilic genra of fungi, which include *Aspergillus* and *Trichoderma* are predominant in xylanase production and among thermophilic fungi, it include *Chaetomium thermophile*, *Humicola insolens*, *Humicola lanuginosa*, *Humicola grisea*, *Melanocarpus albomyces*, *Paecylomyces variotii*, *Talaromyces byssochlamydoides*, *Talaromyces emersonii*, *Thermomyces lanuginosus* and *Thermoascus aurantiacus.* The xylanases produced from these group of fungi have optimum temperatures of 60 and 80 °C. Sanghi et al. (2007) reported that the alkalophilic *Bacillus subtilis* ASH produced high levels of xylanase by the use of easily available agricultural waste residues such as wheat bran, wheat straw, rice husk, sawdust, gram bran, groundnut and maize bran in solid-state fermentation (SSF). Production of xylanase from *Bacillus megaterium* can be enhanced by solid state fermentation (Sindhu et al. 2006). Li et al. (2013) produced xylanase by *Aspergillus niger* LPB 326 cultivated on lignocellulosic substrate composed by sugarcane bagasse and soyabean meal in solid state fermentation (Table 9.3).

9.3.4 Pectinase Producing Microorganisms

A large number of microorganisms, such as bacteria, fungi, actinomycetes and yeast produce the pectinase enzyme (Takao et al. 2000; Kapoor et al. 2000; Hayashi et al. 1997; Patil and Dayanand 2006; Blanco et al. 1999). The primary source of industrial

Table 9.3 List of xylanase producing microorganisms

Microroganisms	Xylanase	Cultivation condition	References
Alternaria alternate	23.71 IU/ml	pH 5.5, 30 °C, 7 days	Mielenz (2001)
Paenibacillus sp. *N1*	52.30 IU/ml	3rd day, pH 9.0, 50 °C	Kumar et al. (2015)
Myceliopthora thermophila SH1	203.20 U/g	Vogels medium, pH 5.5,7 day	Sharma and Chand (2013)
Geotrichum sp. *F3*	112.890 U/g	Nakamura medium, 4.0, 55 °C, 10%, substrate concentration 2.25 %	Sharma et al. (2012)
Bacillus tequilensis SH8	41.30 IU/ml	Basal salt medium at 96 h, pH 5.5, temperature 45 °C, inoculums size 10%, carbon source-wheat bran (1.25%)	Kumar et al. (2013)
Pseudomonas sp. *XPB-6*	65 IU/ml	0.5% meat extract, 30 °C, 7.0	Sharma and Chand (2013)
Bacillus axarquiensis P6		30 °C, pH 5.0, Bacillus xylose salt medium	Sharma (2017)
Bacillus altitudinis Kd_1(M)		TGY medium, 30 °C, pH 5.5	Sharma (2017)
Rhizopus delemar F_2		30 °C, 6 days	Kumar et al. (2015)

enzyme are microorganism; 50% from fungi and yeast, 35% from bacteria, while the remaining 15% are either of plant or animal origin. Filamentous microorganisms are most widely used in submerged and solid-state fermentation for pectinase production. The capacity of this type of microbes to colonize the substrate by apical growth and by means of penetration it gives them a ecological advantage over the non-motile bacteria and yeast, which are less able to multiply and colonize on low moisture content (Kapoor et al. 2000). From filamentous fungi genra, three classes namely, phycomycetes such as genera *Rhizopus*, the ascomycetes genera *Aspergillus* and *Basidiomycetes* especially the white and rot fungi have gained the most practical importance in SSF Bacteria and yeasts mainly grows on the solid substrate containing moisture levels in the range of 40–70% ((Young et al 1983). The most commonly used bacteria are; *Bacillus licheniformis*, *Aeromonas cavi* and *Lactobacillus and* yeasts are *Saccharomyces* and *Candida.* In Solid-state fermentation, higher pectinase production has been found by *Aspergillus* strains than in submerged process (Solís-Pereira et al. 1993). *Penicillium janthinellum* sw09 strain isolated from soil produce a significant amount of pectinase, characterized mainly as exo-polygalacturonase (exo-PG) (Hayashi et al. 1997) (Table 9.4).

Table 9.4 Microorganisms secreting pectinase

Source	Type of enzyme	pH/stability and other stability	References
Aspergillus niger	Pectin methyl esterase	pH 3.5, 50 °C	Joshi et al. (2011)
Aspergillus fumigatus (ITCC 6915).	Polygalacturonase	72 h, 30 °C, 2.5% pectin	Gupta and Lakhanpal (2013)
Aspergillus niger	Exopolygalacturonase	30 °C, 15% inoculum size, citus fruit peel	Kapoor et al. (2000)
Aspergillus foetidus MTCC 151 (pectinase)	Pectinase	72 h, 30 °C	Chatanta et al. (2014)
Stenotrophomonas maltophilia P9	Pectinase	72 h, 30 °C	Sharma (2017)
Brevibacillus parabrevis C_1 *Streptomyces violaceoruber*	Pectinase		Handa et al. (2016)

Table 9.5 Microorganisms producing lipase

Source	Type of enzyme	References
Bacillus methylotrophicus PS3	Lipase	Sharma (2017)
Bacillus halotolerans PS4	Lipase	Sharma (2017)

9.3.5 Lipase Producing Microorganisms

Lipases are being produced by many microorganisms and higher eukaryotes. Most commonly used lipases are of microbial origin. Lipases of bacterial and fungal origin most commonly used in biodiesel production are *Aspergillus niger*, *Candida antarctica*, *Candida rugosa*, *Chromobacterium viscosum*, *Mucor miehei*, *Pseudomonas cepacia*, *Pseudomonas fluorescens*, *Photobacterium lipolyticum*, *Rhizopus oryzae*, *Streptomyces* sp. and *Thermomyces lanuginose* (Yahya et al. 1998). Most commonly used microorganism for lipase production is yeast, which is named as *Candida rugosa* (Freire and Castilho 2001). Recently in 2012, Cho et al. reported *Streptomyces* sp. as a potential source for lipase production for biodiesel production (Table 9.5).

9.4 Enzymatic Hydrolysis of Different Substrates for Clean Sugar Production

Traditionally obtained enzymes of industrial interest from submerged fermentation (SmF) are easy to handle and control. For any industrial enzyme production, inexpensive substrate and efficient process of fermentation are greatly required for its commercial viability. SSF is defined as the cultivation of microorganisms on moist solid supports, either on inert carriers or on insoluble substrates that can, in addition, be used as carbon and energy source. Solid-state fermentation (SSF) is found advantageous over the submerged fermentation (SmF), reason being the use of small volume of solvent which is required for product recovery, which results in the higher production per unit, low contamination and foaming problems (Van and Pletschke 2012). Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully selected micro organisms in closed vessels containing a rich broth of nutrients and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution. Enzymatic hydrolysis requires enzymes to hydrolyse the feedstocks into fermentable sugars. Three types of enzymes that are commonly used for cellulose breakdown such as endo-β—1,4-glucanases, cellobiohydrolases and β-glucosidases. The factors affecting the cellulase enzyme activity are the concentration and its source. Degradation of cellulose tales place to the formation of reducing sugars under mild conditions having pH 4.8–5.0 and temperature 45–50 °C. Enzymatic hydrolysis's efficiency is greatly affected by conditions like temperature, time, pH, enzyme loading and substrate concentration (Van and Pletschke 2012). The amount of fermentable sugar obtained increases as the enzyme load increases while cellulose load decreases. The limitation of using enzymes in hydrolysis is because they are too expensive for the economical production of ethanol from biomass. Enzymatic hydrolysis include the utilization of enzymes to produce the fermentable sugars from the biomass. Enzymatic saccharification processes include the utilization of cellulases, amylases and glucoamylases. Enzymatic hydrolysis is reported to have many advantages over chemical hydrolysis, like as lower equipment costs. Shanavas et al. (2011) reported Spezyme (a highly powerful α-amylase) as a liquefying enzyme treatment followed by saccharification and fermentation of cassava starch (10% w/v slurry concentration) was the best process strategy. Muktham et al. (2016) obtained 558 g ethanol per kg cassava starch within 48.5 h of duration using Stargen enzyme (granular starch hydrolyzing enzyme) at 1:100 w/w ratio of the enzyme to cassava starch and dried baker's yeast as fermenting organism at 30 °C. Liquefied cassava flour at temperature 80 °C for 90 min using α-amylase and β-glucanase subjected to SSF at 30 °C with simultaneous addition of glucoamylase and active dry yeast. The ethanol content achieved at lab and pilot scale were 17.2% (v/v) and 16.5% (v/v) corresponding to 86.1% and 83.6% of the theoretical ethanol yield, respectively (Zhang et al. 2011). Moshi et al. (2015) reported an ethanol titer of 33 gL^{-1} corresponding to 85% of the theoretical ethanol yield from α-amylase and β-glucanase treated cassava subjected to fed-batch

fermentation under high hydrogen pressure using a thermoanaerobe, *Caloramator boliviensis* at 60 °C. The microwave pretreatment followed by enzymatic hydrolysis showed maximum reducing sugar yield of 64.27% in the mixed fruit pulps, followed by the banana fruit pulp (57.58%). The banana fruit peels also yielded a maximum reducing sugar content of 36.67% where as the lowest of 31.29% was observed in mango fruit peels. The fermentation of the DAPhydrolysate of mixed fruit pulps showed maximum ethanol production of 35.86% corresponding to a fermentation efficiency of 70.31% at 48 h of incubation (Arumugam and Manikandan 2011).

Wheat straw used in this study contained 388 ± 05% cellulose and 310 ± 03% hemicellulose. The effects of temperature (160–240 °C, 5 min) and duration (5–20 min at 200 °C) of microwave pretreatment of wheat straw (8.6%, w/v, in water) on its enzymatic saccharification to fermentable sugars were evaluated by Saha and Cotta (2011). The yield of monomeric sugars from microwave (200 °C, 10 min) pretreated wheat straw (8.6%, w/v, in water) after enzymatic saccharification (45 °C, pH 5.0, 120 h) using a cocktail of 3 commercial enzyme preparations (cellulase, glucosidase, and hemicellulase) at the dose level of 0.15 ml of each enzyme preparation per g wheat straw was 544 ± 7 mg/g straw (glucose, 320 ± 14 mg; xylose, 189 ± 7 mg; arabinose, 21 ± 1 mg; galactose, 10 ± 0 mg; 70% yield). Pretreatment of BPS was performed at different alkali concentration, liquid-solid ratio, temperature and microwave exposure time. Enzymatic hydrolysis of pretreated BPS was done at constant cellulase enzyme loading and yield of reducing sugars (YRS) with respect to time was observed. It was found that when BPS was pretreated by 10% NaOH with 4:1 liquid to solid ratio at 90 °C for 8 min, the yield of reducing sugars reached 84% by enzymatic hydrolysis of 110 h with cellulase enzyme loading of 30 FPU/g of solid (Yuping et al. 2016). The agricultural residues, wheat bran and rice hulls, were used as substrates for cellulase production with *Trichoderma* sp. 3.2942 by solid-state fermentation, substrates pretreated by 450 W microwave for 3 min. The maximum filter paper activity, carboximethylcellulase (CMC)ase, and RSC were increased by 35.2, 21.4, and 13%, respectively (Zhang et al. 2010).

Choi et al. (2013) explored the effects of two different commercial enzymes (including amylase from *B. licheniformis* and glucoamylases from *Aspergillus niger*) on the bioethanol conversion efficiency of *Chlamydomonas reinhardtii* biomass with a carbohydrate content of about 59.7% dry weight base. The results showed that when algal biomass was hydrolyzed at pH 4.5 and 55 °C for 30 min, better sugar conversion of 0.57 g sugar/g algal biomass was obtained Trivedi et al. (2013) studied the potential of green seaweed *Ulva* as a feedstock for production of ethanol following enzymatic hydrolysis. Among the different cellulases investigated for efficient saccharification, cellulase 22,119 showed the highest conversion efficiency of biomass into reducing sugars than Viscozyme L, Cellulase 22,086 and 22,128. Pre-heat treatment of biomass in aqueous medium at 120 °C for 1 h followed by incubation in 2% (v/v) enzyme for 36 h at 45 °C gave a maximum yield of sugar 206.82 ± 14.96 mg/g. The fermentation of hydrolysate gave ethanol yield of 0.45 g/g reducing sugar accounting for 88.2% conversion efficiency. Ghazal et al. (2016) studied a green seaweed *Ulva fasciata* was selected as a potential feedstock for cellulase hydrolysis for the aim of producing bioethanol and the remaining treated algae were subjected to lipid

extraction for biodiesel production. Five marine-derived fungal strains (*Aspergillus niger*, *Aspergillus flavus*, *Penicillium oryzae*, *Penicillium chrysogenum*, and *Rhizopus oryzae*) were screened to produce cellulase for breaking down the algal cell wall. Moreover, commercial cellulase (CMCase; EC 3.2.1.4) also applied for algal cell wall hydrolysis and its efficiency was compared to the *Aspergillus niger* crude enzyme. Cellulase produced from the marine fungus *Cladosporium sphaerospermum* through solid state fermentation (SSF) was investigated for its saccharification potential of seaweed biomass using the common green seaweed *Ulva fasciata*. The seaweed substrate, containing inoculated fungus with 60% moisture content, cultured at 25 °C and pH 4 for four days, showed optimum enzyme production. The enzyme, assayed for carboxymethyl cellulase and filter paper assay, showed an activity of 10.20 ± 0.40 U/g and 9.60 ± 0.64 U/g on a dry weight basis, respectively. The hydrolysis of *U. fasciata* feedstock with enzyme (10 U/g) for 24 h at 40 °C and pH 4 gave maximum yield of sugar 112 ± 10 mg/g dry weight(Trivedi et al. 2015).

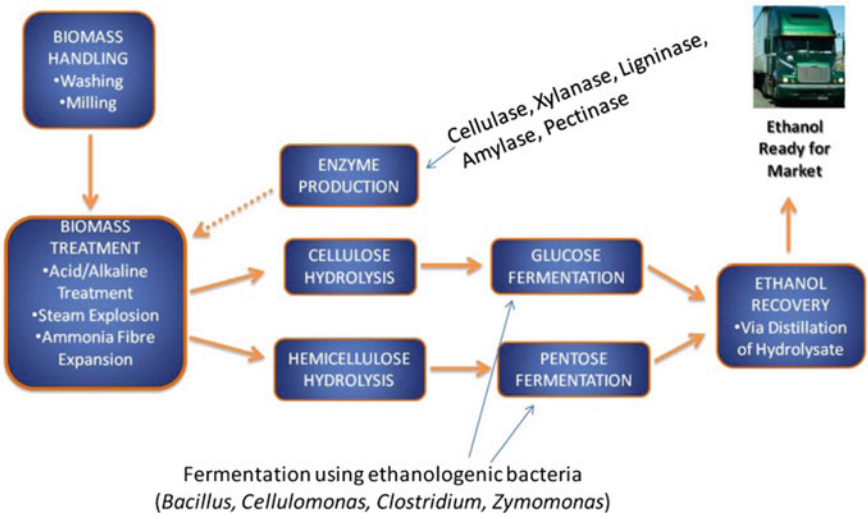
9.5 Fermentation

The pretreatment of any biomass is crucial before enzymatic hydrolysis, after the hydrolysis the sugars have to be fermented to ethanol and hydrolysate now contains various hexoses, mainly glucose and pentoses, mainly xylose which can be easily utilized by the fermenting microbes for ethanol production. The saccharified biomass is used for fermentation by several microorganisms. For these reasons, ideal organisms for fermentation of substrate to bioethanol must have certain features: High ethanol yield and productivity, High ethanol tolerance, Broad range of substrate utilization (both pentoses and hexoses, even in the presence of glucose), Withstand inhibitory products, Oxygen tolerance, Low fermentation pH, High shear tolerance (Chandrakant and Bisaria 1998, Taherzadeh and Karimi 2007).

Chemical equation below shows the glucose conversion to ethanol with the help of zymase respectively:

$$C_6H_{12}O_6 + \text{Zymase} \rightarrow 2C_2H_5OH + 2CO_2$$

Some microorganisms like *Saccharomyces cerevisiae*, *Kluyveromyces. Pichia kudriavzevii*, *Escherichia coli*, *Klebsiella oxytoca*, *Clostridium thermocellum*, *Zymomonas mobilis* have benn extensively studied for bioethanol production. But *S. cerevisiae* is most common choice for fermentation due to its greater efficiency and tolerance to high levels of alcohol and also holds the GRAS status. Zymase, an enzyme complex catalyzes the fermentation of sugars into ethanol and carbon dioxide and found generally in yeasts, but activity of zymase varies among different yeast strain however only utilize glucose, sucrose and fructose (Lin and Tanaka 2006).



9.5.1 *Separate Hydrolysis and Fermentation (SHF) and Simultaneous Saccharification and Fermentation (SSF)*

Fermentation is generally carried out in two ways: Separate hydrolysis and fermentation (SHF) and Simultaneous saccharification and fermentation (SSF). In Separate hydrolysis and fermentation (SHF), enzymatic hydrolysis is performed separate from fermentation (Devarapalli and Atiyeh 2015). SHF offers various benefits like ability of enzyme to work at higher temperature, optimization of utilization of sugars (Menetrez 2014). In SSF, the enzymatic hydrolysis and fermentation can be performed separately. Simultaneous saccharification and fermentation (SSF) of pretreated feedstock is an excellent choice for process integration. It offers certain advantages over separate hydro-lysis and fermentation (SHF) in the production of ethanol from substrate (Alfani et al. 2012; Ohgren et al. 2013; Olofsson et al. 2008; Tomas-Pejo et al. 2008).

9.6 Market Landscape for Different Enzymes

Many enzymes are being used in different steps for producing the biofuel from feedstock of biomass. Amylase enzymes are utilized for the production of ethanol, which is starch based. Similarly for the production of cellulosic ethanol, cellulase and xylanase enzymes are used and lipase enzymes are also used for the production of biodiesel. Cellulase, amylase, and xylanase enzymes are utilized in biogas pro-

duction. Other types of enzymes like protease and lysomax have their application in many processes such as distillation, hydrolysis, and oil degumming during biofuel production. Biofuel enzyme production has increased along with biofuel growth. In case of amylase growth, the demand of the fuel ethanol is the main driving force and its market is expected to grow at 9.4% CAGR with revenue of $408.3 million by 2020. The total revenue of cellulase market is $169.1 million in 2013 and will reach up to $205.3 million in 2014. With the use of advance enzymes and improved processing technology, the market has showed the healthy growth in previous years. The market is projected to reach a value of $365.6 million with a CAGR rise of 11.5% by 2020. The market of xylanase had revenue of $92.3 million in 2013, which rose to $110.9 million in 2014 and its demand is growing with the increased cellulosic ethanol production. This market will reach up to the revenue of $177.2 million by 2020 with CAGR of 9.1%. Market of lipase enzyme reached from $7.9 million in 2013 to $26.4 million in 2015. Lipase market is growing rapidly in Europe as biodiesel is the major alternative fuel used in that region. With a significant CAGR of 16.2%, the lipase market is expected to reach $56 million by 2020. Other enzymes like protease, acyl transferase etc. had revenue of $30.3 million in 2013. This market depends on the biofuel market (http://blog.bccresearch.com/biofuel-enzymes-market-surge-spurred-by-second-gen-feedstock-enzymes).

9.7 Limitations of Enzymes for Sustainable Fuel Production

9.7.1 Enzyme Cost

Pretreated biomass is deconstructed with mixtures of enzymes. For making enzymes cost-effectively, the cost of enzyme should be $0.10 per gallon of biofuel (NREL estimate). During previous 15 years, intense research on enzyme production have provided us with fungal enzyme mixtures that do not meet these cost requirements and in fact also require a huge infrastructure for production. Other research efforts are in multifunctional enzymes and combined bioprocessing organisms, the latter of which can decompose plant polymers as well as ferment them into biofuels (http://www.biofuelsdigest.com/bdigest/2016/06/06/catalysts-and-enzymes-in-biofuel-production/).

9.7.2 Enzyme Recycling

As the biomass degrading enzymes are costlier, so they should ne recycled for reducing the processing cost (Jin et al. 2012; Weiss et al. 2013). But there are some limitations of this method. Many accessory enzymes lack CBMs (βG, xylanase, xylosidase, etc.) with their action on soluble substrate. Sometime, enzymes having

cellulose binding modules (CMBs) reabsorb from substrate after a fixed period of time (Gao et al. 2011a). Moreover, the enzymes can be deactivated due to thermal denaturation or shear stress. Sometime after the hydrolysis process their activity also get lost after hydrolysis. Another method for recycling of the enzyme include immobilized enzymes on nanoparticles or polymeric matrices, ion exchange adsorption and ultrafiltration (Ansari and Husain 2012; Mackenzie and Francis 2013; Wu et al. 2010; Qi et al. 2011)

9.7.3 Unproductive Oligosaccharide Production

After enzymatic hydrolysis, 15–25% of the released sugars are in the form of gluco- and xylo-oligosaccharides. With the increase in the solid loading, concentration of oligomeric sugar also increases due to inhibition from high concentrations of monomeric sugar and degradation products. In the process of pretreatment which donot solubilize hemicellulose (e.g., dilute ammonia pretreatment, AFEX, etc.), more xylo-oligomers are present in comparison to the gluco-oligomers. These oligomeric sugars are treated as unproductive as most of the microbes used in the fermentation can consume only monomeric sugars (Bowman et al. 2012).

9.7.4 Enzymatic Hydrolysis Time

Time required for the completion of hydrolyse biomass into monomeric sugars highly depends on many factors like, lignin content of biomass, pretreatment effectiveness, cellulose crystallinity, substrate concentration, and enzyme activity (Zeng et al. 2014, Jeoh et al. 2007; Nguyen et al. 2014). For reducing this problem of long timing of hydrolysis process, a new approach is developed where biomass is initially hydrolyzed for 24 h. After this process, sugars are removed and fermented separately, whereas residual solids desire more hydrolysis time, with they are added to fresh pretreated substrate in the same tank along with fresh enzymes for further hydrolysis. This technique is greatly helpful in reducing the biomass to sugar processing time (Jin et al. 2012).

9.8 Conclusion

This chapter highlights the applications of different enzymes in sectors of sustainable fuels (ethanol, biodiesel) and their production strategies from different potential microbes to commercialize the biofuel production technology (pretreatment, hydrolysis, microbial fermentation, and biofuel separation). The ongoing progress and interest in enzymes provide further success in areas of biofuels. Big corporations

and companies that have capital to align different novel process technologies for enzymatic hydrolysis, still have several separation and purification challenges to overcome. The choice of enzymes and biomass could be decided based on the availability of sufficient quantity of catalyst and feedstock in that region. Once appropriate feedstock and enzymes are combined to produce cheap and clean sugars. Furthermore, in order to compete with the cost of petroleum fuels, the cost of biofuel processing should be kept as low as possible using energy efficient technologies and using less water which could be possible due to application of enzymes. Producing enzymes as many as possible will help to reduce the cost of biofuel production. Many future investigations will use combinations of engineered and de novo designed enzymes coupled with chemistry to generate more (and most likely new) cheaper (and renewable) resources, which will consequently contribute to establishing a bio-based economy and achieving low carbon green growth. Due to the topics discussed in this paper, it is anticipated that there may be a considerable improvement in production of sustainable fuels from different feed stocks.

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Chapter 10
The Realm of Lipases in Biodiesel Production

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Abstract Lipases are the enzymes known for the hydrolytic activity on carboxylic fatty ester bonds. The industrial interest in lipases is due to their application in a wide array of products: in detergents and cleaning products, in pharmaceutical applications, in the food industry, and on the production of biodiesel. Biodiesel, i.e. short-chain-acyl fatty ester, is mainly produced via the transesterification of fatty-acyl glycerides or esterification of fatty acids, both reactions with a short chain alcohol. Lipases can catalyze both said reactions with high specificity, producing biodiesel at high yields at low temperature. With the significant advances in biodiesel production over the last decades, coupled with a strong industrial partnership, the costs of utilizing lipases as catalysts have dropped significantly. The production of lipases became popularized in the industry due to advances not only in the reaction mechanisms, and in better understanding of lipase-producing microorganisms, but to cost-effective utilization practices. Immobilization is the practice responsible for the initial breakthrough innovation that allowed efficient reutilization of lipases, thus reducing the cost per batch. There was, and still there is, numerous advances in the development of immobilizing matrices and novel utilization pathways of immobilized enzymes available in the literature. More recently, other methods of using lipase in biodiesel production have been developed, e.g. via the utilization of whole-cell and fermented solid with lypolytic activity, and by the use of lipase in liquid formulations. Over the last years, there has been an increased interest in developing next-generation biodiesel, i.e., the one produced from alternative lipid feedstock, such as microbial and residual lipids, and by utilizing ethanol as acyl agent, instead of methanol. There has also been prominent advances in the reactor engineering aspect of lipase-derived biodiesel, by promoting more efficient batch processes, and the development of lower-cost continuous processing. The present chapter reviews the recent literature

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in the important field of using lipases in biodiesel production, and critically describes the opportunities and challenges present in such applications.

Keywords Lipase · Biodiesel · Immobilization · Transesterification
Hydro esterification · Batch and continuous processes

10.1 Introduction

Lipases (glycerol ester hydrolases EC 3.1.1.3) are part of the family of hydrolases that act on the carboxylic ester chain and do not require any cofactors. Under conditions in which the availability of water in the medium is reduced, most lipases are capable of catalyzing reverse reactions such as esterification and interesterification (transesterification, alcoholysis and acidolysis), as well as the hydrolysis of triacylglycerols, among others (Hasan et al. 2009). The behavior of the induced-fit type of these enzymes makes it possible to convert a significant variety of artificial substrates, which often do not have naturally common structures-triacylglycerols (Faber 2011). Thus, lipases are among the most important biocatalysts used in chemical reactions in both aqueous and non-aqueous media. The reason of their importance is mainly due to their ability to utilize a broad spectrum of substrates, in addition to their robustness to operate within a wide range of temperature, pH and organic solvents, and their chemo, regio and enantioselectivities (De Castro et al. 2004; Faber 2011).

Daiha et al. (2015) analyzed the number of publications and patents related to the use of lipases based on some industrial sectors at different stages of development and with different technological levels. According to this publication, the use of lipases as biocatalysts has remained relevant to the industrial segment since the discovery of its potential with a projected increase in world demand of 6.2% per year, reaching USD 345 million in 2017. Among the possible applications of these robust biocatalysts, the enzymatic production of biodiesel is the one with the highest number of publications and records, including patents and scientific articles. Such data are also in agreement with other surveys that evaluated the different types of catalysts used in biodiesel synthesis. According to Pinto et al. (2005) and Quintella et al. (2009), the use of lipase as biocatalyst numerically exceeds the sum of the articles and patents referring to obtaining this fuel by all other types of catalysts.

Biodiesel is defined as the mono-alkyl ester derivative of long-chain fatty acids from lipid feedstocks (oils and fats). The renewability of biodiesel is associated with the replacement of fossil fuels in compression ignition engines or diesel engines (Knothe et al. 2010). The most widely used biodiesel production route in the industry is based on the alcoholysis reaction, also known as transesterification, of a lipid material with a short chain alcohol (e.g. methanol and ethanol). This reaction is considered the most industrially accepted route because it yields a product (biodiesel) with characteristics such as viscosity and cetane number close to those of diesel. The oils and fats when subjected to the transesterification process have their viscosity

values decreased significantly, so that the fuel obtained can be burned directly in diesel engines without the need for adaptation or modification of traditional diesel engines (Knothe et al. 2010). In order to obtain a high-quality biofuel, some technical characteristics are essential, such as: the transesterification reaction must be complete reflecting the total absence of remaining fatty acids, and the biodiesel produced must be of high purity, not containing traces of residual glycerin or excess alcohol from the transesterification reaction (Knothe et al. 2010).

Like any other enzyme-mediated reaction, lipase-catalyzed biodiesel production has a number of advantages over the chemical reaction, mainly due to the specificity and selectivity of the biomolecule, which makes the process less energy-intensive with respect to the raw materials and the reaction conditions (Gog et al. 2012; Meunier et al. 2017). In addition to high selectivity, lipases do not form soap as a by-product, and can be esterified free fatty acids and reused in more than one reaction cycle. The reaction requires little to no heat, since it occurs under mild pressure and temperature conditions, and it does not require costly purification costs (Gog et al. 2012; Christopher et al. 2014). At the end of the process, glycerol (lower phase) is separated from the biofuel (upper phase) by simple decantation. Deodorization and neutralization of the final product is usually not required as well (Ranganathan et al. 2008). The enzyme transesterification is applicable to crude and refined vegetable oils, fats, tallow and other fat residues, and various alcohols, such as methanol, ethanol, propanol, isopropanol, butanol, and isobutanol (Ranganathan et al. 2008; Gog et al. 2012; Christopher et al. 2014). The free fatty acids present in the oil are esterified and do not require purification steps of the raw material, therefore, oils containing triacylglycerols and free fatty acids are enzymatically converted into biodiesel because the lipases catalyze the transesterification and esterification simultaneously (Ranganathan et al. 2008; Gog et al. 2012; Meunier et al. 2017). The addition of organic solvent *(tert*-butanol, hexane, *n*-heptane, chloroform, 1,4-dioxane, isooctane) in the reaction medium can assist in solubility between the alcohol and the oil, facilitating mass transfer and enzymatic catalysis. Addition of solvent can also minimize the possible inhibitory effect of alcohol on lipase (Iso et al. 2001; Fu and Vasudevan 2009).

The search for enzymatic catalysts that promote reactions that can act competitively with the well-established chemical pathway has led increasingly to the establishment of new forms of lipase presentation, which are mostly characterized as being free, i.e. soluble, immobilized, bound to the mycelium, i.e. whole cells, or in the form of fermented solids with lipolytic activity. In this context, there is a consensus that obtaining more stable biocatalysts with properties that allow their reuse for several cycles, with the effective maintenance of the catalytic activity is the key to development of the activities in the field. In the case of the enzyme used in the soluble form, the search for solutions for this type of technology arises with the development of genetic engineering and cultivation techniques. Such advances have contributed to the reduction of the cost of liquid lipase, which allows the enzyme to be used only once, with results comparable to the conventional process, but more economically feasible (Zeng et al. 2017). In the vast majority of studies, besides the enzyme, there is a concern in the determination of the conditions optimized for the

reaction, with evaluation of the factors that directly interfere in the process. Thus, the literature related to the production of biodiesel by lipase is quite extensive and dynamic. The research in lipase-catalyzed biodiesel, motivated by the importance and opportunities that biofuel represents within sustainable development, is a major factor to the development of energy security alternatives and an energy grid based on renewable fuels. For these reasons, this chapter reviews the latest technologies within the area, presenting some examples, in order to highlight the great advance in related research (Fig. 10.1).

10.2 Lipase Properties for Biodiesel Synthesis

Lipases are a broad group of enzymes with several industrial applications. These biomolecules are characterized by their versatility of catalysis of hydrolysis and synthesis reactions, often in a chemo, regional and enantioselective way (Kapoor and

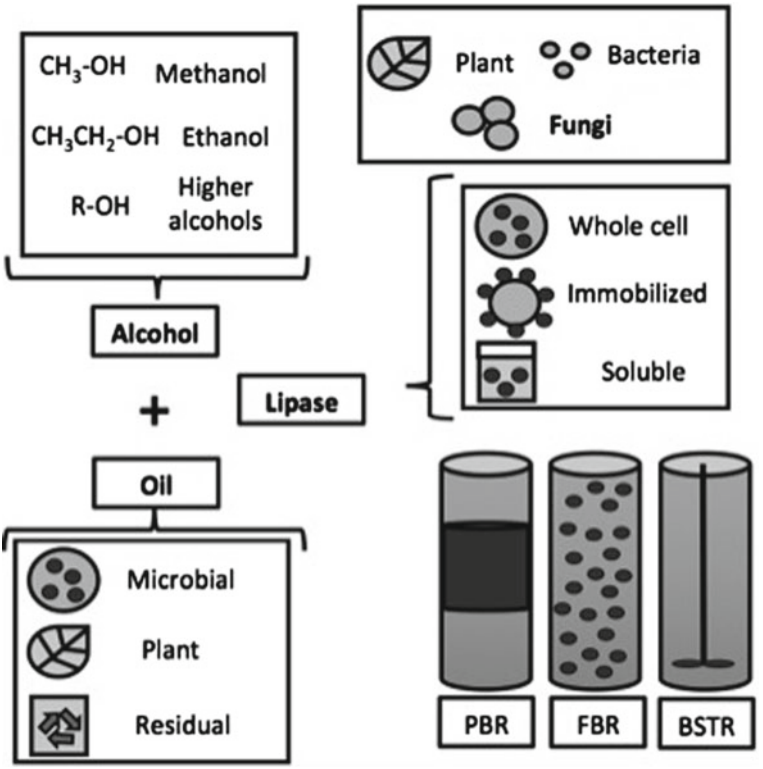

Fig. 10.1 The worldwide energy consumption for different time period (Adapted and modified from IEO 2017, an open source article)

Gupta 2012). Lipases can be found in animal and plant tissues, as well as in microorganisms (Ribeiro et al. 2011; Freire and Castilho 2008). Among the lipase-producing sources, the microbial one is the most industrially used, due to simpler isolation procedures from the fermentation broth, and by the fact that they are generally more stable and have more diversified properties than lipases from other sources (Jaeger and Reetz 1998). Lipases can be produced by bacteria, yeasts, actinomycetes and fungi, the latter being the most used in industrial processes. Microorganisms with the potential to produce lipases originate from a variety of habitats, including the marine environment, the Antarctic environment, vegetable oils and residual oils and dairy industries, contaminated soils, plants, and rotten foods. In this way, nature offers an extraordinary and potential collection of sources of microbial lipases (Cortez et al. 2017). Among the species using in the industrial scale, a few fungi belonging to the *Aspergilllus*, *Mucor*, *Rhizopus*, *Geotrichum*, *Penicillium*, and *Thermomyces* genus, as well as the *Candida* yeasts, and, *Bacillus*, *Pseudomonas* and *Burkholderia* bacteria stand out when compared to other strains (Treichel et al. 2010). With respect to the animal source of lipase, porcine pancreatic tissue is the most commonly found, mainly due to the stability of the isolated enzyme (Mendes et al. 2012). In the case of plant sources, a variety of seeds and beans from oil crops and cereals (Barros et al. 2010), as well as in latex plant tissues, e.g. from *Carica papaya* (Villeneuve 2003; Mazou et al. 2016), can be utilized as a feedstock to lipase extraction. Comparatively speaking, the use of plant lipases is much less developed than those from microbial origin, but plant-based enzymes can also be envisaged as biocatalysts for lipid bioconversions. Lipases are available in large amounts in the latex of some plant species, which can yield a relatively inexpensive source, though still underdeveloped (Mazou et al. 2016). However, the industrial biodiesel production by plant lipase is still a challenge due to the slow transesterification kinetics and lower yield when compared with microbial lipases (Cambon et al. 2009; Mounguengui et al. 2013).

Specificity is an important feature of lipases, being controlled by their molecular properties, substrate structure and by factors that affect enzyme-substrate binding (Antczak et al. 2009). Lipases can then be classified according to their positional specificity, i.e. non-specific or 1,3-specific, or according to their fatty acid specificity (Lotti and Alberghina 2007; Antczak et al. 2009). Substrate specificity consists in the ability to distinguish structural features of fatty acid chains such as the length, number, position or configuration of unsaturated bonds, the presence of branching, as well as the nature of the fatty acid chain, i.e. fatty acid, alkyl ester or glycerol ester. In the reaction of triacylglycerols and alcohols, lipases also distinguish the size and type of alcohol used in the reaction (Antczak et al. 2009; Faber 2011). The *sn*-1,3-specific lipases such as those produced by *Rhizopus oryzae* (Ban et al. 2002), *T. lanuginosus* (Nordblad et al. 2014) and *M. circinelloides* (Carvalho et al. 2015a) have been reported to efficiently catalyze transesterification of vegetable oils with conversion yields greater than 90%. Such high conversion of transesterification reactions is due to the efficient transfer from the residue acyl to specific positions on the glycerol molecule. Though being *sn*-1,3-specific enzymes, such lipases are effective for cleaving TAG fatty acids in the *sn*-1,3 positions promoting the migration of the acyl residues in the *sn*-2 position terminal (*sn*-1 and *sn*-3) in

glycerol (Antczak et al. 2009). On the other hand, the migration of the acyl group may also be influenced by the polarity of the solvent used in the transesterification reaction. According to Li et al. (2010) the reduction of solvent polarity increases the acyl group migration rate constants due to the favorable influence of the dispersion of the charge on the transition state, leading to varied yields of biodiesel.

The mechanism of lipases, unlike other enzymes, is significantly complex and dependent on certain structures typical of the biomolecule. In addition, the water content has a primary effect on its behavior, directly affecting the hydration of the enzyme or indirectly altering the nature of the reaction medium (Salis et al. 2007). Thus, the selection of suitable conditions for the performance of a lipase catalyzed reaction must follow a careful manipulation of the environment of the biocatalyst in such a way that the productivity of the system is maximized by the total potentiality of the enzyme activity (Reis et al. 2009). Using appropriate solvents and controlling the water content in the reaction medium can efficiently increase the activity levels close to their maximum potential. When water is replaced by an organic solvent, changes in the native conformation of the enzyme can occur both in the tertiary structure and in the more prominent secondary structures (α-helix and β-sheet), thus causing its destabilization. In order to ensure a catalytically active enzymatic conformation in organic media, the enzyme molecule must have a defined hydration layer, separating the solvent from contact with the surface of the protein and contributing to the increase of its internal flexibility (Klibanov 2001). Another way to protect the native configuration of the enzyme is through its immobilization on solid supports (Villeneuve et al. 2000; Hanefeld et al. 2009). Immobilization refers to the location or confinement of the enzyme. The selection of the immobilization method should be based on certain parameters, such as global activity of the immobilized derivative, regeneration and inactivation characteristics, cost of the immobilization procedure, toxicity of the immobilization reagents and desired final properties of the immobilized enzyme (Guisán 2006; De Castro et al. 2008; Hanefeld et al. 2009; Meunier et al. 2017).

The use of immobilized enzymes is known to offer several advantages compared to their use in free form (Sheldon 2007). In addition to a more convenient approach of handling the enzyme, it provides ease of separation from the reaction mixture and enables its use in repeated and continuous runs (Sheldon 2007; Adlercreutz 2013). Furthermore, immobilization is often linked to enhanced thermal stability and it is essential to perform on non-conventional medium reactions (Sheldon 2007; Adlercreutz 2013; Sheldon and Van Pelt 2013). Enzymes have been immobilized by different techniques, including adsorption, covalent attachment or entrapment (Ansari and Husain 2012; Meunier et al. 2017) on several matrixes. The factors involved on the features are directly related to the potential industrial applications, such as the required mechanical strength, chemical and physical stability, hydrophobic character, enzyme load capacity and cost for each application (Adlercreutz 2013; Es et al. 2015). In addition, the properties of supported enzyme preparations are governed by the properties of both the enzyme and the carrier material. The interaction between the two provides an immobilized enzyme with specific chemical, biochemical, mechanical and kinetic properties (Tacias-Pascacio et al. 2017). A wide

variety of natural, organic or inorganic synthetic materials with different characteristics such as size, shape, porosity, hydrophobicity and density have been tested for lipase immobilization (De Castro et al. 2008, 2010; Cazaban et al. 2017). However, comparative studies show significant differences in the performance of immobilized lipases in different substrates and show that the immobilization of lipases is still a complex challenge, since the extent of immobilization depends on the structure of the enzyme, on the immobilization method and on the type of support (De Castro et al. 2008, 2010; Tacias-Pascacio et al. 2017). In many cases, supports that provide high activity and stability of the enzyme have serious limitations of mechanical resistance and pressure drop, which make them unviable for use in some types of reactors (Yahya et al. 1998; Poppe et al. 2015a). Supports with high mechanical strength are desirable particularly in systems with agitation. The presence of solvents may require supports with high chemical resistance. The application of substrates with adequate internal geometries is quite attractive, since it reduces the diffusional limitation effects of the substrates to the active sites of the immobilized enzyme (Zanin and Moraes 2014; Poppe et al. 2015a). Considering that the loss of activity is a matter of time, the support must also be easily regenerated and reused (Yahya et al. 1998; De Castro et al. 2008).

The most recommended supports for immobilization of lipases for their subsequent use as a biocatalyst in biodiesel synthesis are hydrophobic, microporous styrene-divinylbenzene copolymer (STY-DVB) (Dizge et al. 2009), polypropylene (Bosley and Moore 1994), Sepharose (Villeneuve et al 2000), silica functionalized with organosilanes (Cazaban et al. 2017) matrices, among others. The amount of enzyme adsorbed on such supports is generally high, and an increase in adsorption is usually followed by increase in observed enzyme activities. Hydrophilic supports tend to compete with the enzyme for the water available in the reaction. When the lipase and the support are fully hydrated, the hydrophilic support leads to a higher water concentration in the environment of the enzyme favoring hydrolytic reactions (Villeneuve et al. 2000). Another benefit of the hydrophobic matrix is its ability to limit the adsorption of glycerol as byproduct formed during the transesterification reaction (Lima et al. 2015). It has become common knowledge that glycerol has a negative effect on lipase activity and stability by reducing the diffusion of the hydrophobic substrate to the active site of the lipase (Dossat et al. 1999). This undesirable effect of glycerol greatly shortens the operational stability of the catalyst and consequently influences the economic viability of the process. Since the glycerol issue could increase the production cost and affect the process design, it needs to be taken into account when immobilized lipases are used for large scale biodiesel production. Several articles and reports available in the literature deal with different lipase immobilization techniques, characterization of the activated complexes and applications in reactions in non-aqueous medium, according to the reviews published by Adlercreutz (2013), Es et al. (2015), Hanefeld et al. (2009), Villeneuve et al. (2000) and textbooks (Guisán 2006; De Castro et al. 2008; Zanin and Moraes 2014).

10.3 Production of Biodiesel Catalyzed by Lipases

Biodiesel is produced by a sequence of reversible reactions: (i) from triacylglycerol (TAG) to diacylglycerol (DAG), (ii) from DAG to monoacylglycerol (MAG), and (iii) from MAG to alkyl ester (biodiesel), generating glycerol as a co-product. At each step of reaction, an alkyl ester molecule is released. The stoichiometric ratio of the reaction corresponds to 1 mol of TAG for 3 mols of alcohol. Empirically, it has been proven that alcohol excess usually ensures the equilibrium in the direction of product formation (Knothe et al. 2010). Lee JH et al. (2013) propose that the enzymatic biodiesel production consists of three steps. The first is the rate-determining step, in which interfacial reaction occurs due to the insolubility of low-chain alcohols and oils. As the reaction progresses, the products (fatty acid alkyl esters and glycerol) act the emulsifiers and the interface disappears, becoming a homogeneous phase, which increases the reaction rate. Lastly, the glycerol concentration builds up, the alcohol moves to the glycerol layer and the rate of reaction is again decreased. The lipase-mediated transesterification reaction involves the catalytic triad of the enzyme, consisted of aspartic or glutamic acids, histidine and serine amino acids. In the first stage of the reaction, the hydroxyl group (alcohol) of the serine acts as nucleophile by the action of histidine that attracts the proton of the hydroxyl forming an oxyanion. The oxyanion of the serine attacks the carbon of a carbonyl of the substrate, forming the tetrahedral intermediate 1. Then the electrons of the oxyanion are pushed back to the carbonyl carbon, and the proton in the histidine fraction is transferred to the diacylglycerol, which is subsequently released. The formed serine ester reacts with the alcohol to complete the transesterification. The histidine nitrogen removes the hydrogen from the alcohol molecule to form the alkyl oxide anion. Such structure attacks the carbonyl carbon, and the intermediate oxyanion is stabilized by a hydrogen bond (tetrahedral intermediate 2). The following step is composed by an electron push back to the carbonyl carbon, and the free fatty acid is formed. Serine oxygen recovers the hydrogen located in the histidine to reestablish the hydrogen bond network. Aspartic acid serves to extract the positive charge of histidine during the times when it is fully protonated (Jegannathan et al. 2008).

Most of the research published in the field uses methanol as acyl group acceptor. Due to the high hydrophilicity of the C1-alcohol, the reactions are usually carried out in medium containing organic solvent, in generally within high proportions (of the order of 50–90% relative to the total mass of reagents involved). From the economic point of view, methanol also stands out as one of the cheapest alcohols. With the replacement of methanol by other alcohols with, for example, ethanol, propanol and butanol, the use of solvents becomes unnecessary, which can make the biodiesel production more feasible, reducing solvent costs and distillation steps, reducing the energy consumption (Iso et al. 2001). However, in this case, the biodiesel yield may be lower and the reaction time longer (Mittelbach 1990). The use of ethanol as acyl acceptor has increased significantly within the last decades. The production of fatty acid ethyl esters through the use of bio-ethanol provides a process with little to no dependency of fossil fuels depending on the energy requirements of the process.

According to Firdaus et al. (2014), biodiesel standards in Brazil and the USA (specification according to ASTM D6751) are applicable for both fatty acid methyl esters and fatty acid ethyl esters (FAME and FAEE, respectively). The replacement of the methyl route by ethanol in Brazil is quite attractive due to the great agricultural capacity and the already consolidated ethanol industry in the country, currently the second largest producer in the world (Reis and Hu 2017). Even considering some technical disadvantages in production (slower reaction, higher alcohol consumption and greater difficulty of separation), ethyl biodiesel has slightly higher viscosity than methyl biodiesel, promoting greater lubricity in relation to methyl biodiesel. Furthermore, FAEE usually presents lower opacity and better burning qualities than FAME, as well as requiring lower combustion temperatures, potentially reducing NO_x and CO emissions (Knothe et al. 2010; Firdaus et al. 2014). The source of lipid feedstock depends greatly on the geographic scale of production. In conditions which oils are inserted in the food chain, local policies tend to lead to the search for alternative raw materials (non-edible feedstocks) for the production of biodiesel, such as perennial crops oils (Ramos et al. 2009; Perez et al. 2014) and microbial oils (Patel et al. 2017; Talebi et al. 2013).

10.4 Main Aspects of Lipase Utilization Methods for Biodiesel Production

10.4.1 Immobilized Lipases

The first report on the enzymatic production of alkyl esters was published by Mittelbach (1990) using sunflower oil and different alcohols in the presence and absence of solvent (petroleum ether). Among the tested lipases, only immobilized enzymes (Lipase SP 382, Lipozyme® RM-IM) showed satisfactory results even in the absence of solvent, while free lipases did not provide acceptable conversions. In the following years, most biodiesel production published references employed lipase in its immobilized form. However, in the last decade efforts have been directed towards the application of lipases in their free form, which will be discussed later in this chapter. Immobilized lipases can be obtained by industrial companies, for example as the most prevalent in the current market: *Candida antarctica* lipase B (Novozym® 435) and lipase from *Thermomyces lanuginosus* (Lipozyme® TL-IM), *Rhizomucor miehei* (Lipozyme® RM-IM) and *Burkholderia cepacia* (Lipase PS-IM). Table 10.1 shows some biodiesel production data using commercial immobilized lipases.

Among the first reports, an investigation on the transesterification reaction of vegetable oils and beef tallow using primary and secondary alcohols and various lipases was reported with promising results (Nelson et al. 1996). The results with highest conversion yields were obtained from the alcoholysis of tallow oil with methanol and ethanol catalyzed by Lipozyme RM-IM®. The yields obtained with hydrated ethanol were higher than that of anhydrous ethanol. Using secondary alcohols, Novozym®

Table 10.1 Examples of biodiesel production by transesterification process catalyzed by commercial lipase immobilized

Lipase	Feedstock	Acyl acceptor	Solvent	Yield (%)	Reference
Lipase SP 382, Lipozyme® RM-IM	Sunflower oil	Primary and secundary alcohols	Petroleum ether	≥90	Mittelbach (1990)
Lipozyme IM60 (Lipozyme® RM-IM)	Beef tallow	Ethanol and isobutanol	*n*-Hexane	≥98	Nelson et al. (1996)
Novozym® 435	Mixture of soybean and rapeseed oils	Methanol	Free	≈98	Shimada et al. (1999)
Novozym® 435	Soybean oil	Metyl acetate	Free	92	Du et al. (2004)
Novozym® 435, Lipozyme® RM-IM	Palm, cashew nut, papaya, rambutan oils	Methanol	Free	≥80	Winayanuwattikun et al. (2008)
Lipozyme® TL-IM	Waste cooking oil	Methanol	*tert*-Butanol	92	Wang et al. (2008)
Novozym® 435	Waste cooking palm oil	Methanol	*tert*-Butanol	≈80	Halim et al. (2009)
Novozym® 435	Soybean oil	Methanol	*tert*-Butanol	97	Zheng et al. (2009)
Lipozyme® TL-IM	Crude palm oil	Methanol	*tert*-Butanol	≈96	Sim et al. (2010)
Novozym® 435, Lipozyme® TL-IM, Lipozyme® RM-IM	Canola oil	Methanol	Free	≈93	Yücel and Demir (2012)
Novozym® 435, Lipozyme® TL-IM, Lipase PS-IM	Andiroba, babassu, jatropha, palm oils	Ethanol	Free	≈100	Tiosso et al. (2014)
Novozym® 435	Waste frying oil	Methanol	*tert*-Butanol	>80	Azócar et al. (2014)
Novozym® 435	Microbial oil from *Mucor circinelloides*	Ethanol	Isooctane	≈93	Carvalho et al. (2015b)
Lipozyme® RM-IM	Spent coffee ground oil	Ethanol	Hexane	≈92	Caetano et al. (2017)

435 was the most efficient. The optimization of the reaction of transesterification of soybean and rapeseed oils with methanol using Novozym® 435 has also been reported (Shimada et al. 1999). The Novozym® 435-catalyzed reaction was performed with three equivalents of methanol needed for each oil equivalent, but it was noticed that addition of increased methanol molar equivalent, i.e., greater than 1.5, deactivated the enzyme at the start of the process. Therefore reactions were performed with the addition of alcohol in fed batch system, yielding an overall yield of 98.4% methyl esters. Another approach to reduce the negative effect of methanol on enzyme activity has been reported to be related to the methanol replacement for methyl acetate as acyl group acceptor, obtaining yields greater than 92% on methyl esters in a 12:

1 molar ratio of acetate to oil (Du et al. 2004). The main advantage of this process was the non-formation of glycerol as a by-product, which as previously mentioned have inhibitory effects on lipase activity.

Residual oils have also been employed with relative success as feedstocks in biodiesel synthesis. The transesterification of corn oil with methanol catalyzed by Lipozyme® TL-IM was conducted in the presence of *tert*-butanol to reduce inactivation of the biocatalyst resulting in conversion into methyl esters 92.0% after 12 h of reaction (Wang et al. 2008). The synthesis of methyl esters using waste or waste frying cooking palm oil was catalyzed by Novozym® 435 and the maximum conversion achieved was approximately 80% (Halim et al. 2009; Azócar et al. 2014). An extensive work involving the sorting of vegetable oils was carried out by Winayanuwattikun et al. (2008) using the methyl route and Novozym® 435, and Lipozyme® RM-IM lipases. Among the 27 oils investigated, only palm, cashew nut, and rambutan oils provided biodiesel samples with suitable properties to be used as biofuel. More recently, it has been reported yields of ethyl esters near 100% from non-edible vegetable oils in a solvent-free system. Better performances were obtained with PS-IM and Novozym® 435 lipases (Tiosso et al. 2014). It has been observed that samples from the biodiesel and jatropha babassu oils presented viscosity in accordance with those values predicted by the technical standards of ASTM D6751 (1.9–6.0 mm^2/s) (Tiosso et al. 2014).

Quality of the biodiesel obtained by enzymatic route depends on the reaction system to be used (type of oil and acyl acceptor), origin of the enzymatic preparation, immobilizing matrix, among others. In some cases, the process presents technical potential, but the product does not always meet the specifications set by ASTM D6751 and EN 14214. However, in most cases the immobilized system maintained satisfactory activity over several recycles with a slight decrease on the biodiesel yields. Some examples of biodiesel synthesis using lipase immobilized on different supports and procedures are presented in Table 10.2.

The physical properties of high tensile strengths silica carriers make them robust and resistant to breakage through mechanical shear in the reactor running, thus producing a product suitable for multiple reuses (Cazaban et al. 2017). Another type of support that has been the focus of researchers is silica xerogel, obtained by sol-gel technique involving hydrolysis and condensation of $Si(OR)_2$ in the presence of a trialkoxysilane (Pierre 2004; Kandimalla et al. 2006). In such kind of functionalization, a material of the type $xSiO_2 \cdot SiO_{3/2}–(CH_2)_n–L$ is obtained, in conditions in which it is possible to control the density of ligand anchored to the silica surface. This technique has been mainly used for the immobilization of lipase to present good retention of activity (Reetz et al. 1996; Meunier and Legge 2012). The combination of inorganic and organic components, constitute also an alternative approach to produce matrixes having specific features for a specific application or supports with properties that cannot be found in conventional materials (Samuneva et al. 2008; Pandey and Mishra 2011). For example, the biocompatibility of the silane precursor with tetraethoxysilane (TEOS) and polyvinyl alcohol has been successfully tested for the immobilization of different sources of lipase, such as porcine pancreatic (Paula et al. 2007), *Pseudomonas fluorescens* (Moreira et al. 2007) and *Burkholderia cepa-*

Table 10.2 Examples of biodiesel production by lipase immobilized on different supports and procedures

Lipase	Support	Immobilization method	Feedstock	Acyl acceptor	Solvent	Yield (%)	Reference
Pseudomonas fluorescens (Lipase AK)	Kaolinite particles (Toyonite 200-M)	Physical adsorption	Safflower oil	1-Propanol	Free	100	Iso et al. (2001)
Chromobacterium viscosum	Celite-545 particles	Physical adsorption	Jatropha oil	Ethanol	Free	92	Shah et al. (2004)
P. fluorescens (Lipase AK)	Epoxy SiO_2-PVA	Covalent	Palm oil	Ethanol	Free	≈98	Moreira et al. (2007)
Porcine pancreatic	SiO_2-PVA particles activated with glutaraldehyde	Covalent	Babassu oil	Ethanol, 1-propanol, 1-butanol	Free	75–95	Paula et al. (2007)
P. fluorescens (Lipase AK)	Macroporous polypropylene particles	Physical adsorption	Soybean oil	Methanol	Free	98	Salis et al. (2008)
Burkholderia cepacia (Lipase PS)	SiO_2-PVA particles activated with epichlorohydrin	Covalent	Andiroba, babassu, jatropha, macaw palm, palm oils, beef tallow	Ethanol	Free	92–100	Carvalho et al. (2013)
Rhizomucor miehei (Palatase®)	Liposome nanospheres covered by porous silica shell	Encapsulation	Triolein	Methanol	Free	≈90	Macario et al. (2013)

(continued)

Table 10.2 (continued)

Lipase	Support	Immobilization method	Feedstock	Acyl acceptor	Solvent	Yield (%)	Reference
Pseudomonas mendocina cells	Fe_3O_4-chitosan microspheres	Covalent	Soybean oil	Methanol	Free	≈87	Chen et al. (2016)
B. cepacian (Lipase PS)	Silicone microspheres	Encapsulation	Soybean oil	Ethanol	*n*-Hexane	≈95	Ma et al. (2016)
B. cepacia (Lipase PS) *Rhizopus oryzae*	PEI microcapsules modified by carbon nanotubes	Encapsulation	Soybean oil	Ethanol	Free	>90%	Su et al. (2016)
B. cepacian (Lipase PS)	Magnetic carbon nanotubes modified by PAMAM dendrimers	Covalent	Soybean oil	Methanol	Free	≈93	Fan et al. (2017)

SiO_2-PVA silica-polyvinyl alcohol; *PAMAM* polyamidoamine; *PEI* polyethyleneimine

cia (Carvalho et al. 2013). In these cases, conversions into biodiesel above 95% were found using non-edible oils as feedstocks.

Lipase from *Chromobacterium viscosum* immobilized on Celite-545 particles by physical adsorption was used as a biocatalyst in the ethanolysis of Jatropha oil resulting in a conversion of 92% (Shah et al. 2004). Also via physical adsorption process, lipase from *P. fluorescens* was immobilized on kaolinite particles and the biocatalyst was used in the transesterification of safflower oil with 1-propanol. The authors obtained 100% oil conversion after 10 h of reaction. Salis et al. (2008) performed immobilization of *P. fluorescens* on macroporous polypropylene particles. By using a biocatalyst for the transesterification of soybean oil with methanol, the authors obtained high FAME yields (98%) in 70 h. It has been reported that using octyl functionalized silica, glycerol was not absorbed on the support surface (Lima et al 2015). This proves to be an important advantage for batch and continuous use, since glycerol accumulation on the surface is a concern for mass transfer and enzymatic activities (Dossat et al. 1999; Xu et al. 2011; Costa-Silva et al. 2016).

The literature also points to a variety of innovative technologies for lipase immobilization to mediate the synthesis of biodiesel. With larger specific area, less diffusion limitation, and many other advantages, nanostructured materials (nanoparticles, nanotubes and nanofibrous membrane) have been used as novel and promise support (Shuai et al. 2017). The immobilization of both *R. oryzae* and *B. cepacia* lipases in polyethyleneimine (PEI) microcapsules was assessed by Su et al. (2016). The authors also evaluated the modification of the carbon nanotubes with microcapsules with the aim of improving the enzymatic activity by increasing the emulsion interface of oil and water to reduce toxicity of PEI on the biomolecule (reduction of positive charges on the polymer). The results showed a support with high stability and retention of the enzyme, which may be located mainly in the microcapsule wall. Evaluating other immobilization technique using lipase from *B. cepacia* as an enzyme model, carbon nanotubes with magnetic properties modified by polyamidoamine dendrimers were synthesized (Fan et al. 2017). The modification aimed at increasing the effective loading of the enzyme, among other factors. According to the authors, the immobilization technique enhanced 17 times the catalytic activity compared to free enzyme, and made it more stable to pH and temperature variations. The biocatalyst also presented catalytic activity retention of about 90% after 20 cycles and easy removal from the reaction medium by using a magnetic field.

The amphiphilic liposome composition also permits the formation of biocompatible structures, containing inside an aqueous microenvironment, which is suitable for the encapsulation of a variety of hydrophilic substances, including enzymes. For example, in their study, Macario et al. (2013) found that the immobilization of lipase on liposome nanospheres, the enzyme remained stable for five consecutive reaction cycles.

Various other alternative forms of immobilization have gained increasing attention being developed to ensure better interact with the enzyme reaction medium to increase the interfacial area required for full activity of lipase and facilitate separation of the biocatalyst from the medium. When immobilized, lipase from *B. cepacia* in silicone microspheres, Ma et al. (2016) observed an excellent thermal and mechanical

stability of the biocatalyst. In addition, the biocatalyst was recycle over 15 batch runs while maintaining biodiesel yields greater than 70%. Chen et al. (2016) produced magnetic whole cell biocatalysts constructed by immobilizing *Pseudomonas mendocina* cells into Fe_3O_4-chitosan microspheres to be applied for biodiesel production. A yield of 87.32% was obtained under optimum operating conditions (biocatalyst concentration of 10 wt%, water content of 10 wt%, 35 °C, methanol-to-oil molar ratio of 4:1 and a four-step addition of methanol) for 48 h. The biocatalyst had an excellent reusability and still gave a biodiesel yield of 83.57% after 10 cycles, which was higher than that of Fe_3O_4-uncontained whole cell biocatalysts (74.1%). Moreover, the biocatalyst could be separated and be recycled easily due to their superparamagnetism.

10.4.2 Soluble Lipases

Liquid enzyme formulations are composed of a given enzyme in liquid solution with added stabilizers to prevent denaturation of the biomolecule (for example, glycerol or sorbitol), or additives to prevent microbial growth (e.g., benzoate) (Nielsen et al. 2008). With the use of biocatalyst in soluble form, and to avoid the cost of immobilization procedure on solid supports, some limitations are reduced, such as those related to mass transfer. Furthermore, the use of the enzyme in the soluble form prevents the insertion of a third phase (solid) in the reaction system and recovery (and recycle) is based on amphiphilic property of the biomolecule. Due to being active in the oil and water interface, lipase is found concentrated in the emulsified phase between the ester and glycerol phases, and the products can go through separation by centrifugation or natural gravity phase decantation (Nielsen et al. 2008, 2016; Nielsen and Rancke-Madsen 2011).

Novozymes laboratories initiated the industrial-scale innovation in the use of soluble lipase enzyme instead of immobilized for biodiesel production in 2006 (Nielsen 2014). From the following years until recently, a number of collaborative partnerships among Novozymes, the Danish Advanced Technology Foundation, universities and biodiesel producers aiming not only to develop a competitive and less demanding biocatalyst with respect to raw materials, but also to be effectively used in the production scale. As a result, lipase formulations were developed from engineered variants of *Thermomyces lanuginosus* by Novozymes with patent registration enabling the implementation of the first industrial process for enzymatic production of biodiesel in a refinery located in the United States (Fig. 10.2).

A proof-of-concept transesterification reaction was carried out with soluble lipase batch reactor, with the formation of an oil emulsion with the addition of a small amount of water and alcohol (methanol) and the enzyme under constant stirring. The remainder of the ethanol (a total of 1.4–1.5 molar equivalents of alcohol to fatty acid) was step fed in order to decrease inactivation of the biocatalyst, and the reaction occurred in the temperature range of 35–45 °C for 4–24 h, depending on the amount of enzyme added (Nielsen and Rancke-Madsen 2011; Nielsen 2014). The methyl esters

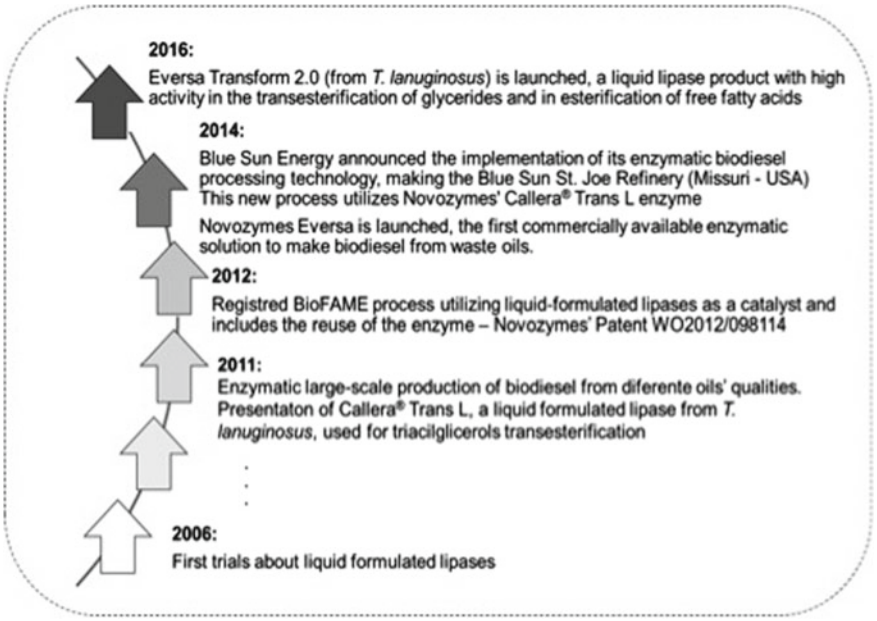

Fig. 10.2 World energy consumption by various sectors for different time period (Adapted and modified from IEO 2017, an open source article)

and glycerol phases were separated by centrifugation and the enzyme at this stage was recycled. Tests conducted in bench level allowed to verify the mode of action and the potential of this type of biocatalyst, as well as the challenges of the process, also describing ways to circumvent the limitations in order to produce a compound, which fit specifications among the specifications of existing standards. Cesarini et al. (2013) were the first group to report high yield conversion (96%) of crude feedstock (non-degummed soybean oil) into fatty acid methyl esters (FAMEs) from with stepwise addition of methanol in the presence of water (3–15%). In this study, the authors used Callera® Trans L and noted the importance of water to maintain the enzyme activity and found that this lipase has a specific mode of action. Evidence was obtained that TAGs are hydrolyzed by Callera Trans L into DAGs, MAGs and FFAs during the first 5 h of reaction for all water concentrations. This hydrolysis process was also favored by the low initial MeOH concentration, added step-wise during the reaction. The release of FAMEs during the first 5 h is probably due to a true transesterification activity of the enzyme, whereas at longer reaction times, when TAGs have almost disappeared, esterification activity is predominant and FAMEs formation derives from the FFAs generated by the complete hydrolysis of TAGs, DAGs and MAGs. This effect was particularly evident at 3–5% water concentrations, where FAMEs production by Callera Trans L was more effective.

In practice, the separation procedure at the end of the reaction typically results in a light ester phase, but the procedure may difficult to obtain a clean glycerine phase and

recovering the emulsified layer containing the enzyme may require energy-intensive separation procedures. A strategy adopted consists in removing only those esters out of the reactor, which results in lower observed losses of catalytic activity, but with progressive accumulation of glycerol, limiting the use of the enzyme only in 3 to 4 batches. With recent advances and the price reduction of the enzymes, the trend therefore is to consider the use of biocatalyst in only one-step in process conducted in a single reactor, in conditions, which ensure the product is within the required specifications (Nielsen et al. 2016). Based on this principle, Nielsen et al. (2016) proposed a process for obtaining biodiesel employing Eversa® Transform, a recent version of Callera® Trans formulation. After initial stage of transesterification, the enzyme was added into the medium containing excess methanol, and NaOH was added to saponify the remaining free fatty acids (FFA). As a result, a high yield of biodiesel to 97% was achieved, with a reduction in FFA levels (<0.25%) and MAG (0.9–0.6%). In addition, phase separation was facilitated by the lack of phase between the ester phase and the heavy phase. Yet according to the authors, this process has been expanded to production scale operation. However, up to date there are no reports of using Eversa® Transform 2.0 formulation on other scales.

10.4.3 Mycellium-Bound Lipase (Whole Cells)

The first example of using intact cells (whole cells) as biocatalyst in biodiesel production has been reported by Ban et al. (2001), who used immobilized cells of *Rhizopus oryzae* for transesterification of soybean oil with methanol. Since then, extensive studies have been reported the use of whole cells as a biocatalysts in order to reduce the cost of biodiesel production as reviewed by Fukuda et al. (2008) and Cortez et al. (2017). Whole cells have attracted considerable attention, mainly because it avoids the added costs of purifying enzymes and further separations. The whole cell acts as support, avoiding the steps of extraction, isolation and purification of enzymes, which are typically labor intensive and contribute to make the process cost-inhibitive. Moreover, the natural environment of intact cells ensures the stability of the enzyme because its optimal spatial location remains intact (Milner and Maguire 2012; Cortez et al. 2017). However, whole cells have low operational stability in solvent free systems (Li et al. 2008), a condition which can be modified with cell immobilization. In addition to promoting greater stability, immobilization facilitates the handling and the separation of the reaction medium cells (Perkins et al. 2015). The cell immobilization procedure occurs as a natural phenomenon or by artificial procedures following the widely used procedures for enzyme immobilization (adsorption, covalent binding, cross-linking and entrapment or encapsulation), but with some criteria imposed by cell morphology (Cortez et al. 2017). The cells are usually treated before or after the immobilization procedure to lose the multiplication capacity (viability), but with the assurance that the enzyme system remains stable and active.

A growing number of studies published in the literature on the use of whole cells from filamentous fungi immobilized in the production of biodiesel is found

throughout the years (Ban et al. 2001; Xiao et al. 2010; Andrade et al. 2012; Carvalho et al. 2015a; Soares et al. 2017). Despite by the fact that whole cells reduce the number of steps to obtain a purified biocatalyst, causing a reduction of the final cost of the product, some disadvantages must be taken into consideration. Cell immobilization makes the synthesis of biodiesel slower than the isolated enzyme-based process (Robles-Medina et al. 2009). In this regard, various studies are being performed aiming at enhancing the operational stability of whole cells in order to reduce the deactivation associated to mycelium-bound lipases caused by the excess methanol in the reaction medium. This, in addition to proposing new process strategies aimed at increasing conversion efficiency and productivity gains has been the key drivers of investigations in this research field. Table 10.3 shows some examples of biodiesel production catalyzed by immobilized whole cells of filamentous fungi.

It is clearly seen that the majority of published studies favors the use of whole cells of *R. oryzae* immobilized on polyurethane foam particles due to the catalytic efficiency of this lipase, with the operational simplicity of immobilization technique and support characteristics (inert material with good mechanical properties, low cost, high porosity and surface adsorption). In this case, the immobilization is by entrapment into preformed porous matrix, characterized by the diffusion of cells (hyphae) through the pores and surface adhesion (Cortez et al. 2017). Table 10.3 also presents strategies used to favor the production of biodiesel by transesterification using whole cells.

10.4.4 Fermented Solids with Lipase Activity

The use of fermented solids containing lipase is also low-cost alternative to reduce biodiesel production costs (Christopher et al. 2014). In this case, the producing organisms can be grown on low cost substrates such as agro-industrial residues maintaining low moisture content. At the end of cultivation, the fermented solid is dried and dilapidated to remove fatty compounds deriving from the fermentation and then used directly as biocatalyst (Fernandes et al. 2007; Salum et al. 2010; Aguieiras et al. 2014). The first work using this type of biocatalyst was reported by Fernandes et al. (2007). In this study, the authors described promising results using the freeze-dried fermented solid (corn bran) containing *Burkholderia cepacia* LTEB11 for transesterification of corn oil with ethanol as acylant agent and ester conversions were in the range of 83–95% after 120 h, depending on the experimental conditions. Subsequently, Salum et al. (2010) produced a solid fermented lipase from the same microorganism (*B. cepacia* LTEB11) in sugarcane bagasse and sunflower seed meal to catalyze the biodiesel synthesis in a packed-bed reactor running on substrate based on soybean oil. Their results showed a high conversion of 95% after 46 h at 50 °C, with an ethanol-to-oil molar ratio of 3:1. Another specie of *Burkholderia* genus (*B. contaminas*) was used as lipase source also in a form of fermented solid (sugarcane bagasse) to catalyze biodiesel production by solvent-free ethanolysis of palm oil (Galeano et al. 2017). By using a packed-bed reactor in batch mode with

Table 10.3 Examples of biodiesel production by whole cells immobilized of filamentous fungi (mycelium-bound lipase)

Filamentous fungi	Strategies	Results	Reference
R. oryzae	Addition of substrate-related compounds to the culture medium; Stepwise additions of methanol, in the presence of 15% water	Olive oil was as the most suitable compound to enhance intracellular methanolysis activity Methyl esters (MEs) content in the reaction mixture reached 90%	Ban et al. (2001)
R. oryzae	Addition of ionic liquid to the reaction medium; Reuse of biocatalyst;	Yields higher than 90% and decrease to 60% from the second recycle	Arai et al. (2010)
R. oryzae	Oil degumming and use of hexane as solvent	Yield of 78% after 73 h of reaction	Ganesan et al. (2012)
R. oryzae, *M. circinelloides*, *P. citrinum*	Screening of lipase-producing fungus isolated from different sources and immobilization and utilization of the whole cells for biodiesel production by transesterification process	The highest performance was attained by *M. circinelloides* immobilized on polyurethane foam particles, giving 83.22 ± 3.68% ester yield in less than 96 h reaction	Andrade et al. (2012)
A. nominus	Addition of solvent (*tert*-butanol) in the reaction medium; Reuse of the biocatalyst	Addition of the solvent descrease the enzyme inactivation and yield of 95.3% in esters was obtained after 40 h reaction	Talukder et al. (2013)
R. oryzae	Packed bed reactor running on batch mode with substrate recycle with periodic feeding of methanol	Conversions were maintaining in 80% for four consecutive cycles for 200 h	Kyeong and Yeom (2014)
R. oryzae	Two steps reaction: previous hydrolysis of the vegetable oil following the esterification of the free fatty acids	Yields in methyl esters 88.6%, after 42 h with 79% retention of the activity after six recycles.	Zhou et al. (2015)

(continued)

Table 10.3 (continued)

Filamentous fungi	Strategies	Results	Reference
M. circinelloides	Screening of non-edible vegetable oils	High yields were obtained with coconut oil (97%) and macaw palm oil (95%)	Carvalho et al. (2015a)
M. circinelloides	Assessment of polyurethane foams synthetized with different types of polyol to be used as a low-cost support to immobilize whole cells	The type of polyol influenced the the pore diameter, water sorption and solvent absorption; Hydrophobic and hydrophilic character of the matrix influenced the attachment of the microorganism and substrate transfers;Ethyl esters varied from 60 to 90% at 120 h	Souza et al. (2017)

recirculation in a closed-loop system and stepwise ethanol addition (alcohol: oil ratio of 5.5:1), a conversion of 89% was attained after 30 h.

Using a fermented solid composed by *Rhizopus microsporus* in a mixture of sugarcane bagasse and sunflower seed meal, Zago et al. (2014) reported 91% conversion of corn oil into ethyl esters in the presence of *n*-heptane, at 48 h in shake flasks. More recently, a promising simultaneous esterification/transesterification method for FAEE production from macaw palm oil containing high acid level was proposed by Aguieiras et al. 2017 using fermented solid *Rhizomucor miehei* on dry babassu cake. The biocatalyst was able to convert oils with different acidities into ethyl esters (biodiesel) in a single reaction step. FAEE content above 85% was achieved at 96 h of reaction with enzyme loading of 13 U per g of oil, 120 mmol of hydrous ethanol (95% ethanol and 5% water)/ 20 mmol of oil (molar ratio ethanol:oil of 6:1), at 40 °C. After two consecutive enzymatic reactions, 90.8 wt% FAEE content was obtained. Although the composition of the final product did not meet the required quality to be used as a fuel, according to the authors the process has potential to decrease the costs of enzymatic biodiesel production.

10.5 Lipase-Catalyzed Production of Biodiesel in Continuous Operations

The transesterification of vegetable oils is substantially faster and more economically viable in continuous reactors than in batch reactors, even considering the higher ini-

Table 10.4 Enzymatic transesterification for biodiesel production under continuous flow

Lipase	Feedstock	Acyl acceptor	Solvent	Reference
Novozym® 435	Residual oil	Methanol	Free	Shimada et al. (2002)
B. cepacian	Residual oil	Ethanol	Free	Hsu et al. (2004)
Immobilized Candida sp. in cotton membrane	Vegetable oil Residual oil	Methanol	Éter de petróleo/água	Nie et al. (2006)
Novozym® 435	Cotton oil	Methanol	*tert*-Butanol	Royon et al. (2007)
Novozym® 435	Soybean oil	Methanol	*n*-Hexane *tert*-Butanol	Shaw et al. (2008)
Novozym® 435	Waste cooking palm oil	Methanol	*tert*-Butanol	Halim et al. (2009)
Novozym® 435	Sunflower oil	Methanol	Free	Ognjanovic et al. (2009)
Novozym® 435	Soybean oil	Iso-propanol	Free	Chang et al. (2009)
Novozym® 435	Soybean oil	Methanol	*tert*-Butanol	Chen et al. (2011)
Lipase *P. fluorescens* imobilizada em SiO_2-PVA	Palm oil	Ethanol	*tert*-Butanol	Dors et al. (2012)
Lipase *B. cepacia* imobilizada em SiO_2-PVA	Macaw palm oil	Ethanol	Free	Costa-Silva et al. (2014)
Lipase *B. cepacia* imobilizada em SiO_2-PVA	Babassu oil	Ethanol	Free	Simões et al. (2015)
Whole cells of *M. circinelloides* immobilized	Coconut oil	Ethanol	*tert*-Butanol	Carvalho et al. (2015a)

tial capital expenditures (Chisti 2006; Zanin and Moraes 2014; Poppe et al. 2015a). In this sense, the use of continuous reactors has been widely reported in the literature due to its advantages, such as cost and volumetric productivity, which can be adjusted according to the operating levels. In addition, greater amount of biodiesel per unit volume can be obtained and easier control of reaction conditions in terms of optimizing the product quality (Poppe et al. 2015a; Christopher et al. 2014; Meunier et al. 2017). Table 10.4 lists some published references on the production of biodiesel reactors operating in continuous flow.

Nie et al. (2006) conducted experiments aimed at optimizing the methanolysis of vegetable oils and residual oil mediate by *Candida* sp. 99–125 lipase immobilized on cotton membrane under continuous runs. A three-step transesterification reactions

were carried out by using reactors in series, in which each reactor was fed with methanol and at the same time an apparatus facility (hydrocyclone) was used to on-line separate the formed byproduct (glycerol). The conversion of the continuous process was 90% for vegetable oil and 92% for the residual oil. The operational stability of the immobilized lipase was reported to be close to optimum values for over 20 days.

Royon et al. (2007) reported the production of biodiesel through methanolysis of cottonseed oil using Novozym® 435 catalyst and *tert*–butanol as solvent. Yields as high as 95% were obtained using a continuous packed bed reactor (6 × 180 mm) with a flow rate of 9.6 mL h^{-1}. The system operated continuously for over 500 h without showing significant reduction in the yield of esters.

The continuous process of methanolysis of soybean oil catalyzed by Novozym® 435 in the presence of co-solvent (mixture of *n*-hexane: *tert*-butanol) was investigated by Shaw et al. (2008). The packed bed reactor consisted of a stainless-steel tube (25 × 250 mm) packed with 1 g of enzyme. Response surface methodology was used to evaluate the effects of parameters on the reaction conversion. According to the authors, the best conditions were: temperature 52 °C, flow 0.1 mL min^{-1} and molar ratio of methanol to soybean oil = 4.3:1. The predicted value for the conversion was 74.2% and the experimental value of 75.2%. Halim et al. (2009) studied the methanolysis of residual palm oil catalyzed by Novozym® 435 to determine an optimal continuous procedure in a packed bed reactor to investigate the possibility of larger scale production. The two columns with dimensions of 180 mm × 10 mm were operated in an up-flow regime. Two important process variables were analyzed, height of the bed and substrate flow rate. The optimum conditions for transesterification were as follows: 105.3 mm bed height and volumetric flow rate 0.57 mL·min^{-1} of substrate, which promoted FAME yield of 79.0%, having a predicted value of 80.3% through statistical design of experiments.

Ognjanovic et al. (2009) demonstrated the possibility of using Novozym® 435 in biodiesel synthesis from sunflower oil and methanol in a solvent free system in a packed bed reactor, obtaining a conversion of 93.6% for a special-time of 8 h.

The production of biodiesel in a packed bed reactor (stainless-steel, 250 mm × 46 mm) catalyzed by Novozym® 435 lipase using soybean oil with isopropanol in a solvent-free system was investigated by Chang et al. (2009). To determine the optimal conditions, a statistical experiment design was used, and under optimized conditions (flow rate = 0.10 mL min^{-1}, temperature = 51.5 °C and molar ratio of 1:4.14 (oil: alcohol), 1.7 g of lipase) concentrations as high as 75% isopropyl esters were obtained. The lipase showed excellent operational stability allowing operating the system for seven days without reduction in the concentrations of isopropyl esters. Chen et al. (2011) also employed the statistical design as a tool to establish the conditions for biodiesel synthesis from soybean oil and methanol in the presence of *tert*-butanol (32.5 wt% relative to oil) mediated by Novozym® 435. The analysis of results was made by response surface methodology, investigating the influence of the independent variables (temperature of reaction, volumetric flow rate and molar ratio of the substrate) in the response variable (mol conversion). The optimal conditions for a packed bed reactor operating in continuous mode provided maximum conversion

of 83.31% with a volumetric flow rate of 0.1 mL min^{-1}, 52.1 °C and molar ratio of 4:1 (ethanol:oil). The bed reactor operated for more than 30 days without significant loss in the substrate conversion.

Dors et al. (2012) evaluated the continuous alcoholysis reaction mediated by lipase of palm oil with ethanol in presence and absence of solvent (*tert*-butanol), using *P. fluorescens* immobilized in a hybrid matrix of polysiloxane-polyvinyl alcohol (SiO_2-PVA) in a packed bed reactor. The best performance was found for the reactor running in the presence of *tert*-butanol, which resulted in a stable operating system and an average yield of 87.6 ± 2.5%. This strategy also gave high biocatalyst operational stability, revealing a half-life of 48 days and an inactivation constant of 0.6×10^{-3} h^{-1}. Simões et al. (2015) assessed the transesterification reaction of babassu oil with ethanol mediated by *Burkholderia cepacia* lipase immobilized on SiO_2-PVA composite in a packed bed reactor running in the continuous mode. Experiments were performed in a solvent-free system at 50 °C. The performance of the reactor (14 mm × 210 mm) was evaluated using babassu oil and ethanol at two molar ratios of 1:7 and 1:12, respectively, and operational limits in terms of substrate flow rate were determined. Based on the results obtained, the best reactor performance was achieved for runs in which the oil to alcohol molar ratio of 1:12 was used. Under such condition, and at space time greater than or equal to 11 h, an average transesterification yield of 96.0 ± 0.9% and a productivity of 41.1 ± 1.6 $mg_{ester}\ g^{-1}_{catalyst}\ h^{-1}$ were achieved. This also resulted in biodiesel samples with viscosity values (average 4.3 ± 0.7 $mm^2\ s^{-1}$) complying with the international standard for biodiesel viscosity i.e. ASTM 6751-02 (1.0 < kinematic viscosity of B100 < 6.0 $mm^2\ s^{-1}$).

It is important to highlight that in processes conducted in a continuous flow, the effect of the glycerol, byproduct of the reaction, is more pronounced on the efficiency of the process than in the batch process. Glycerol makes it difficult for the substrate to diffuse onto the lipase molecule, thus reducing reaction efficiency (Shimada et al. 2002). This is due to glycerol being a hydrophilic viscous liquid that easily adsorbs onto the surface of the immobilized enzyme, forming a hydrophilic layer, limiting the enzyme performance in hydrophobic substrates, and consequently impairing the transesterification yield (Hama et al. 2011; Xu et al. 2011). Thus, from the standpoint of planning and operation of an industrial plant, the efficient removal of glycerol may be an obstacle in the implementation of continuously operating reactors (Hama et al. 2011). In order to minimize the negative effects of glycerol different strategies have been investigated (Table 10.5).

Dossat et al. (1999) used silica gel and other adsorbent substances to extract glycerol. Watanabe et al. (2000) developed a continuous methanolysis system in a three columns (15 mm × 80 mm) packed with the immobilized enzyme (Novozym® 435 at 30 °C and flow rate of 6 mL h^{-1}), in which the addition of methanol was made in three steps, one third molar equivalent added to each column. Each column had their effluent treated to remove glycerol, and the subsequent column was fed. The conversions of the vegetable oil into FAMES obtained in each reactor were respectively 33, 66 and 93% and the immobilized lipase was reported to be used by more than 100 days without activity reduction. Li et al. (2006) and Royon et al. (2007) investigated the

Table 10.5 Removal glycerol strategies in processes carried out under continuous flow

Lipase	Glycerol removal Strategy	Results	Reference
Lipozyme® RM-IM	Use of silica gel to adsorb the produced glycerol	In a continuous plug flow reactor the addition of silica resulted in a partitioning of glycerol between silica and support. About 0.05 g/L of glycerol was removed from the reaction medium and this extended the biocatalyst half-life	Dossat et al. (1999)
Novozym® 435	Residual oil methanol	Addition of methanol was made in three steps, one third molar equivalent added to each column. Each column had their effluent treated to remove glycerol, and the subsequent column was fed.	Watanabe et al. (2000)
Novozym® 435	Fluidized bed reactor coupling with a column packed with Lewatit GF202 for continuous glycerol removal	The best performance was obtained by running the reactor with biocatalyst loading of 12% and a space-time of 8 h, attaining an average yield of 98.1% and productivity of 9.9 $mol_{ester}\ g_{cat}^{-1}\ min^{-1}$ System under stable conditions for 30 days without any loss of biocatalyst activity	Fidalgo et al. (2016)
Burkholderia cepacia immobilized on SiO_2-PVA	Two-stage packed-bed reactor incorporating a column with Lewatit GF 202 to remove the glycerol	At space-time of 14 h, a FAEE content of 58.5 ± 0.87 wt% was achieved, corresponding FAEE yields of 97.3 ± 1.9% and productivities of 41.6 ± 1.0 $mg_{ester}\ g_{medium}^{-1}\ h^{-1}$. The immobilized lipase was found to be stable, showing half-life time ($t_{1/2}$) ~ 1540 h	Costa-Silva et al. (2016)
Burkholderia sp. lipase immobilized on alkyl-celite	Series of three packed-bed reactors integrated with glycerol removal devices	In the first column, TAG was converted to FAME, glycerol and intermediate products. The second and third columns continuously converted intermediate products to FAME with the supply of methanol and without the accumulation of glycerol. A biodiesel yield of 85% was achieved	Tran et al. (2016)

(continued)

Table 10.5 (continued)

Lipase	Glycerol removal Strategy	Results	Reference
Burkholderia cepacia immobilized on SiO_2-PVA	Two-stage packed-bed reactor incorporating a column with Lewatit GF 202 to remove the glycerol	Reactors with different height-to-diameter ratios (l/d) were used for continuous runs carried out using an oil-to-ethanol molar ratio of 1:12 at a fixed space-time (14 h). The best performance was attained by using reactor with an l/d of 15, which was further used to perform runs in a two-stage PBR by incorporating a column with cationic resin. The system operation for a space-time of 16 h resulted in a productivity of 37.9 ± 2.4 m_{gester} g_{medium}^{-1} h^{-1} (biodiesel yield = $96.3 \pm 2.1\%$)	Ramos et al. (2017)

addition of organic solvents (*n*-hexane or *tert*-butanol) to ensure homogeneity of the reaction mixture, thereby reducing the viscosity of the reaction medium. Glycerol extracting columns packing with Lewatit GF 202 have been evaluated aiming at maintaining the catalytic activity of the immobilized lipases (commercial available -Novozym 435 or homemade-*B. cepacia* immobilized on SiO_2-PVA) during the continuous ethanolysis of lauric oils for longer periods. In both cases, yields in biodiesel higher than 95% were reached and greater biocatalyst stabilities were verified.

10.6 Strategies for Enzymatic Production of Biodiesel

The importance of biodiesel as a renewable energy source makes its production, especially by enzymatic route, to be constantly challenged to obtain technologies that result in processes that are more efficient. In this respect, several strategies have been and continue to be developed in order to extend the biocatalyst half-lives. Some examples are shown in Table 10.6. The addition of a co-solvent to the reaction medium can assist in reducing the oil viscosity, the dissolution of the formed glycerol, and a significant improve in the mass transfer coefficients. Moreover, the co-solvent may assist in enzyme protection against the inhibitory effects of acyl acceptor and glycerol itself. The major drawbacks are related to process economics. Although the addition of co-solvent (e.g. *tert*-butanol) can contribute to increase the half-life time of the enzyme, the operational cost of this system is high due to the recovery of solvent. In this sense, a solvent-free system is often regards as a better choice for the enzymatic production of biodiesel. As an alternative to the high costs involved in solvents, there are increasing reports on the replacement of organic

solvents with other types of technology that act in a similar fashion (Hama et al. 2013). A common example is the case of ionic liquids. Ha et al. (2007) described the process of methanolysis of soybean oil catalyzed by Novozym® 435. This study gave 80% of conversion after 12 h of reaction at 50 °C, in addition to other benefits derived from the supplementation of the [Emin][TfO] into the reaction medium. Gamba et al. (2008) demonstrated that *B. cepacia* lipase supported in 1-n-butyl-3-methylimidazolium bis (trifluoromethylsulfonyl) imide ionic liquid (BMI-NTf_2) can be a green alternative method for the production of biodiesel from the alcoholysis of soybean oil. The ionic liquid provides the ideal medium for the stabilization of the enzyme and for the removal of by-product glycerol, with increased biodiesel yields. The transesterification can be performed at room temperature, in the presence of water and without the use of organic solvents. It is also compatible with various alcohols. The biodiesel is separated by simple decantation and the recovered ionic liquid/enzyme catalytic system can be reused at least four times without loss of catalytic activity and selectivity. Another option is to perform the reactions under supercritical or near-critical conditions (Lee et al. 2009; Lee M et al. 2013). Using near-critical CO_2, Lee M et al. (2013) achieved yields close to 100% in short period of time, and reported that the enzyme maintained 90% of its original activity after 20 recycles.

The association of lipases is another approach to improve the biodiesel production process. The synergistic effect based on the specificity of each enzyme has been evaluated by a number of researchers (Hama et al. 2009; Guan et al. 2010; Tongboriboon et al. 2010; Adachi et al. 2011; Lee et al. 2011; Yücel and Demir 2012; Poppe et al. 2015b; Su et al. 2015; Amoah et al. 2016), which inferred that a combination of different types of lipases allows attaining high biodiesel quality (low contents of mono and diacylglycerols). Hama et al. (2009) described a decrease in the amount of accumulated intermediate with the use of mono- and diacylglycerol lipase from *A. oryzae* mixed with 1,3-regiospecific lipase. A biodiesel synthesis from palm oil with ethanol in solvent free medium was carried out in two-stage packed bed reactor using a mixture of two non-specific lipases (*Pseudomonas fluorescens*-AK and *Candida rugosa*-AY) was proposed by Tongboriboon et al. (2010). The highest biodiesel yield (>80%) was achieved using the combination of 50% of each lipase under the following conditions: 2% of water content, 10% enzyme dosage and 1/3 molar ratio of palm oil to ethanol. The mixed lipases could be repeatedly used under the optimal conditions for 15 times with a relative activity higher than 50%.

In another study, 1,3-specific *R. miehei* lipase and mono- and diacylglycerol lipase from *Penicillium cyclopium* were separately expressed in *Pichia pastoris* (Guan et al. 2010). The authors used the free enzymes (extract without purification) for the transesterification of soybean oil with methanol. When used in combination, conversion to biodiesel achieved yields greater than 95%. Adachi et al. (2011) developed an immobilized recombinant *A. oryzae* co-expressing triglyceride and partial glyceride lipases that attained methyl ester yields of 98% with low contents of residual glycerides.

The use of raw materials with a high concentration of free fatty acids usually requires modification of the traditional process. Based on previous studies, Watanabe

Table 10.6 Examples of combined process for the enzymatic production of biodiesel

Strategy	Technology	Lipase	Main characteristics of process and Results	Reference
Co –solvent	Ionic liquid	Novozym® 435	Methanolysis of soybean oil, using [Emim](TfO) ionic liquid as cosolvent. Production yield (80%), eight times higher than conventional solvent-free system and ≈15% higher than system using *tert*-butanol	Ha et al. (2007)
		B. cepacia	The use of lipase supported in BMI·NTf_2 ionic liquid was used to produce biodiesel from soybean oil. The best conversion (96%, 48 h) was obtained using 0.6 g lipase in 8.2 mmol BMI·NTf_2, 70:30 41.2 mmol methanol:water, 3.4 mmol oil, 30 °C	Gamba et al. (2008)
	Supercritical carbon dioxide	*Candida antarctica* lipase B	Methanolysis of olive oil under CO_2 environment, with stepwise addition of alcohol. Biodiesel conversion of ≈99% after 6 h. Mass and thermal transfer was increased, with a faster reaction rate than can occur at atmosphere pressure	Lee et al. (2009)
	Near-critical carbon dioxide	Lipozyme® TL-IM	Methanolysis of canola oil under CO_2 environment, with stepwise addition of alcohol. Conversion of ≈99.9% after 4.5 h. Biodiesel conformed to the fuel standard (EU) even without additional downstream processing, other than glycerol separation and drying	Lee M et al. (2013)
Combined lipases	Lipases with different specificities	*P. fluorescens* lipase and *C. rugosa* lipase	Ethanolysis of palm oil by continuous process on a packed-bed reactor. The mixed lipases could be used in 15 replicates with retained relative activity >50%. In a continuous system using mixed lipases packed in bed reactor, >67% of biodiesel was achieved	Tongboriboon et al. (2010)
		R. oryzae lipase and *C. rugosa* lipase	Methanolysis of soybean oil under CO_2 environment, with stepwise addition of alcohol. Yield conversion of ≈100% at 2 h, and yield of 85% after 20 reuses	Lee et al. (2011)
		Novozym® 435 and Lipozyme® RM-IM	Methanolysis of canola oil, with stepwise addition of alcohol. Ester yields of 97.2%	Yücel and Demir (2012)
		R. oryzae lipase immobilized and Novozym® 435	Ethanolysis of soybean oil, with stepwise addition of alcohol. Yield > 98.3%, with reaction time shortened from 60 to 21 h. Yield retained (≈80%) after 20 cycles in a solvent-free system	Su et al. (2015)
		Novozym® 435 and Lipozyme® TL-IM and Lipozyme® RM-IM	Ethanolysis of olive oil by combi-lipase (mixture of three immobilized lipases). Conversion efficiency of 95% in 18 h, up from 50% for Novozym®435. Biocatalyst systems could be used for at least seven cycles keeping higher than 80% of their initial activities	Poppe et al. (2015b)

(continued)

Table 10.6 (continued)

Strategy	Technology	Lipase	Main characteristics of process and Results	Reference
		Callera® Trans L. and *C. rugosa* lipase	Methanolysis of soybean oil added with phospholipid, with stepwise addition of alcohol. Methyl esters yield more than 95% at 6h. The lipase system could be useful for the conversion of unrefined oils	Amoah et al. (2016)
		Whole cells immobilized of *R. oryzae* and *Aspergillus oryzae*	Methanolysis of soybean oil in packed-bed reactor (PBR) system, with stepwise addition of alcohol. Ten repeated-batch methanolysis cycles in the PBR maintained methyl ester content >90%, with MAG and DAG at 0.08–0.69 and 0.22 1.45%, respectively	Hama et al. (2009)
		Rhizomucor miehei lipase and *Penicillium cyclopium* lipase	Methanolysis of soybean oil, with stepwise addition of alcohol. Conversion of 99.7% after 24 h	Guan et al. (2010)
		Wholes cells immobilized of *A. oryzae*	Methanolysis of soybean oil, with stepwise addition of alcohol. By using recombinant whole-cells, the methyl ester content (98%) was superior to the attained with lipase-mixing and two-step reactions	Adachi et al. (2011)
Two stages reactions	Enzymatic hydro-esterification	*C. rugosa* lipase and Novozym® 435	Hydrolysis of high acid oil followed by esterification of resulting FFA with methanol. After hydrolysis the resulting oil composition was 91.5 wt% FFA, 0.8 wt% TAG, 0.4 wt% DAG. The total esterification reached 99% after 24 h	Watanabe et al. (2007)
		C. rugosa lipase and Novozym® 435	Crude palm oil was first hydrolyzed in the presence of isooctane and the FFA esterified with stoichiometric excess of methanol. Higher biodiesel yield (98%) was attained the single-step Novozym® 435 catalyzed methanolysis (92%) and the solvent-free system with three successive additions of methanol (92%)	Talukder et al. (2010a)
		Vegetable lipase (dormant castor seeds) and fermented solid (*Rhizomucor miehei* lipase)	Hydrolysis of macaw palm oil followed by esterification of released FFA with ethanol. Hydrolysis produced 99.6% of FFA after 6 h. Esterification yielded 91% after 8 h in a solvent-free system	Aguieiras et al. (2014)
	Enzymatic hydrolysis following by chemical esterification	*C. rugosa* lipase immobilized and sulfuric acid	Hydrolysis of soybean oil followed by esterification with methanol. Biodiesel conversion of 99% was obtained after 12 h. The product met the ASTM standard	Ting et al. (2008)
		Physic nut (*Jatropha curcas* L.) lipase niobic acid	Hydrolysis of the physic nut oil, and subsequent esterification of the generated FFA with methanol. The resulting biodiesel was of excellent quality: viscosity (5.5 $mm^2.s^{-1}$), ester content (97.1%), total glycerol (0.09 % w/w), max. methanol (0.05 % w/w)	De Souza et al. (2010)

(continued)

Table 10.6 (continued)

Strategy	Technology	Lipase	Main characteristics of process and Results	Reference
		C. rugosa lipase and Amberlyst® 15	Hydrolysis of waste cooking oil followed by esterification of FA with methanol, in the presence of isooctane. The activity of *C. rugosa* lipase slightly decreased with recycling, and FA yield after five cycles was 92%. Amberlyst 15 was repeatedly used for 100 cycles without loosing its activity	Talukder et al. (2010b)
Two stages reactions	Enzymatic esterification following by alkaline transesterification	Novozym® 435 and alkaline catalyst	Esterification of palm fatty acid distillate with methanol in packed-bed reactor, using a two steps process: first with small excess of methanol and after, with water removal. The resulting product is followed to typical alkaline transesterification step. Both reaction steps in the esterification process are relatively fast, resulting in 15% FFA after column 1 and 5% FFA after column 2. The product can then typically be blended with the deodorized oil and continue through to alkaline transesterification with <0.3% total FFA	Brask et al. (2011)
	Transesterification and esterification	Experimental immobilized lipase (NS 88001) and Novozym® 435	Transesterification of rapessed oil with ethanol, with stepwise addition of alcohol, followed by esterification of resultant product with ethanol (polishing the biodiesel). Separation of the glycerol and water between passes. By using a packed bed reactor system, the second stage brought the biodiesel composition to 'in-spec' levels according to the European specifications. The overall productivity of the proposed two-stage process was 1.56 kg FAEE (kg catalyst)$^{-1}$ h^{-1}	Xu et al. (2012)
	Two stages of transesterification	Lipozyme® RM IM	Butanolysis of low quality rapeseed oil in reaction carried out in two steps: first, glycerol/enzyme is removed and the product washed; second, product and enzyme are returned to the system with addition of more biocatalyst and alcohol. Strategy resulted in a 96.6% butyl ester yield from oil rich in FFA with longer-chain alcohol	Sendzikiene et al. (2016)
Unconventional heating	Reaction assisted by ultrasound	*C. rugosa*	Methanolysis of canola oil, with ultrasonic horn inserted in the reaction mixture to provide sonication. Ultrasonic assisted reaction resulted in complete conversion in 90 min reaction while in its absence, the biodiesel yield was close to 99% after about 24 h. Enhanced mass transfer as a result of cavitation bubble collapse increases the transesterification reaction rates by increasing the collision frequency between reactants	Bhangu et al. (2017)
		Lipozyme RM® IM	Ethanolysis of soybean oil using an ultrasonic water bath equipped with a transducer having longitudinal vibrations. Reaction yield (≈90%) was obtained in a relatively short reaction time (4 h) at mild irradiation power supply (~100 W) and 60°C. The repeated use of the enzyme resulted in a decay in both enzyme activity and product conversion after two cycles	Batistella et al. (2012)

(continued)

Table 10.6 (continued)

Strategy	Technology	Lipase	Main characteristics of process and Results	Reference
		Novozym® 435	Ethanolysis solvent-free of macaw palm oil, using an ultrasonic water bath equipped with a transducer having longitudinal vibrations. Reaction yields ≈70 wt% were obtained at mild irradiation power supply (~132 W) and 65 °C within 30 min. Reutilization of enzyme showed that it may be advantageously employed up to 5 reuse cycles	Michelin et al. (2015)
	Transesterification assisted by microwave	Immobilized *B. cepacia* lipase	Ethanolysis of babassu oil, in reaction performed in a microwave reactor. Under optimal conditions, full conversion was attained at 10 h. The purified product contained no glycerol-bound and properties, such as specific gravity and viscosity, are in accordance with standard fuel specifications	Da Rós et al. (2014)
		Lipase PS immobilized	Ethanolysis of Jatropha oil in a solvent-free system, in reaction performed in a microwave reactor. Ethyl ester yields 98.3% were attained at 72 h of reaction. The operational stability of immobilized lipase PS was determined in repeated batch runs under conventional and microwave heating systems, revealing half-life times of 430.4 and 23.5 h, respectively	Souza et al. (2016)
		Novozym® 435	Ethanolysis of algal oil with isooctane as solvent, in reaction performed in a microwave reactor. The viscosity values for the microbial oil (51.9 $mm^2\ s^{-1}$) sharply decreased to 10.7 $mm^2\ s^{-1}$, upon the progress of transesterification reaction. The maximum FAEE yield (80%) achieved was due to the presence of non-lipid compounds, which may have affected the biocatalyst activity	Da Rós et al. (2017)
Other technologies	In situ transesterification of of microbial biomass bearing oil	Novozym® 435	Direct enzymatic transesterification of microalgae biomass using methanol (added at stepwise) and tert-butanol as solvent. Maximum FAME conversion of 99.5% (56 h). FAME conversion decreased by 40% when a lipase batch was used to catalyze three successive reactions	López et al. (2016)
		Novozym® 435	Enzymatic biodiesel production (ethanolysis) from microalgae biomass using propane as pressurized fluid. Conversion of 75.8% was achieved using (20 wt.%) of the enzyme with mild temperature demonstrating that microalgae biomass could be potentially used in biodiesel production	Marcon et al. (2017)
	Blend of alcohols (methanol + ethanol) as acyl acceptors	Novozym® 435	Transesterification of soybean oil with blended alcohols by lipase Among six proportions tested, 0 (100 mol% ethanol), 20, 40, and 60 mol% methanol in the blended alcohols exhibited high yields of biodiesel. For the optimum temperature (30 °C), the highest yield of biodiesel (>95 wt%) was obtained at an enzyme loading of 5–10 wt%	Zhao et al. (2014)

et al. (2007) proposed an enzymatically hydroesterification using different lipases at each stage: (i) hydrolysis of acylglycerols by *C. rugosa* lipase, (ii) followed by esterification of resulting oil fatty acid with immobilized *C. antarctica* lipase. The methyl esterification of fatty acids proceeds on a much higher rate than the triglyceride methanolysis. Other process combinations have been proposed, as chemoenzymatic hydroesterification (Ting et al. 2008; De Souza et al. 2010; Talukder et al. 2010a; Brask et al. 2011), which is consisted on an enzymatic transesterification followed by esterification of the resultant product to improve the biodiesel yield by converting FFA and partial glycerides to FAEE (Xu et al. 2012). These also consider two steps of transesterification for more efficient conversion of low quality oils using higher chain alcohols (Sendzikiene et al. 2016). In addition to high conversion into biodiesel in a short time reaction, these technologies allow low-risk conditions of inactivation of the biocatalyst, obtaining a final product within normative specifications.

The benefit of ultrasound and microwave irradiation on biodiesel production catalyzed by lipase has also been reported (Batistella et al. 2012; Da Rós et al. 2014; Michelin et al. 2015; Souza et al. 2016; Bhangu et al. 2017). Among the major benefits of using this type of technology, include the use of solvent-free systems and reduction of the reaction time. The theory of ultrasonication and its application in many reacting systems has been widely reported as an important factor in endothermic reactions, as the transesterification reaction to produce biodiesel. The reason of using ultrasound in reaction systems is due to the mechanical energy applied to the system, which induces mixing effects needed to initiate reaction (Koh and Ghazi 2011). In transesterification specifically, sonication causes cavitation bubbles near the boundary between the alcohol and oil phases leading to intensive mixing of the system. The cavitation leads to a localized increase in temperature, and due to the formation of micro-jets, neither heating nor agitation are required (Santos et al. 2009). As reviewed by Koh and Ghazi (2011), ultrasonication increases the chemical reaction speed, the efficient molar ratio of methanol-to-oil, and the yield of transesterification of vegetable oils and animal fats into biodiesel. Such method clearly works with lower energy consumption compared to the conventional mechanical stirring method. Studies have shown that increased biodiesel yields under ultrasonic irradiation are mostly attributable to the efficiency of cavitation irradiation, which is dependent on frequency. This enhances the mass transfer between the reacting mixtures, thereby increasing the reaction rate (Aransiola et al. 2014; Lerin et al. 2014).

Ultrasound irradiations have been investigated towards the enzymatic methanolysis by Lerin et al. (2014) and the conversion to fatty acid methyl esters was greater than 85% within 4 h reaction. The major drawback associated with this method, however, is the possibility of fragmenting the immobilizing support (Rufino et al. 2010).

On the other hand, the use of microwave irradiation can overcome the low speed of the enzymatic reaction with conservation of the morphological properties of the immobilized enzyme (Souza et al. 2017). Microwaves, electromagnetic waves with frequencies ranging from 300 MHz to 300 GHz, induce molecular rotation of dipolar species accompanied by intermolecular friction and energy dissipation, resulting in

volumetric heating without affecting the molecular structure. Microwave heating is a process of direct energy absorption by the irradiated material (liquid) with a uniformly distributed heat sources which prevents convection due to thermal gradients, a common phenomenon in conventional heating (Da Rós et al. 2013). The effect of overheating, i.e. by heating a given substance above its boiling point, is assigned as the major factor for accelerating reactions heated by microwave (Lidström et al. 2001). The Arrhenius equation ($k = Ae^{\frac{-\Delta G}{RT}}$, in which k = rate constant A = pre-exponential constant AG = free energy of activation, R = ideal gas constant, T = temperature) describes the rate constant for any system. Thereby, microwaves may act in three ways to increase the rate constant of a reaction: (i) by increasing the vibration frequency, thereby increasing the molecular mobility that is related to the pre-exponential factor A (dependent on the vibrational frequency), (ii) by changing the exponential factor which would cause changes of activation energy, (iii) by heat generated into the system, which causes are more generally applicable to the increased speed of reactions in a microwave. The rapid heating and power distribution cannot be achieved by conventional heating, since the latter can change the selectivity of reagents (Lidström et al. 2001).

The direct use of microbial biomass containing lipid feedstock (López et al. 2016; Marcon et al. 2017) to obtain biodiesel by transesterification have been discussed in order to develop more streamlined processes and lower operating costs. Using *Nannchloropsis gaditana* microalgal biomass, López et al. (2016) obtained a conversion of 99.5% FAME by direct transesterification employing the enzyme Novozym® 435.

Methanol and ethanol, which are the most accepted acyl acceptors for synthesis of biodiesel, have their own advantages and disadvantages. Thus, using a blended alcohol (methanol and ethanol) as an acyl acceptor for lipase-catalyzed transesterification could be an innovative strategy for overcoming the drawbacks of each alcohol (Zhao et al. 2014). According to the authors, the use of blended alcohols engendered the successful results, although proportions of methanol higher than 60 mol% in the alcohol blended adversely affected the biodiesel yield. On the other hand, the reactivity of methanol in the transesterification was higher than ethanol. The results importantly indicate that higher methanol consumption results in more ethanol remaining in the reaction mixture and show possible extension of lipase activity. It also increases the solubility of the oil in the alcohol, yielding faster reaction rate. Thus, the employment of the blended alcohols of methanol and ethanol as an acyl acceptor for the transesterification has positive effect on the enzymatic biodiesel production.

10.7 Challenges and Opportunities for Lipases in the Realm of Biodiesel Production

Despite the innumerous advances in the field of lipase-catalyzed biodiesel, the commercialization of biotechnological approaches towards a competitive market is still a challenge to be overcome. There have been significant novel approaches towards

manufacturing and optimizing lipases including the production of liquid lipases, innovative immobilization materials, and the utilization of fermentation broth as an enzymatic-active material. The recent interest of adding value to wastewater from both agricultural and food industries via cultivation of microorganisms have lead to low-cost alternatives for production of many different metabolites, including enzymes (Reis and Hu 2017). The utilization of waste materials regarded as nutrient sources for cultivation of enzyme-producing microorganisms may eventually lead to further cost cuts in the lipase production industry. It is unlikely a biodiesel plant to operate its own factory with lipases that inhibits a costly-effective process. However, the bio-valorization of "waste" via lipase-producing microorganisms promotes simultaneously the partial or full treatment of such material and the production of a valuable resource, i.e., lipases. The world overall interest in biodiesel has not maintained steady over the past few years for a number of reasons, (i): one of the major energy users and producers in the world, China, has strict policies regarding the utilization of food crops towards energy production. As China is now one of the most influential countries when it comes to innovation in the biotechnological industry, its own policies often reflect on the degree of innovation that it exports to the world. Despite being home to ambitious programs in green and sustainable energy development and security in the world, China has shifted much of its focus towards the production of other forms of renewable energy, as wind and solar. (ii): the price of fossil fuels has significantly dropped within the last decade, especially those of natural gas. The production of biodiesel became, in many parts of the world, not feasible techno-economically and has been ever since a forgotten alternative to liquid fossil fuels. (iii): life cycle assessment of first-generation biodiesel has shown that its emissions and environmental impacts may often be higher than initially expected. It has been suggested by (Hill et al. 2006) that lowering greenhouse gas emissions and water usage, as well as other environmental impact indicators, is required in order to place biodiesel as a green alternative towards the world energy grid again. The production of biodiesel spans a full generation of research, and has a key driver in developing economies as a novel source of income and energy security. Such efforts, though now several seen as over dated, should not be discarded, nor should biodiesel.

Lipases, as well as other enzymes, have been explored to their capacity and have been on their way to become a commodity "chemical", instead of the past and current label of specialty catalyst. As reviewed in this chapter, the utilization of enzymes not only is preferable to chemical reactions due to lower energy requirements and higher specificity and formation of by-products, but also due to its reduction of waste and other added resources that do not add value to the final product. Furthermore, the utilization of lipases is a direct application of several of the 12 principles of green chemistry (Anastas and Eghbali 2010), and should be directly reflected in companies and countries that are driven towards sustainable development. Therefore, instead of crediting biodiesel a label of alternative fuel, it should be seen as a feasible way of adding value to waste lipid resources, from restaurant-waste (Canakci 2007) to scum in wastewater treatment plants (Bi et al. 2015). The utilization of lipases should not be seen as the cost-inhibitive step in the process, as many still credit its own use today, but rather an alternative to add value to agricultural wastes. The decades of research

in the field of biodiesel and on lipases have advanced many of the technical and optimization steps necessary towards the development of an efficient process, and the previously developed technology should now be transferred to current platform feedstocks in order to make them economically feasible. Factors that were problematic decades ago, as resilience, solvent use, and reusability now have been overcome by the development of high-efficiency immobilization techniques, green solvents and supercritical fluids, and robust immobilization or liquid enzymes, respectively. Thus, lipases may the answer to part of the complicated energy situation in the world, and the steps required to their full implementation not only rely on the implementation of policies, but also to "connecting-the-dots" on all the impressive research done over the past decades in the field of lipases for biodiesel production.

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Chapter 11
Nanotechnology-Based Developments in Biofuel Production: Current Trends and Applications

Avinash P. Ingle, Priti Paralikar, Silvio Silverio da Silva and Mahendra Rai

Abstract The extensive consumption of fossil fuels due to ever increasing global population leads to the depletion in its resources all over the world. Moreover, these fuels are playing a major role in creating environmental pollution. As a renewable energy alternative resources, utilization of biomass resources for the production of biofuels attracted a great deal of attention from every corner of the world. Various conventional approaches including chemical, thermochemical, biological methods, etc. have been developed but certain limitations in the smooth application of these methods create pressing need to investigate rapid and environment friendly approaches for sustainable biofuel production. In this context, nanotechnological approaches are found as more promising. Nanotechnologies represent one of the most fascinating techno-scientific revolutions ever undertaken in various sectors including biofuel and bioenergy. Various nanomaterials in the form nanocatalysts play an important role in catalytic degradation of different lignocellulosic biomass into fermentable sugars, which are further used for bioethanol production. Similarly, the production of biodiesel and biogas through nanotechnological approaches has attained a great deal of attention. In this chapter, we have mainly focused on recent trends and applications of nanotechnology in biofuel production. In addition, conventional methods commonly used for biofuel production are also discussed in brief.

Keywords Nanotechnology · Biofuel · Nanomaterials · Nanoparticles
Biodiesel · Bioethanol

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11.1 Introduction

Constant increasing global population and urbanization increases the worldwide demand of energy. If it continues with same pace then energy requirements will become an area of prime concern very soon for many countries including the most developed nations of the world (Akia et al. 2014). It has been noticed that energy crisis has become one of the major problems, which is significantly affecting overall economic development of various countries around the globe. However, such problems are considered far more serious in the perspective of all developing nations where there is a significant pressure mounted on the available natural sources of energy (Malik and Sangwan 2012).

According to International Energy Outlook-2017 (IEO-2017) the world energy consumption has been continuously increasing every year from 1990 to till date; and it is predicted to increase in future also. It was reported that, the world energy consumption in 1990 was 355 quadrillion British thermal units (Btu) which increased to 575 quadrillion Btu in 2015 and forecasted to increase to 663 quadrillion Btu by 2030 and then to 736 quadrillion Btu by 2040 (Fig. 11.1). Further, it is expected that most of the increase in energy demand will come from nations, which are not the member of Organization for Economic Cooperation and Development (OECD) (i.e. non-OECD countries) due to their strong economic growth and rapidly growing populations. It was estimated that energy consumption in non-OECD countries will increase 41% between 2015 and 2040 in contrast to a 9% increase in OECD countries. Among all non-OECD countries, the countries from Asia (including China and India) alone accounts for more than half of the world's total increase in energy consumption over the 2015–2040 projection period (IEO 2017).

The energy is mostly utilized for the purpose of building (household), transportation and industrial use. It was reported that, among the industrial sectors (viz. mining, manufacturing, agriculture and construction) accounts for the largest share (50%) of

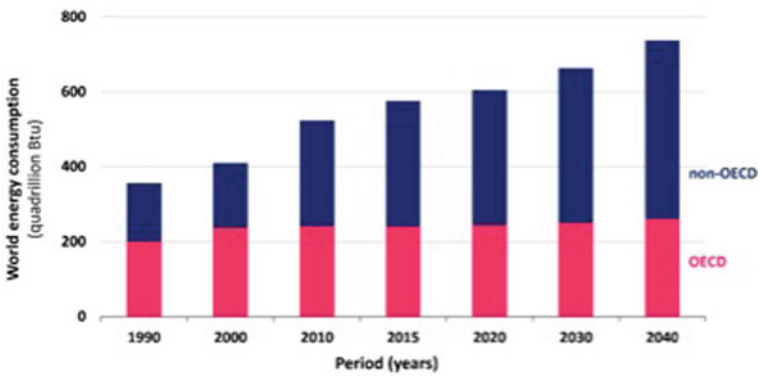

Fig. 11.1 The worldwide energy consumption for different time period (Adapted and modified from IEO 2017, an open source article)

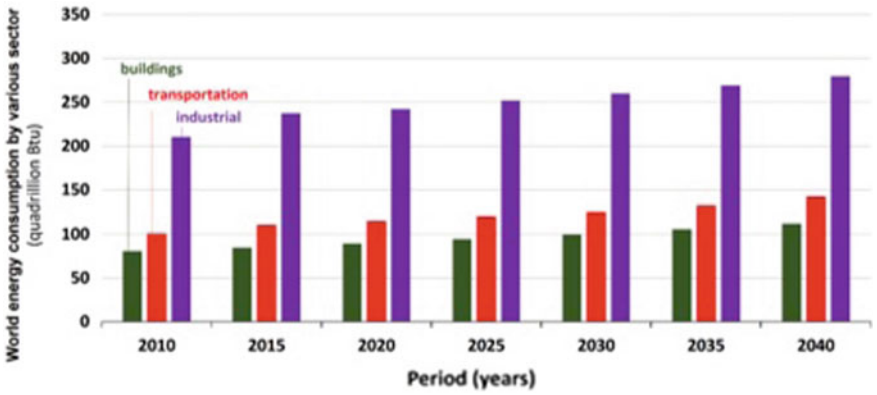

Fig. 11.2 World energy consumption by various sectors for different time period (Adapted and modified from IEO 2017, an open source article)

energy consumption over the entire projection period. It is estimated that use of energy in industrial sector all over the world will increase from 237 quadrillion Btu in 2015 to 280 quadrillion Btu in 2040. Similarly, in case of building sector it is expected to increase from 85 quadrillion Btu in 2015 to 112 quadrillion Btu in 2040 and for transportation sector it is expected to increase from 110 quadrillion Btu in 2015 to 142 quadrillion Btu in 2040 (Fig. 11.2) (IEO 2017).

The excessive dependence on the conventional energy sources like coal and petroleum which are directly or indirectly obtained from fossil fuels lead to rapid depletion of resources of fossil fuels in the nature. In addition, it also causes environmental pollution thereby affecting the quality of environment and creates most serious environmental problems like global warming (Demirbas 2008). Due to these serious issues mankind is forced to search and explore renewable alternative sources for energy particularly in the developing nations. Generally, solar energy, energy obtained from water and wind and energy derived from biomass (bioenergy) are considered as common replacement sources for fossil fuels. However, among these sources, energy derived from biomass is gaining a great deal of attention due to abundant presence of biomass on the planet earth (Antunes et al. 2017).

Bioenergy or biofuels are ecofriendly, which does not increase levels of carbon dioxide (CO_2) in the environment and produce very low amounts of sulfur. According to Demirbas (2008), approximately 27 billion tons of CO_2 is emitted annually from the burning of fossil fuels and it is predicted to increase about 60% by 2030. In such situation, production and use of biofuels is only crucial alternative to reduce the carbon footprint. Moreover, it is postulates that biofuels can pave one half of the total energy demand in developing countries by 2050.

Various biofuels like bioethanol, biodiesel and biogas have been commonly used. Bioethanol is considered as principle fuel used as a petroleum substitute. It is mainly produced by the sugar fermentation process, although it can also be produced by the chemical process of reacting ethylene with steam. However, production of bioethanol

via sugar fermentation process is considered as more convenient. The main crops used as rich source of sugar include maize, corn and wheat, waste straw, willow, sawdust, reed canary grass, cord grasses, jerusalem artichoke, myscanthus and sorghum plants. In addition, there are reports on the use of municipal solid wastes to produce bioethanol (http://www.makebiofuel.co.uk/bioethanol-production/). In addition to bioethanol, biodiesel is another alternative renewable source of biofuels, which has the potential to substitute and replace fossil fuels. Biodiesel is defined as a biofuel containing mono-alkyl esters of long chain fatty acids, produced from renewable biolipids using transesterification process. It is generally produced from plant oils and animal fats and has many advantages like it is a clean burning and eco-friendly alternative fuel. Among the various feed stocks used for the production of biodiesel, jatropha, karanja, mahua and castor oils are often used as sources of non-edible oils. In addition, edible oils including soybean, sunflower, rapeseed, palm, etc. are also commonly used for biodiesel production (Hashmi et al. 2016). Similar to bioethanol and biodiesel, biogas is also another source of renewable energy. It is commonly referred to as a mixture of different gases produced by the breakdown of organic matter anaerobically. Various raw materials like agricultural waste, manure, municipal waste, plant material, sewage, green waste, food waste, etc. can be used for the production of biogas. The available approaches for the production of biogas include anaerobic digestion with anaerobic organisms, which digest material inside a closed system, or fermentation of biodegradable materials.

However, there are various conventional approaches being developed for the production of these biofuels from variety of biomass resources like wastes from agricultural, forest, urban sectors (municipal and industrial wastes) (Akia et al. 2014; Ben-Iwo et al. 2016) and organisms like algae (Mondal et al. 2017). Palm trunk and empty fruit bunch, corncobs, wheat straw, sugarcane bagasse, corn stover, coconut husks, wheat rice, etc. are the common agricultural wastes. However, forest residues include hardwood, softwood and switch grass (Limayem and Ricke 2012; Mood et al. 2013; Lee et al. 2014). Despite of huge potential, the utilization of above mentioned biomass for biofuel production is highly limited. Actually, it is mainly due to unavailability of efficient biochemical modification techniques through which biomass can be harvested in a profitable manner (Malik and Sangwan 2012). Therefore, such situation necessitates optimization and development of novel approaches for the sustainable biofuels production. In this context, nanotechnology can play a key role because it is proved to be a blessing in this regard and has extraordinary potential towards the improvement in the efficiency of conventional strategies.

The present chapter is mainly focused on conventional approaches for the production of biofuel. It also summarizes the current trends in nanotechnological advancements and major breakthroughs in the production of biofuels. In addition, risks associated with utilization of various nanomaterials have also been described.

11.2 Conventional Approaches for the Production of Biofuel from Various Biomass

Generally, thermochemical and biochemical approaches are used for the conversion of various biomass to biofuels. Thermochemical conversion is considered as potential route for the production of bio-methanol, biodiesel, bio-oil, bio-syngas and bio-hydrogen. However, biochemical or biological conversion are mainly used for the production of liquid or gaseous fuels using various biological agents through fermentation, anaerobic respiration, etc. (Kumar et al. 2009; Mitrovi et al. 2012). The production of biofuels from lignocellulosic biomass can be divided into two steps. In first step, whole biomass is decomposed to get upgradeable gaseous or liquid platforms. This step is usually performed through thermochemical conversion to produce synthesis gas (by gasification) or bio-oils (by pyrolysis or liquefaction), or through the hydrolysis of lignocellulosic biomass to produce sugar monomers, which are further converted to biofuel like bioethanol through the biochemical conversion (Akia et al. 2014).

As mentioned above, thermochemical conversion methods are mainly achieved through gasification or direct liquefaction. Gasification process has potential to convert various highly distributed and low-value lignocellulosic biomass into synthetic gas, which can be further used for the production of electricity, heat, liquid fuels, synthetic chemicals and hydrogen (H_2) production. The gasification process is considered of prime importance because variety of lignocellulosic biomass can be considered appropriate for this process (Luo and Zhou 2012). Gasification of biomass can be carried out by two different ways i.e. low-temperature gasification (LTG) and high-temperature gasification (HTG). However, use of specific gasification approach depends on the production of type of biofuel (Ozaki et al. 2012).

The process in which biomass can be converted to liquefied products with the help of combinations of various physical and chemical reactions is called as direct liquefaction. In this approach specific biomass macromolecules are decomposed to small molecules by heating and sometimes in the presence of a catalyst. Moreover, direct liquefaction is generally achieved by liquefaction and pyrolysis methods. The operation temperature for liquefaction method is in between 250–325 °C, whereas, for pyrolysis methods it is in the range of 377–527 °C (Akia et al. 2014).

In addition, various biological and biochemical approaches have been developed for the production of biofuels. Different biological agents such as plants and microorganisms are commonly used directly or indirectly for efficient biofuel production. Among, microorganisms, photosynthetic organisms such as cyanobacteria and algae have also attracted a great attention for third generation biofuel production, but utilization of these photosynthetic organisms has certain limitations like cell growth rate is quite low, and thus the productivity of the metabolites is significantly low (Sheehan 2009; Sarkar and Shimizu 2015). Similarly, other microorganisms like *Saccharomyces cerevisiae*, *Hanseniaspora uvarum* and *Starmerella bacillaris* (Wang et al. 2014; Ingle et al. 2017). *Zymomonas mobilis* (Galbe and Zacchi 2007), *Pichia stipites*, *Kluyveromyces marxianus* (ex fragilis) (Sheikh et al. 2016), *Aspergillus*

niger and *Mucor mucedo* (Oyeleke and Okansanmi 2008; Li et al. 2009) are most frequently used for fermenting ethanol from various plant based raw materials in industrial processes. Among the plants, edible and non-edible oil seed crops viz. soybeans, rapeseed, canola, mustard, camelina, cotton, seasem, olive, castor bean, safflower, sunflower, jatropha, etc. (Ahmad et al. 2011; Ardebili et al. 2011; Chang et al. 2017) are commonly used for biofuel production. However, use of edible and non-edible oils is not economically viable and also low yield of biofuel is major concern associated with it. Hence, the development of economically viable, safe and most efficient strategies are essentially required. Considering the recent nanotechnological developments in the field of biofuel and bioenergy production, researchers around the globe are trying to utilize various nanomaterials for rapid and efficient production of biofuels.

11.3 Role of Nanotechnology in Biofuel Production

Nanotechnology is gaining great deal of attention in field of environmental sciences for sustainable development. It is the promising branch of science, which is applied for assessment of new technological replacements. Hence, researchers around the globe showed considerable interest in the use of nanomaterials for process development, which is aimed at exploiting the unique phenomenon associated with nano scale size materials to improve their function (Puri et al. 2013). Recent studies have revealed the various nanotechnological breakthroughs, which have improved the efficiency of bioresources as an energy source (Malik and Sangwan 2012). Moreover, nanotechnology can provide promising solutions for the production of various biofuels including bioethanol, biodiesel and biogas (Antunes et al. 2017). However, different nanomaterials, such as nanoparticles, metal oxide nanoparticles, carbon nanotubes, etc. are most commonly used as nanocatalysts for sustainable biofuels production (Rai et al. 2016; Palaniappan 2017).

Among nanoparticles, magnetic nanoparticles, metal nanoparticles, acid functionalized nanoparticles, etc. have been successfully exploited directly or indirectly for the production of different biofuels mentioned above. Among these, magnetic nanoparticles are the foremost choice of researchers due to their ability to reuse repeatedly because of magnetic properties. The application of all the nanoparticles in the production of biofuels has been briefly discussed here.

11.3.1 Magnetic Nanoparticles

Generally, cellulases and lipases are the two most important candidates involved in the large-scale enzymatic biofuel production (Trans et al. 2012; Verma et al. 2013). Enzyme-based hydrolysis of lignocellulosic biomass can be improved economically by increasing thermal stability, efficiency and reusability of enzymes through the

immobilization of enzymes on support matrixes like nanomaterials (Puri et al. 2012; Zhang et al. 2012). The available reports suggested that immobilization of such enzymes on magnetic nanoparticles play significant role in the synthesis of biofuels. Moreover, due to their supermagnetic properties, thus immobilized enzymes can be easily recovered by applying simple magnetic field and recycled and reuse (Alftren and Hobley 2013).

Various attempts have been made for the immobilization of cellulase on magnetic nanoparticles and used for enzymatic hydrolysis of various biomass. It was reported that immobilization of cellulase isolated from *Aspergillus niger* on β-cyclodextrin-conjugated magnetic nanoparticles can be effectively used for the degradation of rice straw. The results obtained showed that immobilized cellulase hydrolysed higher concentration of glucose as compared to free cellulase. Moreover, 85% of immobilized enzyme can be recovered by applying magnetic field and reused for continuous hydrolysis (Huang et al. 2015). Similarly, Song et al. (2016) demonstrated that immobilization of β-glucosidase A and cellobiohydrolase D on magnetic nanoparticles effectively used for the conversion of cellulosic biomass into sugar for bioethanol production. Moreover, immobilization of β-glucosidase on magnetic nanoparticles and their efficacy towards the hydrolysis of cellobiose was studied by Verma et al. (2013). From the results obtained it was revealed that immobilization of enzyme increases the catalytic activity of enzyme. Moreover, after magnetic recovery about 50% of catalytic activity was maintained up to 16th cycle hydrolysis. Zang et al. (2014) demonstrated the significant efficacy of magnetic chitosan (Fe_3O_4-chitosan) nanoparticles for immobilization of cellulase through covalent bonding using gluteraldehyde as a coupling agent at pH 5 and 50 °C.

However, some of the recent studies also proved that immobilization of enzyme on magnetic nanoparticles provide considerable stability to the enzyme and increase their catalytic activity. Manasa et al. (2017) reported that cellulase immobilized on zinc ferrite nanoparticles significantly increases enzymatic hydrolysis of previously pretreated *Crotalaria juncea* biomass. About 74% binding efficacy of enzyme was reported at pH 5 and temperature 60 °C using glutaraldehyde. Moreover, it was observed that immobilization provide thermal stability to the enzyme which remain stable at 60 °C and retain its activity up to 3 recycles. Similarly, cellulase recovered from *Trichoderma reesei* and immobilized on chitosan-coated magnetic nanoparticles by covalent bonding using glutaraldehyde can retain about 80% of its activity even after 15 cycles of repeated use in the hydrolysis of carboxymethylcellulose (Sanchez-Ramirez et al. 2017).

Like cellulases, lipases isolated from various sources have been immobilized on different magnetic nanoparticles and utilized for the production of biodiesel. The immobilization of lipase on modified magnetic Fe_3O_4NPs (amino-functionalized) via covalent bonding using glutaraldehyde as a coupling reagent showed potential catalytic activity. It was found that 70% of immobilized enzyme can be recovered by applying magnetic field, besides this, it showed over 90% of conversion of soybean oil at 60% binding efficacy. Further study showed that the immobilized lipase could be used four times without significant decrease of activity (Xie and Ma 2009). In another similar study, Xie and Wang (2014) demonstrated the significant application

of immobilized lipase on Fe_3O_4/Poly (styrene-methacrylic acid) magnetic microsphere for the enhanced production of biodiesel from soybean oil. Karimi (2016) confirmed the application of immobilized lipase on functionalized superparamagnetic iron oxide nanoparticles for the production of biodiesel. In this study, the authors immobilized lipase enzyme isolated from *Burkholderia cepacia* on silica coated iron oxide nanoparticles and applied for enzymatic transesterification of waste cooking oil. It was reported that the conversion of waste cooking oil to biodiesel reached to 91% in methanol: oil molar ratio of 6:1, immobilized lipase at concentration of 25 wt%, *n*-hexane content of 10 wt%, water content of 10 wt%, reaction temperature of 35 °C, and reaction time of 35 h. The findings reported in all of the above studies confirmed that the magnetic nanomaterials can be used as potential nanocatalysts for the sustainable production of biofuels likes bioethanol and biodiesel.

11.3.2 Metal and Other Nanoparticles

Compared to magnetic nanoparticles other metal nanoparticles are very rarely used in biofuel production. In this context, an attempt was made on immobilization of two types of mesoporous silica nanoparticles having different particle size, pore size and surface area by physical adsorption and chemical binding. Further, it was reported that cellulase immobilized on mesoporous silica nanoparticles by covalent bonding and having large pore size showed effective cellulose-to-glucose conversion exceeding 80% yield and excellent stability (Chang et al. 2011). However, there is another report on immobilization of cellulase on silver and gold nanoparticles. It was observed that immobilized enzyme can be recovered and reused up to 6 times with 73–78% retaining activity for the hydrolysis various cellulosic materials (Mishra and Sardar 2015). Nanoparticles such as nickel has also been reported to act as catalyst in the conversion of lignocellulosic material into bioethanol (Srivastava et al. 2017).

It is well known fact that methanogenic bacteria essentially require iron, cobalt and nickel for the anaerobic digestion in traces; in this context, Feng et al. (2010) proposed the possibility of replacing such elements with their respective nanoparticles for the production of biogas. Abdelsalam et al. (2015) demonstrated the efficacy of cobalt and nickel nanoparticles towards enhanced methane gas production. It has been also reported that the cobalt and nickel nanoparticles at different concentrations, enhance the anaerobic process which increase the biogas production. Major findings of the study revealed that the addition of cobalt and nickel nanoparticles reduced the time of biogas production by stimulating the activity of methanogenic bacteria (Abdelsalam et al. 2017). Further, studies on comparative evaluation of efficacy of Fe and Fe_3O_4NPs, it was observed that Fe_3O_4NPs showed better catalytic activity and yields of the highest biogas and methane production from anaerobic digestion of cattle dung (Abdelsalam et al. 2016). Casals et al. (2014) reported the enhancement of the activity of disintegration and also increasing in the yield of methane and biogas production when Fe_3O_4NPs were applied to the organic waste in the anaerobic digester.

11.3.3 Acid Functionalized Nanoparticles

The potential conventional pretreatment methods for lignocellulosic biomass includes acid and alkali based approaches. In this context, acid-functionalized nanoparticles are believed to play key role in hydrolysis of various biomasses, which are further used for biofuel production. Some of the studies carried out revealed that acid-functionalized magnetic nanoparticles like sulfamic and sulfonic silica-coated crystalline Fe/Fe_3O_4 core/shell can be effectively used in biodiesel production via transesterification of glyceryl trioleate. Of these, sulfamic acid-functionalized nanocatalysts showed comparatively higher activity as compared to sulfonic acid-functionalized nanocatalysts (Wang et al. 2015). Moreover, it was demonstrated that silica-coated nanoparticles functionalized with three different acid such as perfluoropropyl-sulfonic acid, carboxylic acid and propyl-sulfonic acid also efficiently hydrolyse the β-1,4 glycosidic bond of the cellobiose molecule. Among these, propyl-sulfonic and perfluoropropyl-sulfonic acid functionalized nanoparticles showed significant catalysis of wheat straw hemicelluloses with glucose yield of 90 and 58% respectively (Duque 2013).

Similarly, various other acid functionalized nanoparticles such as silica-coated magnetic nanoparticles functionalized with alkylsulfonic acid (AS-SiMNPs) and perfluoroalkyl-sulfonic acid (PS-SiMNPs) (Gill et al. 2007), silica-coated cobalt spinel ferrite nanoparticles (Pena et al. 2011), acid-functionalized perfluoroalkyl-sulfonic acid, alkylsulfonic acid and butylcarboxylic acid (Pena et al. 2012), silica-coated nanoparticles functionalized with propyl-sulfonic acid (Pena et al. 2014), etc. are reported to play a vital role in the conversion of biomass into fermentable sugars, which further can be easily converted to bioethanol.

11.3.4 Carbon Nanotubes (CNTs)

Although, CNTs have desirable properties like chemical stability, high surface area, low toxicity, etc. required for an ideal catalyst, a few attempts have been made towards the utilization of CNTs in biofuels production. However, studies performed showed that application of carbon based catalyst for biodiesel production is promising because use of carbon material is cost–effective as their precursors are renewable (Peng et al. 2005). Recently, Guana et al. (2017) developed the facile technique for synthesis of sulfonated multi-walled CNTs as a solid acid catalyst for biodiesel production. The results indicated that the sulfonated multi-walled CNTs showed significant catalytic activity for transesterification of triglycerides in biodiesel production because of its suitable interval porosity size, high dispersion, high acid sites and surface area.

11.3.5 Metal-Oxide Nanoparticles

In addition to various nanoparticles mentioned above, a variety of metal oxide nanoparticles (naked or after functionalization) have also been exploited for the production different biofuels like bioethanol, biodiesel and biogas. As discussed earlier, enzyme-like cellulases and hemicellulases plays a crucial role in the bioethanol production. In this context, Srivastava et al. (2016) demonstrated that immobilization of cellulase enzyme recovered from *Aspergillus fumigatus* on zinc oxide nanoparticles (ZnONPs) provide thermal and pH stability to such crude enzyme. It was observed that immobilization of cellulase can sustain the enzyme thermal stability up to 65 °C for 10 h and also showed pH stability in the alkaline pH range and retained its 53% of relative activity at pH 10.5. In another study, it was reported that immobilization of cellulase on functionalized Fe_3O_4 magnetic nanosphere increases the stability of enzyme and also retain about 87% native activity (Zhang et al. 2015).

Similarly, biodiesel which is known for less polluting, renewable and biodegradable properties can be conventionally produced by the alkali-catalyzed transesterification of triglycerides. However, the use of nanocatalysts can make the production process cost–effective and environmentally friendly. It was demonstrated that calcium oxide nanoparticles (CaONPs) and magnesium oxide nanoparticles (MgONPs) prepared by sol-gel and sol-gel self-combustion methods respectively can be potentially used for production of biodiesel. The results obtained revealed that CaONPs nanoparticles showed significant increase in the biodiesel yield compared to MgONPs (Tahvildari et al. 2015). Moreover, in another study, it was reported that CaONPs synthesized from calcium nitrate and Snail shell showed efficient transesterification of soybean oil and enhance biodiesel yield from 93 to 96%; however, the optimum conditions for the highest yield were 8 wt% catalyst loading, 65 °C temperature, 12:1 methanol/oil molar ratio, and 6 h for reaction time (Gupta and Agarwal 2015).

Considering the advantage of nanocatalysts, Alves et al. (2014) demonstrated the use of mixture of magnetic iron/cadmium and iron/tin oxide NPs synthesized by co-precipitation method for the production of biodiesel from soybean oil. The nanocatalysts thus used showed significant potential towards hydrolysis, transesterification and esterification of soybean oil and their fatty acids. It was reported that among these two, iron/tin oxide NPs showed maximum efficacy with 84% yield of biodiesel.

In another study, Qiu et al. (2011) used zirconia dioxide (ZrO_2) nanocatalyst (10–40 nm) loaded with potassium bitartrate ($C_4H_4O_6HK$) for the production of biodiesel through the transesterification of soybean oil and methanol. Various parameters such as concentration of nanocatalyst, reaction temperature, time of reaction, ratio of soybean oil and methanol were optimized. Interestingly, it was observed that, methanol and soybean oil in the ratio 16:1 having 6.0% nanocatalyst at 60 °C for 2.0 h showed maximum biodiesel yield of about 98.03%. Moreover, it has been reported that λ-Al_2O_3-supported catalysts showed high activity in heterogeneous reactions including transesterification for biodiesel production (Noiroj et al. 2009).

Recently, Tang et al. (2017) developed a solid base catalyst, nano KF/Al_2O_3, for glycerol free production of biodiesel using λ-Al_2O_3 nanoparticles as support, through the tri-component coupling transesterification of methanol, canola oil, and dimethyl carbonate (DMC). The results obtained showed maximum yield of biodiesel (98.8%), at optimum obtained such as KF loading of 10.0 wt%, calcination temperature of 400 °C, 2 h of reaction time at 338 K, 5.0 wt% catalysts and molar ratio of methanol/oil/DMC of 8:1:1. In addition, it was proposed that the yield of biodiesel can be greatly enhanced by the increasing the surface area of λ -Al_2O_3-supported nanoparticles.

Kumar et al. (2012) impregnated potassium ions in calcium oxide nanoparticles (CaONPs) to promote its basicity and prepared a solid base nanocatalyst for the production of biodiesel. However, in another study, Puna et al. (2014) developed nanocrystalline Li/CaO catalyst using wet impregnation technique, followed by calcinations at 575 and 800 °C and used for the production of biodiesel by transesterification of soybean oil. Feyzi and Norouzi (2016) synthesized a magnetic nanocatalyst Ca/Fe_3O_4@SiO_2 using sol-gel and incipient wetness impregnation approaches. Further, these nanocatalysts were evaluated for the production of biodiesel and it was reported that it yield of about 97% of biodiesel from sunflower oil. In another study, Reddy et al. (2016) demonstrated the efficacy of calcium oxide (CaO) nanocatalyst synthesized from seashell, *Polymesoda erosa*. The results obtained suggested that CaO nanocatalyst can be effectively used for the conversion of Jatropha oil into biodiesel with maximum yield of 98.5%. In addition, various other studies have been performed using different nanocatalysts for biodiesel production these includes CaO/SiO_2 (Moradi et al. 2014), and CaO on alumina (Umdu et al. 2009). Recently, Abdel-Razek et al. (2017) have demonstrated the efficacy of aluminium oxide nanoparticles having size of about 10 nm towards the production of biodiesel from Jatropha plant.

For the production of biogas, Abdelsalam et al. (2016) reported that nanoparticles such as Fe, Fe_3O_4, nickel (Ni), and cobalt (Co) can yield the highest biogas and methane from anaerobic digestion of cattle dung. Also, Casals et al. (2014) reported that when Fe_3O_4 nanoparticles were applied to the organic waste in the anaerobic digester, enhancement of the activity of disintegration as well as increasing yield of methane and biogas production was observed. Moreover, nanocatalysts which are extensively used for the production of biofuels have been summarized in Table 11.1.

11.4 Conclusions

The rapid and continuous depletion of fossil fuel resources and environmental problems associated with the extensive consumption of these fuels are the prime concerns all over the world. Although, in recent era various attempts have been made towards the utilization of renewable bioresources like lignocellulosic materials for the sustainable production of biofuels, these attempts failed to fulfil the global demand of

Table 11.1 Different nanocatalysts and feedstocks used for the production of various biofuels

Nanocatalysts	Types of biofuels	Feedstocks	References
CaO–MgO	Biodiesel	Rapeseed oil	Yan et al. (2008)
MgO	Biodiesel	Sunflower oil and rapeseed oil	Verziu et al. (2008)
$CaO–Al_2O_3$	Biodiesel	Palm oil	Zabeti et al. (2009)
Li–CaO	Biodiesel	Sunflower oil	Alonso et al. (2009)
Fe_3O_4NPs	Biodiesel	Soybean oil	Xie and Ma (2009)
CaO–ZnO	Biodiesel	Sunflower oil	Alba-Rubio et al. (2010)
Li–CaO	Biodiesel	Karanja oil and Jatropha oil	Kaur and Ali (2011)
$MgO–TiO_2$	Biodiesel	Soybean oil	Mguni et al. (2012)
TiO_2–ZnO	Biodiesel	Palm oil	Madhuvilakku and Piraman (2013)
ZnO	Biodiesel	Olive oil	Molina (2013)
Pd on carbon, Pd on alumina, silica NPs, hydroxyl-functionalized single-walled CNTS, alumina, and Fe_3O_4NPs	Bioethanol	Biomass	Kim et al. (2014)
$CaO–Al_2O_3$ and $MgO–Al_2O_3$	Biodiesel	*Nannochloropsis oculata* (Microalgae)	Chang et al. (2014)
$CaO–SiO_2$	Biodiesel	Corn oil	Moradi et al. (Moradi et al. 2014)
Magnetic composite poly(styrene-methacrylic acid) microsphere,	Biodiesel	Soybean oil	Xie and Wang (2014)
ZnO/zeolite and PbO/zeolite	Biodiesel	Jatropha oil	Singh et al. (2014)
CoNPs and NiNPs	Biogas and Methane	Raw manure (feces and urine)	Abdelsalam et al. (2015), Abdelsalam et al. (2017)
Fe and Fe_3O_4NPs	Methane	Cattle dung	Abdelsalam et al. (2016)
Iron oxide nanoparticles	Biodiesel	Waste cooking oil	Karimi (2016)
$Ca/Fe_3O_4@SiO_2$	Biodiesel	Sunflower oil	Feyzi and Norouzi (2016)
$CaO–Al_2O_3$	Biodiesel	Jatropha oil	Hashmi et al. 2016
CaO	Biodiesel	Jatropha oil	Reddy et al. (2016)
Fe_3O_4NPs	Methane	Municipal solid waste	Ali et al. 2017

energy. Considering the recent advancement of nanotechnology in the field of biofuel and bioenergy, it is believed that nanotechnology will bring out novel breakthroughs in this field. Various nanomaterials particularly, magnetic nanoparticles provide solid support for the immobilization of enzymes involved in biofuel production, which significantly increases their thermal stability and catalytic efficacies. In addition, immobilization of enzymes on magnetic particles allow the repeated use of same enzyme for more than one cycles, which ultimately helps in the development of cost effective technology for biofuel production. Similarly, other nanomaterials like oxide nanoparticles, CNTs, acid functionalized nanoparticles are also found as promising materials as far as sustainable biofuel production is concerned. Overall, nanotechnological approaches have been found to be more convenient, rapid and eco-friendly. However, more thorough research is required, which should focus on technical bottlenecks such as biocompatibility issues, restricted mass transfer, enzyme leaching upon reuse, toxicity of nanomaterial used, etc.

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Chapter 12
Ester-Based Biofuels from Wastes

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Abstract Nowadays, an increasing worldwide interest in the use of renewable energy sources and the production of biofuels through the use of agro-industrial waste biomass as substrate for biofuels has become a necessity. Therefore several studies have reported the use of wastes for biofuel production including ethanol, methane and hydrogen. Another biorefinery concept for wastes exploitation includes its use as a substrate for anaerobic acidogenesis, to produce organic acids (OAs). Low molecular weight OAs have many applications, including biogas and biodiesel production, and their production through anaerobic fermentation of waste biomass has the advantage of being a cost-effective and environmentally friendly process. The produced OAs can be esterified with the produced ethanol or/and with an added alcohol, to esters that may be used as a new biofuel. The use of such esters in a homogeneous charge compression ignition engine, gave promising results for the use of such alternative liquid biofuel. This chapter presents different aspects of the production of an ester-based biofuel from wastes. Agro-industrial waste is being used as the raw material for organic acid production through microbial processes, which after passing through the esterification process, will lead to the production of a 2nd generation biofuel.

Keywords Wastes · Anaerobic fermentation · Acidogenesis · Esters · Biofuels

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12.1 Introduction

The agro-industrial waste is defined as the organic and non-organic residues generated by the activity of the production and processing of raw materials such as agricultural (coffee, sugar, cereal, etc.), livestock (meat production and packing industries), dairy products etc.

In any case, the production process is complicated and involves steps such as cleaning the raw material, removing any impurities, processing, production and packaging. At each of these stages, waste and by-products are produced which result from losses of raw material, product losses, washing, condensation and cooling water, and mainly from the residues resulting from the processing of the feedstock. Agro-industrial waste contains an organic load in concentrations that vary considerably depending on the raw material used and the type of product produced. Data show that agro-industrial waste accounts for over 30% of global agricultural production (Ugwuanyi et al. 2009). In the international literature there is an extensive reference to the methods of utilization of residues/wastes produced by the industries processing them and processing and producing all kinds of agricultural products. Interest is focused on the fact that when deposited in the environment, agro-industrial waste produces negative environmental, social and economic impacts. The impact of agro-industrial waste on the environment and its method of proper management varies according to the product from which it is produced (Prazeres et al. 2012; Yadav et al. 2015). Utilizing these raw materials, wastes or by-products can reduce unit production costs and at the same time create new raw materials, technologies and products with high added value, leading to new jobs from this progress. This often results in the production of many products from a side-stream in industrial production with the creation of high added value.

At the same time, the need to shift to renewable and more environmentally friendly sources of energy strengthens the development of modern biofuel production methods that meet both small energy consumption and the solution of technical problems in their production unit, as well as an interest in production costs. This chapter was designed and based on these needs, and its main purpose was to present the production of 2nd generation biofuel using agro-industrial waste as the raw material. The biofuel produced from waste is a blend of lower fatty acid esters with small carbon chain alcohols. It can be synthesized by chemical (Marx 2016), enzyme (Vázquez et al. 2016) and microbial processes (Ledesma-Amaro et al. 2016a, b). Anaerobic digestion is mostly used to produce energy through biogas and biohydrogen. However, gas production is more time-consuming than the preceding oxidation stage. The approach of anaerobic digestion in acidogenesis conditions has also been done by other investigators who have sought to investigate the process on synthetic carbon donor glucose substrates (Ren et al. 1997) and anaerobic glucose oxidation in the presence of γ-alumina as a promoter (Syngiridis et al. 2013; Lappa et al. 2015). These studies have revealed the potential for producing ethanol and organic acids at the same time from simple carbohydrates and waste from the food industry. Glucose in this case has been used as a model compound since it is a product of hydrolysis of

cellulose. The transformation to esters production from biomass and agro-industrial side streams, instead to bio-ethanol production is proposed in order to avoid the hydrolysis step of cellulose to glucose, which can be achieved difficult. Esters of low molecular weight aliphatic organic acids have been proved as suitable bio-fuel for engines of automobiles (Contino et al. 2011).

12.2 The Esters of VFAs as Biofuels

In many cases, volatile fatty acid esters belong to the biodiesel category (Westfall and Gardner 2011). It is a fact that microbial production of chemicals and fuels derived from fatty acids is a topic with a variety of research implications due to the limited resources and high variability of conventional fuels. Therefore, the use of certain micro-organisms producing volatile fatty acid esters constitutes an important prospect for biodiesel production.

However, fatty acid biosynthesis is usually not high enough to develop an efficient production scale. For this reason, efforts are directed towards the development of appropriate metabolic strategies for significant fatty acid production and, as a result, of their esters (Valle-Rodríguez et al. 2014). As biodiesel consists of fatty acid esters, not only the fatty acid structure but also the ester itself derived from different alcohols can affect the properties of the fuel. Since the transesterification reaction of an oil or fat leads to the production of biodiesel with properties dependent on the properties of the oil or fat used as feedstock, biodiesel is a mixture of fatty esters wherein each ester contributes to the properties of the fuel. These properties are related to ignition quality, combustion heat, cold flow, oxidative stability, viscosity, etc. (Knothe 2005). Butyl butyrate is compatible with fossil fuels and has similar properties to alternative fuel substitutes such as ethyl acetate, ethyl propionate and ethyl butyrate. At the same time, as interest in alternative fuels and other transport sectors other than terrestrial ones is increasing, it is important that Butyl butyrate also demonstrates compatibility with kerosene. It is, without doubt, a promising additive for the aviation sector.

The challenge in this case is to examine the behavior of these fuels at low temperatures and their effect on the operation of aircraft. At present, the main application of the esters has an auxiliary form as they are used to produce blends with conventional fossil fuels. Contino and co-workers proved that the esters of low molecular weight are suitable to be used as a fuel in automobiles.

12.3 VFAs Production

12.3.1 Anaerobic Digestion

Anaerobic digestion involves the degradation and stabilization of organic materials under anaerobic conditions with microbial organisms and leads to the formation of biogas and microbial biomass (Chen et al. 2008). More specifically, this process is the result of a series of metabolic interactions between different groups of microorganisms. For this reason, anaerobic digestion is considered as a complex process (Khan et al. 2016). Anaerobic digestion is carried out in three stages: (i) hydrolysis/liquefaction, (ii) oxidation and (iii) methanogenesis. The first group of microorganisms secretes enzymes, which hydrolyze polymeric materials in monomers such as glucose and amino acids (Verma 2002). The hydrolysis process is mainly carried out by hydrolytic microorganisms such as *Bacteroides*, *Clostridium*, *Micrococcus*, *Selenomonas*, and *Streptococcus* (Khan et al. 2016). Then the monomers are converted by the oxygenic bacteria into higher volatile fatty acids, H_2, and acetic acid. The final step involves the conversion of H_2, CO_2 and acetates through methanogenic bacteria into CH_4 (Verma 2002).

The treatment through anaerobic digestion helps to reduce pollution from agricultural and industrial activities and contributes substantially to the development of biofuel production methods aiming at gradual release from conventional and non-renewable sources of energy (Chen et al. 2008; Adekunle and Okolie 2015).

12.3.2 Acidogenesis (Mechanism Etc.)

Acidogenesis involves multiple reactions to convert the hydrolysis products to low molecular weight organic acids (OA) and alcohol. The final synthesis of acids and alcohol in this step depends on the amounts of sugars, amino acids and fatty acids produced during hydrolysis. In the case of sugar oxidation, microbes have the ability to shift their metabolism to more reduced organic metabolites, depending on conditions that include pH, hydrogen and partial pressure. It is generally accepted that under conditions of low pH and high levels of hydrogen and formate, more reduced metabolites are produced, such as butyrate, lactate and ethanol.

Acidification of sugars under anaerobic conditions can yield high levels of hydrogen. The degradation of hexoses with mixed anaerobic microbial cultures has been extensively studied and it has been found that hydrogen and various metabolic products, mainly volatile fatty acids (such as acetic, propionic, butyric and lactic acid) and alcohols (butanol and ethanol) depending on the present microbial species and the prevailing conditions. The yield of hydrogen can be stoichiometrically correlated with the final metabolic products with the main reactions describing the individual processes of acidogenesis:

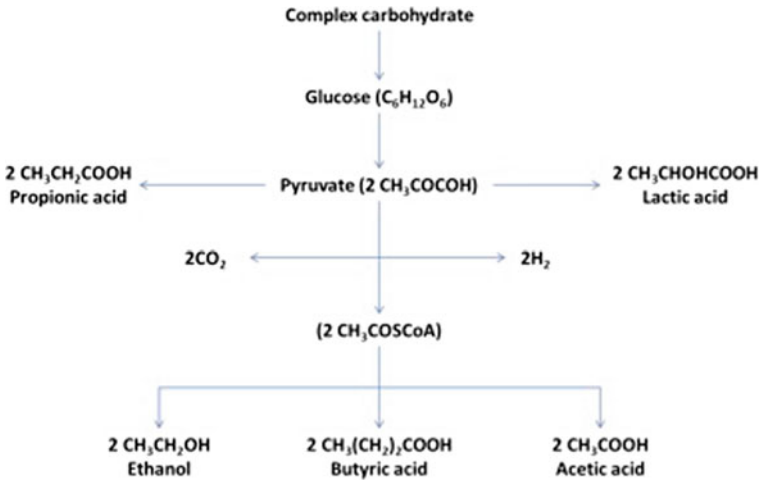

Fig. 12.1 Potential products during acidogenesis (adapted after modification from Ren et al. 1997)

$$C_6H_{12}O_6 + 2H_2O \leftrightarrow 2CH_3COOH + 2CO_2 + 4H_2$$

$$C_6H_{12}O_6 \leftrightarrow CH_3CH_2CH_2COOH + 2CO_2 + 2H_2$$

$$C_6H_{12}O_6 + 2H_2 \leftrightarrow 2CH_3CH_2COOH + 2H_2O$$

The above reactions show that the production of acetic and butyric acid leads to the simultaneous production of hydrogen. The acidogenic step can be adjusted to give hydrogen together with volatile fatty acids and other metabolites. In this process, elimination of hydrogen-consuming microorganisms, pH control and substrate and product concentration (hydrogen) regulation are the key operating factors that drive hydrogen yield (Stamatelatou et al. 2014).

There are two widely known types of fermentation in acidogenesis (butyric and propionic acid). Butyric type fermentation is characterized by the production of butyric acid, acetic acid, CO_2 and H_2, while the propionic type fermentation produces predominantly propionic, acetic, and in some cases valeric acid, without significant gas production (Fig. 12.1).

Available data suggest that fermentation products depend on the substrate used but also on operating conditions such as residence time, organic load, temperature and pH. In fact, pH plays an important role in the oxidation reactor efflux (Ren et al. 1997; Yu and Fang 2003).

12.3.3 Simultaneous Bio-Ethanol and VFAs Production

In order to produce organic acids which are subsequently converted into esters for the production of biofuels, several studies have been carried out using waste as substrates, thus reducing production costs and environmental pollution. Due to the anaerobic digestion of glucose resulted to relatively low concentration of organic acids γ-alumina pellets were used and promoted the rate of fermentation and increased also the final concentration of OA (Syngiridis et al. 2013, 2014). Furthermore, γ-alumina and kissiris promoted the acidogenesis of sucrose and rafinose as model compounds for the acidogenesis of vinasse and proved also the acidogenesis of vinasse after adaptation of mixed anaerobic culture Lappa et al. (2015). Likewise, the volcanic foaming rock kissiris promoted the acidogenesis of lactose and whey (Boura et al. 2017).

12.3.4 Promotion of Acidogenesis

The acid type fermentation that prevails depends on the conditions of the fermentations (Syngiridis et al. 2014; Lappa et al. 2015; Ren et al. 2016; Boura et al. 2017). From the above mechanism it is possible to produce ethanol, acetic acid, propionic acid, butyric acid, lactic acid and hydrogen as well as CO_2. However, in order to have the potential for industrial application of the simultaneous production of organic acids and ethanol required for the production of esters, the concentrations and productivity of organic acids and ethanol should be increased. This increase is mainly obtained by the use of kissiris as promoter of acidogenesis (Dima et al. 2017).

12.3.5 VFAs as Chemicals

In recent years, huge progress has been observed in the production of commercial goods through biotechnological exploitation of renewable raw materials. In particular, the production of organic acids is a rapidly evolving field, related to the wide application of organic acids for direct use, either as polymer building blocks or as products to replace commodity chemicals (López-Garzón and Straathoff 2014).

12.4 Esterification

The acids produced from the acidogenesis will be used for esterification where when recovered from the fermentation broth relatively high molecular weight alcohols were used to avoid mixing with water and making a layer upon extrusion. Thus, the products can be brought into direct esterification (thermal or enzymatic) as such. Of

course, bioethanol produced during oxygenation will be extracted simultaneously and will participate in esterification. Depending on the raw material used in the oxygenation, a different environment from the different acid composition is created (Lappa et al. 2015; Ren et al. 2016; Syngiridis et al. 2014) through of different pH, different substrates used, and other different contiguous components.

The lipase-catalyzed esterification, in non-aqueous organic solvents, has received increasing interest in the last years given both the recovering of pure products and the industrial and biotechnological significance of organic esters. The insufficient activity of the lipases in non-aqueous organic reaction media has been resolved by immobilizing then on inert matrices. The active structures of lipases seem to be thermally stabilized as compared to those of free enzymes depending on the immobilization method. Nevertheless, the efficiency of the catalysis of the esterification depends strongly on the thermodynamics of the operated reaction system; an example is to keep the activity of H_2O as low as required to facilitate esterification to compete the backward hydrolysis reaction (Foukis et al. 2017).

12.5 Economic Analysis

The proposed technology has been applied with very promising results in wastes such as vinasse (Lappa et al. 2015); (Boura et al. 2017) and straw whiskers (Dima et al. 2017) in laboratory scale. However also some experiments have also been performed in semi industrial scale of 70–100 L and these results were used for the economic evaluation of the integrated technology for a new generation biofuel production using agroindustrial liquid and solid wastes as raw materials at industrial scale (Koutinas et al. 2016). The economic analysis showed that is feasible to develop such technology with competitive production and investment costs. The main characteristics of the technology include:

- Kissiris (culture immobilization carrier) better promoted the acidogenic fermentations compared to γ-alumina.
- Butyric, lactic and acetic acids were predominantly produced from vinasse, whey, and cellulose, respectively.
- Butanol-1 found as an efficient solvent for OAs recovery, which could be serve as reagent for their subsequent esterification.

12.6 The Esters in the Group of Biofuels

Volatile fatty acids (VFAs) can be produced from food waste, sludge and a multitude of biodegradable organic wastes under anaerobic conditions and form the basis for a biofuel production platform. Volatile fatty acids are a possible alternative source of carbon for lipid accumulation by oleogenic microorganisms (Park et al. 2014).

The production of volatile acids takes place at the stage of acidogenesis in anaerobic digestion. Volatiles are usually small chain acids (acetic, butyric) and are produced by the contribution of mixed anaerobic microflora. Anaerobic digestion as a process converts all biomass components (carbohydrates, lipids, proteins) in addition to lignin into volatile fatty acids, and is suitable for the management of organic waste without the need for high cost precursors or additional hydrolytic enzymes (Chang et al. 2010).

Volatile fatty acids are generally not used in internal combustion engines because of their resistance to ignition. For this reason they are esterified with ethanol (which is also produced during the early stages of anaerobic digestion) to form ethyl acetate (EtAc), ethyl propionate (EtPr) and ethyl butyrate (EtBu) (Foukis et al. 2017). The process produces a mixture of various esters in proportions that vary according to the fermentation conditions and the type of biomass. Removing undesirable substances or maintaining a constant proportion of the mixture requires more energy consumption during the separation process.

12.7 Molecular Biology for Strain Improvement in Fermentations

Nowadays, there are growing concerns, around the world, about problems caused by climate change and related to petroleum-based industries. For this reason, biorefineries have attracted the interest for the establishment of biosustainability, by replacing the traditional and very pollutant petroleum-based refineries. This change can be made mainly through the use of metabolic engineering to improve microbial hosts in order to overproduce the desired chemical (Cho et al. 2015). Systems metabolic engineering integrates metabolic engineering with systems biology, synthetic biology and evolutionary engineering in the context of the entire bioprocess (Cho et al. 2015). The term "metabolic engineering" introduced in the late 1980s-early 1990s (Bailey 1991), since that time efficient strategies have been developed to improve microbial strains and therefore the range of chemicals and fuels that can be produced has expanded significantly (Lee et al. 2011, 2012; Ledesma-Amaro et al. 2016a, b; Upadhyaya et al. 2014; Yin et al. 2015).

Systems metabolic engineering develops microbial strains in order to fulfill two main requirements: (i) maximizing the production yield and productivity of the desired chemical and (ii) minimizing the cost of the whole process (Cho et al. 2015). For this reason several molecular techniques have been employed and cultivation conditions have been optimized (Table 12.1).

In recent years several works reported the production of various organic acids, by employing systems metabolic engineering (Table 12.2).

Table 12.1 Metabolic engineering of bacterial strains for overproduction of chemicals and fuels: molecular techniques used, cultivation conditions to be considered and future considerations

Metabolic engineering of bacterial strains	References
Examples of molecular techniques employed	
• Conventional gene knockout and overexpression	Jang et al. (2012)
• Construction of a novel metabolic pathway using promiscuous enzymes	Atsumi et al. (2008), Shen et al. (2011)
• Sophisticated downregulation of gene expression levels	Na et al. (2013), Yoo et al. (2013)
• Multiple enzyme targets	Flowers et al. (2013)
• Multiple genome engineering	Isaacs et al. (2011), Wang et al. (2009)
• Synthetic regulatory circuits	Thieffry (2007)
• Omics analysis	Park et al. (2007)
• In silico modeling and simulation	Yim et al. (2011)
Cultivation conditions to be considered	
• Medium composition	Thompson and Trinh (2014)
• Cultivation modes (i.e., batch versus fed-batch)	Park et al. (2011)
• pH	Zhu et al. (2007)
• Aeration	Causey et al. (2003)
Future considerations	
(i) Cost and availability of starting materials (e.g., carbon substrates) (ii) Metabolic route and corresponding genes encoding the enzymes in the pathway to produce the desired product (iii) Most appropriate microbial host (iv) Robust and responsive genetic control system for the desired pathways and chosen host (v) Methods for debugging and debottlenecking the constructed pathway (vi) Ways to maximize yields, titers, and productivities	Keasling (2010)

Table 12.2 Recent examples of organic acids produced using metabolic engineering

Organic acids	Strains	Metabolic engineering strategies	References
Propionic acid	*Propionibacterium freudenreichii* subsp. *shermanii*	Overexpressing the native propionyl-CoA:succinate CoA transferase	Wang et al. (2015)
Butyric acid	*Clostridium tyrobutyricum*	Engineering to ferment mixtures of xylose and glucose as carbon sources	Fu et al. (2017a, b)
Isobutyric acid	*Pseudomonas* sp. strain VLB120	Overexpression of a 2-keto acid decarboxylase encoding gene	Lang et al. (2014)
C4–C6 acids	*Saccharomyces cerevisiae*	chromosome-based combinatorial gene overexpression, deletion of key genes in competing pathways, overexpression of the ATP-binding cassette transporter PDR12	Yu et al. (2016)
Succinic acid	*Yarrowia lipolytica*	deletion of CoA-transferase gene Ylach, overexpressing the key enzymes of oxidative TCA	Cui et al. (2017)
Succinic acid	*Corynebacterium glutamicum*	overexpression of the NCgl0275 gene, increasing the metabolic flux from PEP to OAA	Chung et al. (2017)
Lactic acid	*Saccharomyces cerevisiae*	overexpressing heterologous lactate dehydrogenase (LDH) genes, while attenuating several key pathway genes, including glycerol-3-phosphate dehydrogenase1 (GPD1) and cytochromec oxidoreductase2 (CYB2)	Song et al. (2016)
Lactic acid	*Monascus ruber*	Introducing genes encoding lactate dehydrogenase (LDH), deleting two genes encoding pyruvate decarboxylase (PDC)	Weusthuis et al. (2017)
Malic acid	*Ustilago trichophora* RK089	overexpression of pyruvate carboxylase, two malate dehydrogenases (mdh1, mdh2), and two malate transporters (ssu1, ssu2)	Zambanini et al. (2017)
Citric acid	*Yarrowia lipolytica*	Overexpression of xylose reductase and xylitol dehydrogenase from *Scheffersomyces stipitis*, overexpression of the endogenous xylulokinase	Ledesma-Amaro et al. (2016a, b)

12.7.1 Strain Development Methods in Acid Production

Nowadays there is a need for the development of improved biocatalysts that will make fermentative processes economically competitive with petroleum-based processes. These biocatalysts may have one or more of the following characteristics: high product yield, titer and productivity. One method used so far, with some positive results, is the use of random mutagenesis for strain improvement, which has been used for the production of lactic acid (Bai et al. 2004). However this technology has many limitations, especially for the production of acids, but nowadays we may overcome them using the new trends in recombinant DNA technology, genomic sequencing, metabolic engineering etc. (Liu and Jarboe 2012).

According to Liu and Jarboe (2012) there are three main processes that may be followed for strain improvement for acid production, namely (i) metabolic engineering by genetic manipulations, (ii) omics analysis and (iii) engineering tolerance to product toxicity.

12.7.1.1 Metabolic Engineering by Genetic Manipulations

The first process, metabolic engineering by genetic manipulations, can improve the strains by either overexpressing key enzymes or inactivating competitive pathways or both of them.

Overexpression of Key Enzymes

Overexpression of either native or heterologous enzymes is an usual approach to increase the production of the desirable product and has been used also for the production of several acids through fermentation.

Xiao et al. (2014) reported the engineering of a recently isolated yeast *Issatchenkia orientalis* SD108 that is tolerant to low pH and high concentrations of organic acids, through enhancing the reductive TCA cycle to produce succinic acid. The engineered strain was able to produce succinic acid with a titer of 11.63 g L^{-1}, yield of 0.12 g g^{-1}, and productivity of 0.11 g L^{-1} h^{-1} in batch cultures using shake flasks. In another recent study, carbon catabolite repression in *C. tyrobutyricum* was eliminated by overexpressing three heterologous xylose catabolism genes (xylT, xylA and xlyB) cloned from *C. acetobutylicum*. Compared to the parental strain, the engineered strain produced more butyric acid (37.8 g L^{-1}) from glucose and xylose simultaneously, at a higher xylose utilization rate and efficiency, resulting in a higher butyrate productivity 0.53 g L^{-1} h^{-1} and yield 0.32 g g^{-1} (Fu et al. 2017a, b). The overexpression of the native propionyl-CoA:succinate CoA transferase in *P. shermanii* resulted in up to 10% increase in propionic acid yield (0.62 vs. 0.56 g g^{-1}) and 46% increase in productivity (0.41 vs. 0.28 g L^{-1} h^{-1}), compared to parental strain (Wang et al. 2015).

Inactivation of Competitive Pathways

One of the ways to increase the production of the desirable product through metabolic engineering is the deletion of metabolic pathways that compete with production of the desirable compound. This type of metabolic engineering has been used to increase the production of several acids through fermentation. Thapa et al. (2017) reported the increased production of lactic acid using the engineered strain *E. aerogenes* SUMI01. This strain produced after genetic engineering of *E. aerogenes* ATCC 29007, by deleting the phosphate acetyltransferase (pta) genes, as acetate is the major byproducts during the L-lactic acid fermentation. The deletion of the pta gene increased the production of lactic acid, compared to wild strain, but also decreased the production of bioethanol, acetate, succinate, and 2, 3-butanediol. The production of 2, 3-butanediol decreased in the engineered strain *E. aerogenes* SUMI01, because the deletion of pta gene increased the lactate concentration in the fermentation broth, ceasing the growth of microorganisms.

Combined Approaches

In many cases the application of combined approaches, overexpressing key enzymes but also inactivating competitive pathways, has been proved more sufficient. A combined approach has been applied in *Saccharomyces cerevisiae* for increased production of lactic acid (Song et al. 2016). More specifically this acid-tolerant strain of *Saccharomyces cerevisiae* was engineered by overexpressing heterologous lactate dehydrogenase (LDH) genes, while attenuating several key pathway genes, including glycerol-3-phosphate dehydrogenase1 (GPD1) and cytochrome-c oxidoreductase2 (CYB2). In addition the ethanol production pathway was also attenuated by disrupting the pyruvate decarboxylase1 (PDC1) and alcohol dehydrogenase1 (ADH1) genes. However this resulted to reduced growth rate of the strain. In order to overcome this problem bacterial acetylating acetaldehyde dehydrogenase (A-ALD) enzyme (EC 1.2.1.10) genes were introduced into the lactic acid-producing *S. cerevisiae* and the results showed an increased glucose consumption rate and higher productivity of lactic acid fermentation. The production yield of 0.89 g g^{-1} and productivity of 3.55 g L^{-1} h^{-1} were reached under fed-batch fermentation in bioreactor. In another similar study *Saccharomyces cerevisiae* has been also explored for lactic acid production (Lee et al. 2015). The metabolic engineering of the strain, in this study, was made in three steps. Firstly a L-lactate dehydrogenase gene from *Pelodiscus sinensis* (LDH) was introduced enabling *S. cerevisiae* to accumulate 27.6 g L^{-1} of L-lactic acid. Then competing pathways that lead to ethanol and glycerol formation were attenuated, increasing lactic acid production up to 35 g L^{-1}. Finally the external NADH dehydrogenase genes were deleted leading to further increase of L-lactic acid production up to 117 g L^{-1} in a fed-batch mode with pH controlled at 3.5. Another combined approach was used for the increase of short chain fatty acids (C4–C6) by engineered *S. cerevisiae*. In this study chromosome-based combinatorial gene overexpression, deletion of key genes in competing pathways, and overexpression of the ATP-binding cassette transporter PDR12 were used (Yu et al. 2016).

12.7.1.2 Omics Analysis

Genetic manipulation has been proved a powerful process for increasing the production of several desirable products, including acids; however its application is limited to already known and previously-characterized enzymes and regulators. In order to overcome this limitations omics analysis can provide the global information from disturbed metabolism and find the potential target genes (Liu and Jarboe 2012). Three are the main processes that included in omics analysis, namely transcriptome analysis, proteomics and flux analysis. Transcriptome analysis can be performed either by DNA microarray or sequencing-based quantification, and is used to identify novel target genes that will improve the strain performance (Hirasawa et al. 2010). Proteomics examines the different levels of proteins and their potential changes due to different genetic and environmental conditions. Therefore proteomics provide information for complicated biological processes and posttranslational modifications (Han and Lee 2006). Finally metabolic flux analysis plays a key role in determining biocatalyst behavior. This process helps to understand the metabolic pathways required for production of the desirable compound and controlling the flux through these pathways can be enormously helpful in strain design and modification (Liu and Jarboe 2012). Some examples of these techniques with applications on organic acid production are presented in the next paragraph.

Transcriptome analysis has been used to increase the acid production in several fermentation systems. It has been used to increase lactic acid production using engineered strains. Engineered *Monascus ruber*, in a fed-batch fermentation, resulted in a maximum lactic acid titer of 190 g L^{-1} at pH 3.8 and 129 g L^{-1} at pH 2.8 using glucose (Weusthuis et al. 2017). In addition engineering of sake yeast resulted in increased production of malic and succinic acid (Yano et al. 2003). Comparative transcriptome analysis has also been performed in an engineered strain of *Corynebacterium glutamicum* resulting to remarkably increased succinic acid production, 152.2 g L^{-1}, with a yield of 1.1 g g^{-1} glucose under anaerobic condition (Chung et al. 2017). Proteomics have been used to increase the production of lactic acid by *E. coli* using xylose as substrate (Utrilla et al. 2012) and also to increase the production of succinic acid by the engineered *Mannheimia succiniciproducens* LPK7 (Lee and Lee 2010). The use of ^{13}C metabolic flux analysis was used to identify undesired fluxes in *Basfia succiniciproducens*. Based on this analysis the stain was engineered by deletion of *pflD* and *ldhA* resulted in a succinic acid yield of 0.71 g g^{-1} glucose (Becker et al. 2013). Lee et al. (2005) performed a flux balance analysis based on a genome-scale metabolic model of *E. coli* and predicted that the deletion of *ptsG* and *pykAF* may increase succinic acid production. Indeed this deletion led to an improved strain of *E. coli* with a 100-fold higher succinate production rate than the wild type strain.

12.7.1.3 Engineering Tolerance to Product Toxicity

One of the major problems in the production of products through fermentation is the accumulation of the product and the toxity of it over the strain used. Therefore in more cases high concentrations of the desirable product are formed but they are toxic for the strain and the fermentation is stopped. Therefore the demand for strains with high tolerant in the final product is high. Metabolic engineering has also answers to that problem and several studies have focused in this area. Several studies has been carried out focusing on the engineering of strains in order to make them tolerant against fermentation products like organic acids but also against several environmental stresses (Deparis et al. 2017; Warnecke and Gill 2005). Class I heat shock proteins (HSPs) play an important role in the process of protecting bacteria from sudden changes of extracellular stress by assisting protein folding correctly. In *Clostridium tyrobutyricum* the Class I HSPs *grpE*, *dnaK*, *dnaJ*, *groEL*, *groES*, and *htpG* were significantly upregulated under butyric acid stress. Overexpression of *groESL* and *htpG* could significantly improve the tolerance of *C. tyrobutyricum* to butyric acid while overexpression of *groESL* resulted to increased butyric acid and acetic acid concentrations than the wild-type strain (Suo et al. 2017). In other study the acetic acid tolerance of *Saccharomyces cerevisiae* was improved by overexpression of *HAA1*, which was achieved by introduction of a second copy of the native *HAA1* (Swinnen et al. 2017). Propionibacterium acidipropionici was also engineered in order to improve its ability to growth and produce propionic acid in a high propionic concentration environment (Zhang and Yang 2009).

12.8 Conclusions

Esters based bio-fuel can be produced through of enzymatically catalyzed esterification of OA produced by promoted acidogenesis by the mineral kissiris from agroindustrial liquid wastes. The acidogenesis has to be done by the mixed anaerobic culture under conditions lead to OA production. The preliminary economic analysis of esters based bio-fuel production looks to be cost effective provided that (i) be overcomed all technical issues could exist at the industrial scale-up of the process and (ii) may be necessary research to improve further the acidogenesis. Metabolic engineering by genetic manipulations, omics analysis and engineering tolerance to product toxicity of various microorganisms are technologies could be applied in specific strains uses fixed carbohydrates and cannot be applied with high yields in wastes containing mixed organic compounds each of them needs a different strain.

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Chapter 13
Sustainable Production of Biogas from Renewable Sources: Global Overview, Scale Up Opportunities and Potential Market Trends

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Abstract Anaerobic Digestion (AD), which is the most prominent bioenergy technology worldwide, is a profitable alternative that provides a sustainable solution to treat organic wastes and reduce greenhouse gases emission, while producing energy in the form of methane, improving fertilizer potency and reducing pollution. The most common substrates used in AD process include animal manure and slurry, agricultural residues and their by-products, organic waste from food industries, organic fraction of municipal waste, sewage sludge, and energy crops. However, the feedstocks have different methane yield and they influence the biodigester operational behavior. Thus, many anaerobic biodigesters designs have been implemented, such as anaerobic sequencing batch reactor, continuous stirred tank reactor, anaerobic plug-flow reactor, anaerobic contact reactor, among others. Biogas produced by different sources is mainly composed by CH_4, CO_2, H_2S, NH_3 and water vapour, which have different impacts on biogas utilization. To reduce those impacts, methods of removal of undesirable components in biogas have been applied, such as condensation and absorption. Technologies for the conversion of renewable energy sources in electricity, heat and steam have undergone substantial progress over the past two decades. The total amount of electricity produced from biogas is 63.3 TWh and is estimated that the global power generation capacity will increased more than double in biogas production over the next decade, from 14.5 GW in 2012 to 29.5 GW in 2022.

Keywords Anaerobic digestion · Bioenergy · Biogas · Technology

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13.1 Introduction

One of the main concerns of the society is the environmental impact caused by fossil fuels over the years. The global climate change due to the increase in greenhouse gases concentrations in the atmosphere and the contamination of water, air and soil are well-known consequences of the burning of fossil fuels, especially in the energy sector (van der Ploeg and Rezai 2017). Nonetheless, in recent years, another problem has arisen: the current reserves of fossil fuels are rapidly decreasing, which signifies the importance of looking for alternative sustainable means of energy production to meet the demand of the future generations (Höök and Tang 2013). This also highlights the need for the search for sustainable energy sources, which are economical and as well as less polluting. Biogas production by anaerobic digestion of organic wastes has gained importance as one of the main bioenergy processes due to the high availability of feedstocks, their low cost, relatively simple technology and the different uses that can be given to the biogas (Al Seadi et al. 2008).

Although AD is considered a mature technology, to this day, the process has been subjected to several changes in order to increase its efficiency. These modifications include the use of unconventional wastes as feedstock, the addition of a pretreatment step, the enhancement in reactor design, the alterations in process continuity, the implementation of a cleaning and upgrading step for biogas, and so many others (Li et al. 2011, Ruffino and Zanetti 2017, Sun et al. 2015, Zheng et al. 2014). The research on AD is clearly abundant, and there are numerous reports with promising advances in biogas technology. However, an important aspect that is usually overlooked is the true commercial potential and how a specific alteration can achieve, by fine tuning energy policies and procedures of a given country. Therefore, for every technological modification in AD or downstream process, an economic and political analysis must be carried out to guarantee the favorable impact on biogas production and its conversion to energy (Budzianowski 2016). This chapter describes the potential of biogas as energy source, the current production technology, current contribution to the global energy needs, upgrading and at the same time highlighting the areas of improvement in biogas production technology.

13.2 Biogas as an Energy Option: An Overview

13.2.1 Environment Impact of Fossil Fuels (Fossil Fuels and Renewable Energy)

Nowadays, greenhouse gases emissions have increased due to the burning of fossil fuels (natural gas, petroleum, coal) in many daily activities, which have contributed to climate change and pollution of air, soil and water (van der Ploeg and Rezai 2017). The concentration of carbon dioxide (CO_2) in the atmosphere has risen drastically over the years since the pre-industrial age, from 280 ppm in the 16th century to

401 ppm in the year 2015. This trend is also accompanied with an increase of global temperature. It is expected a rise of 1.6–5.8 °C above the current global temperature (Prasad et al. 2017). Also, with the increase in population and therefore in services, the global energy demand is growing and with the current supply of fossil fuels it won't be possible to cover it for the future generations (Höök and Tang 2013). For these reasons, the search for energy produced from clean renewable sources has become a priority in order to meet the energy demand and to reduce greenhouse gases emissions (Wang et al. 2017).

Although, renewable energy has become an encouraging trend in recent years, consumption of fossil fuels still dominates the energy market, especially in developing countries, as shown in Fig. 13.1.

13.2.2 Organic Wastes as Source for Renewable Energy: Anaerobic Digestion

Energy generation have become a difficult task that seeks practical ways of obtaining energy with minimal environment damage while using accessible and low technologies that guarantee a sustainable development (Divya et al. 2015).

The use of biomass as an energy source is a good option to replace fossil fuels, since it is considered one of the promising environment friendly renewable energy options because of its high availability, relatively cheap production and management, and the reduction of polluting gas emissions during its treatment (Al-Hamamre et al. 2017; Thomas et al. 2017). However, the use of biomass is limited for its complex structure, often requiring the use of additional treatments and technology that increase the costs for energy production (Sanna 2014).

One of the most effective ways to convert biomass into energy is through anaerobic digestion (AD) (Fig. 13.2). This process comprises the oxidation of organic matter in order to obtain a mix of methane (CH_4), CO_2 and other gases, known as biogas. Since

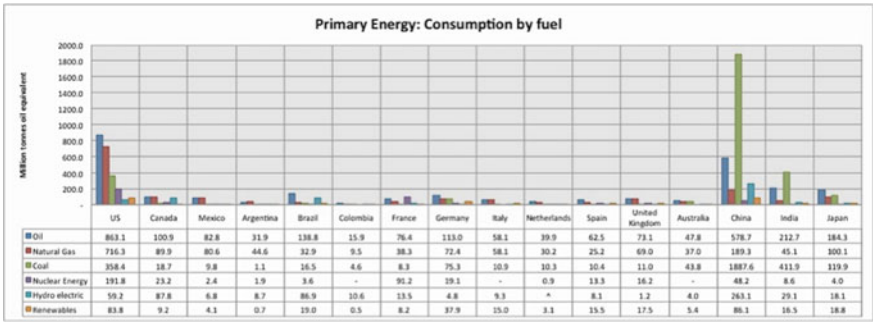

Fig. 13.1 Primary energy: global consumption by fuel. All values in millions of tons of oil equivalent (BP Statistical Review of World Energy June 2017)

the energy stored in biomass remains in the biogas, it can be used as a direct source for heat or it can be converted to electricity (Chynoweth et al. 2001). Also, the solid residue, the effluent, obtained in this process can be employed as an organic fertilizer for several purposes (Nayal et al. 2016; Ruffino and Zanetti 2017). In addition, AD as a source or renewable energy has the potential to improve security of energy supply and help to reduce greenhouse gases (GHG) emissions. It is also useful as an energy source that can be accessed on demand, unlike some other renewables such as wind and solar, which are more intermittent (Whiting and Azapagic 2014).

AD is a process usually divided in four steps: hydrolysis, fermentation or acidogenesis, acetogenesis and methanogenesis. In the first step, complex polymers are hydrolyzed to soluble monomers, which then are converted to volatile fatty acids (VFAs), simple alcohols, hydrogen (H_2) and CO_2 by fermentative bacteria. In the next step, VFAs with a carbon chain longer than 2 are transformed to acetic acid, H_2 and CO_2 by acetogenic bacteria. Finally, methanogens produce methane utilizing as substrates the products from acetogenesis. The overall efficiency of AD depends of several operational parameters such as pH, temperature, organic loading rate, retention time and mixing, as well as of the microbial community composition and dynamics (Chynoweth et al. 2001; Jha and Schmidt 2017).

The equipment of a biogas plant should be able to meet these basic requirements. Therefore, a biogas plant designer should know form the beginning what kind of substrate the plant will feed on so that the right equipment for efficient biogas production can be selected. The process of biogas generation can be characterized by the number of operational steps, the environmental gradients, such as temperature, the

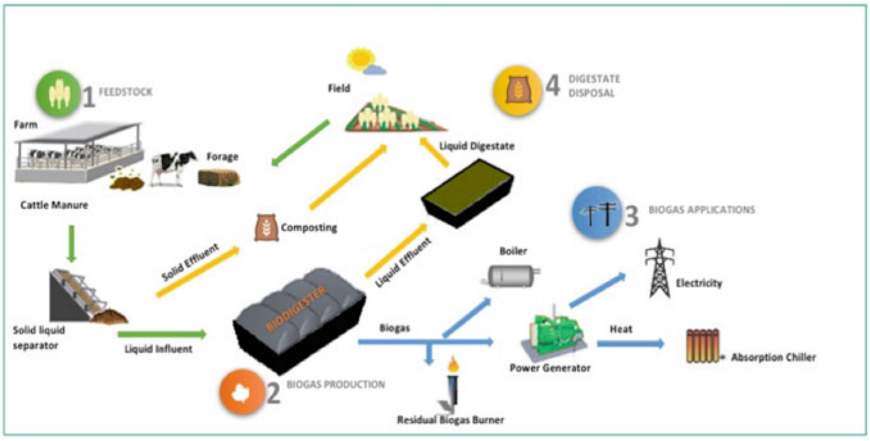

Fig. 13.2 Overview of biogas production and utilization. Biogas production begins with the selection and preparation of feedstock (1). Then, anaerobic digestion takes place (2). Biogas produced is used for heat and electric energy production (3). Finally, effluent of biodigestor is disposed as a fertilizer for crops

dry matter content on the feedstock and the way substrate is fed (Da Costa Gomez 2013).

Anaerobic digestion in the agricultural sector is a very fast growing market around many countries. The success of biogas production will come from the availability at low cost and the broad variety of usable forms of biogas to produce heat, electricity, and for the utilization as a vehicle fuel. Many sources, such as energy crops, industrial wastes, manures, food wastes or algae can be used, also the process can be applied in small and large scales. This allows the production of biogas at any place in the world (Weiland 2010).

13.3 Biomass Resources, Feedstock Treatment and Biogas Production

13.3.1 Biomass Resources for Biogas Production

Almost all types of biomass can be employed as feedstock for AD. The most common substrates include animal manure and slurry, agricultural residues and their by-products, organic waste from food industries, organic fraction of municipal waste, sewage sludge, and energy crops. Traditionally, anaerobic digestions systems have used mainly manure or sewage sludge as substrates because they offer several advantages over other types of biomass. They already have a natural content of anaerobic bacteria and water, and they are cheap with high availability (Al Seadi et al. 2008; Alkanok et al. 2014; Ruffino and Zanetti 2017; Ward et al. 2008).

The selection of feedstock depends of many criteria such as chemical composition, availability, additional pretreatment, carbon to nitrogen ratio (C:N ratio), reactor configurations, and others parameters (Divya et al. 2015). Table 13.1 shows the different characteristics of several biogas feedstocks and their influence in AD process. Other aspect that has to be taken into account during the selection of feedstock is the cost effectiveness of the whole process. According to Budzianowski (2016), AD should be employed for the treatment of wastes that can't be converted into other valuable products than biogas. When the feedstock can go under other type of treatment that yields products with higher value and demand than biogas, AD should not be employed for economical reasons.

The chemical composition of the biomass has a critical influence in the total biogas yield and its composition. Usually, high content of carbohydrates and proteins enhance the degradation rates, but fats are the ones that provide the highest biogas yields (Ware and Power 2016; Weiland 2010). However the use of feedstock rich in fats such as meat industry and slaughterhouse wastes is limited due to the high recalcitrant nature of lipids. They usually generate problems for AD systems like crust formation, which results in pipeline clogging, and bad odor. They also have a negative effect on microorganisms, since they adhere to the cell wall and decrease their degradation rates (Hamawand 2015). The carbon to nitrogen ratio (C:N ratio) is

Table 13.1 Characteristics of some biogas feedstocks and their effect on the AD process (Al Seadi et al. 2008; Steffen et al. 1998)

Type of feedstock	C:N ratio	DM[a] (%)	VS[b] % of DM	VS (%)	Methane yield (m^3 CH_4/kg VS)	Methane production (m^3CH_4/m^3)	Mainly Compound	Digestibility	Process disturbing effects	Process inhibition
Animal wastes										
Pig slurry	7	5	80	4	0.3	12	Proteins	Very good	Foaming	High ammonia concentrations
Pig manure, solid		20	80	16	0.3	48	Proteins	Very good	Foaming	High ammonia concentrations
Cattle slurry	13	8	80	6.4	0.2	12.8	Sugar Starch Cellulose	Very good Very good Poor	Foaming Lignin incrustation	pH decrease
Cattle manure, solid	25	20	80	16	0.2	32	Sugar Starch Cellulose	Very good Very good Poor	Foaming Lignin incrustation	pH decrease
Poultry manure, solid	9	20	80	16	0.30	48	Proteins Cellulose Lignin	Very good Poor Poor	Foaming Lignin incrustation	pH decrease
Stomach/Intestine content, cattle	4	12	80	9.6	0.4	38.4		Very good	Foaming	pH decrease
Stomach/Intestinal content, pig	4	12	80	9.6	0.46	44.2		Very good	Foaming	pH decrease

(continued)

Table 13.1 (continued)

Type of feedstock	C:N ratio	DM[a] (%)	VS[b] % of DM	VS (%)	Methane yield (m^3 CH_4/kg VS)	Methane production (m^3CH_4/m^3)	Mainly Compound	Digestibility	Process disturbing effects	Process inhibition
Plant wastes										
Straw	90	70–90	80–90		0.15–0.35		Cellulose	Poor	Lignin incrustation	pH decrease
Garden wastes	125	60–70	90		0.20–0.50		Cellulose	Poor	Lignin incrustation	pH decrease
Grass	18	20–25	90		0.30–55		Cellulose	Poor	Lignin incrustation	pH decrease
Fruit wastes	35	15–20	75		0.25–0.50		Sugar Starch Cellulose	Very good Very good Poor	Foaming Lignin incrustation	pH decrease
Organic wastes from industries										
Whey		5	90	4.5	0.33	15	Sugar Proteins	Very good Very good	Foaming	pH decrease High ammonia concentrations
Concentrated whey		10	90	9.0	0.54	31.5	Sugar Proteins	Very good Very good	Foaming	pH decrease High ammonia concentrations
Flotation sludge		5	80	4.0	0.54	21.6	Proteins Lipids	Very good Very good	Foaming	High VFA levels Low pH
Fermentation slop	7	1–5	90		0.35–0.78		Sugar	Very good	Foaming	pH decrease

(continued)

Table 13.1 (continued)

Type of feedstock	C:N ratio	DM[a] (%)	VS[b] % of DM	VS (%)	Methane yield (m^3 CH_4/kg VS)	Methane production (m^3CH_4/m^3)	Mainly Compound	Digestibility	Process disturbing effects	Process inhibition
Whole silage (grain)		12.6	91	11.5	0.47	53.9	Sugar Starch Cellulose	Very good Very good Poor	Foaming Lignin incrustation	pH decrease
Thin silage (grain)		8.5	86	7.3	0.50	36.5	Sugar Starch Cellulose	Very good Very good Poor	Foaming Lignin incrustation	pH decrease
Fish oil		90	90	81	0.80	648	Lipids	Very good	Foaming Layering Poor water solubility	pH decrease
Soya oil/margarine		95	90	85.5	0.80	684	Lipids	Very good	Foaming Layering Poor water solubility	High VFA levels Low pH
Olive pulp		24	96	23	0.18	41.4	Cellulose	Poor	Foaming Lignin incrustation	pH decrease
Brewers spent grains		20	90	18	0.33	59.4	Proteins	Very good	Foaming	High ammonia concentrations

(continued)

Table 13.1 (continued)

Type of feedstock	C:N ratio	DM[a] (%)	VS[b] % of DM	VS (%)	Methane yield (m^3 CH_4/kg VS)	Methane production (m^3CH_4/m^3)	Mainly Compound	Digestibility	Process disturbing effects	Process inhibition
Energy crops										
Grass silage		17	15–40	90	<0.45		Cellulose	Poor	Lignin incrustation	pH decrease
Maize silage							Sugar Starch Cellulose	Very good Very good Poor	Foaming Lignin incrustation	pH decrease
Fodder beet silage							Cellulose Lignin	Poor Poor	Foaming Lignin incrustation	pH decrease
Sewage sludge										
Waste water sludge		5	75	3.75	0.4	15		Very good	Foaming	High VFA levels Low pH
Conc. Wastewater sludge		10	75	7.5	0.4	30		Very good	Foaming	High VFA levels Low pH
Food remains		10	80		0.5–0.60			Very good	Foaming	High VFA levels Low pH

[a]Dry matter
[b]Volatile solids

also an important parameter. Too much carbon content could result in inhibition for substrate overload. In the other hand, high concentration of nitrogen may result in ammonia inhibition. Therefore, the C:N ratio should be kept around 15–30 in order to obtain high biogas yield and avoid inhibition (Weiland 2010).

Recently, attention has given to lignocellulosic biomass for biogas production due to its great abundance, availability and carbohydrate content. Lignocellulosic biomass can be divided in two types: wastes from agricultural and industrial activities, and dedicated energy crops (Kabir et al. 2015). The major drawbacks of lignocellulosic biomass are the recalcitrant nature of cellulose and the low degradation rates by anaerobic microorganisms; which limit their use as feedstock for AD. However, these problems can be overcome with the implementation of a pretreatment step or by co-digestion with other substrates, as discussed later.

Dedicated energy crops have become important substrates in the European Union due to their lower content of lignin, compared to lignocellolosic residues, and their high biomass yield; which in turn result in an increased production of biogas. Also, the policies in those countries offer many attractive subsidies in order to cover the cultivation costs. Energy crops such as maize, wheat, triticale, sugar beet and giant reed, among others, are the most popular substrates for conversion to biogas, alone or in co-digestion (Gissén et al. 2014; Sgroi et al. 2015). The main factor that has to be taken into account in order to choose the most appropriate crop is the biogas yield per hectare; however, other considerations have to be studied are the cultivation costs such as water and nutrient demand, fertilization, irrigation systems and machinery, and land availability. Ensiling is another factor that may affect the quality and yield of the biogas from crops, since this pretreatment also increases the production costs (Weiland 2010). Therefore, it is expected that the costs for the implementation of the biogas plants with energy crops as sole feedstock are higher than the ones obtained when crops are mixed with manure or another kind of wastes (Schievano et al. 2009; Sgroi et al. 2015).

Although, algal biomass is better known for the production of biofuels such as bioethanol and biodiesel, its potential as a feedstock for AD has been evaluated in recent years (Korres et al. 2013). However, several problems are associated with this kind of substrate, which have restricted its use even more than lignocellulosic biomass. Among the troubles encountered are the high content of recalcitrant components such as cellulose, lignin, polyphenols and fats that hinder the digestibility, the low C:N ratio that may result in ammonia inhibition, and the presence of sulfur-compounds that both inhibit AD and decrease the biogas quality (Ward et al. 2014). Indeed, it has been proven that AD systems with microalgae as sole substrate are not economical feasible compared with algal biorefineries, mainly because AD requires higher energy consumption. Several adjustments and improvements to AD systems are necessary in order to compete with biodiesel production (Collet et al. 2011).

The scenery changes when AD is implemented within biorefineries. In this case, AD is employed for the treatment of microalgae wastes generated during lipid extraction with the following advantages: (1) Recovery of energy of the wastes that can be re-circulated to the process, (2) Recovery of nutrients like N and P in the digestate, which can be employed later as fertilizer, and (3) if biogas is to be upgraded,

re-circulation CO_2 to algae cultivation as nutrient (Sialve et al. 2009; Wang et al. 2013; Williams and Laurens 2010).

The search for new and profitable feedstocks for AD hasn't ended. As it will be discussed in the following sections, co-digestion has become a popular trend in order to enhance the production and quality of biogas. However, the biogas yield of different and unconventional kind of wastes is still under study. For example, Gupta and Gupta (2014), tested different microbial consortia for the degradation of coal wastes to biogas. Through a series of parameters adjustments, the consortia produced biogas with a methane content of ~90%. This process could in the future, enhance coal exploitation with reduce of polluting wastes. Further research will encourage the use of more types of wastes as AD substrates for a sustainable energy production.

13.3.2 Pretreatment of Substrates

In order to enhance the biogas production of lignocellulosic biomass, it is necessary to implement a suitable pretreatment step, which decreases the recalcitrant nature of cellulose, and in consequence, facilitates the access of the hydrolytic microorganisms to it. According to Budzianowski (2016) several requirements have to be considered when choosing a pretreatment process. An effective method must have low energy demand with minimal use of chemicals and water, in order to reduce costs and environmental impact; it must avoid organic degradation and inhibiting-compounds formation while enhancing polymer digestibility for the microorganisms; and it must be able to work in different types of lignocellulosic biomass. However, almost all pretreatment processes described to this date cannot meet all requirements. Consequently, the choice of pretreatment technology mainly relies in energy demand and costs, and it only becomes economical feasible when it assures high biogas production (Montgomery and Bochmann 2014).

Pretreatment processes can be divided into physical, chemical, and biological methods, with their respective combinations. They are summarized in Table 13.2.

Finally, it must be mentioned that most of the technologies described in literature are not employed in full-scale reactors or are commercially available (Zheng et al. 2014). Different results can be obtained depending of the nature of the study. It is worth to mention that while laboratory-scale experiments render valuable information, the definitive contribution of pretreatments methods to biogas production enhancement should be determined in full-scale AD reactors (Montgomery and Bochmann 2014).

13.3.3 Co-digestion of Substrates

As pointed earlier, organic wastes must meet certain criteria to become suitable for AD process. To this date, there is no substrate that is exempt of troubles or

Table 13.2 Comparison of pretreatment methods for lignocellulosic biomass (Montgomery and Bochmann 2014; Zheng et al. 2014)

Process	Description	Feedstock	Advantages	Disadvantages
Physical comminution	Biomass is submitted to milling or grinding to reduce particle size	Agricultural and forest residues Grass Organic fraction of municipal solid waste (OFMSW)	No production of inhibitory compounds Increases surface area	High energy demand Expensive No lignin removal
Steam explosion	Biomass is heated with high-pressure steam for a short time, and then the pressure is reduced	Agricultural residuals Hardwood and softwood Grass MSW	Hydrolysis of hemicellulose and lignin No need of chemicals Most effective pretreatment method Commercial available Environmental-friendly	Degradation of sugars Energy demand Presence of inhibitory compounds
Liquid hot water	Biomass is heated with hot water at high pressure	Agricultural residuals Grass MSW	Solubilization of hemicellulose and ligning removal High recovery of sugars Low presence of inhibitory compounds No need of chemicals	Possible degradation of by acidification High energy demand
Extrusion	Biomass is feeding to an extruder and then transported along the barrel with a driving screw, where it is exposed to friction heat and shear forces	Agricultural residuals Grass MSW	Hydrolysis of cellulose, hemicellulose and lignin Commercially available	Thermal degradation of sugars Presence of inhibitory compounds Energy demand High maintenance costs
Irradiation	Biomass is treated with microwaves, ultrasound, gamma ray, and electron beam, which result in heat production	Agricultural residuals Grass MSW	Disruption of cell wall structure Low energy demand	Presence of inhibitory compounds Expensive

(continued)

Table 13.2 (continued)

Process	Description	Feedstock	Advantages	Disadvantages
Chemical alkaline	Biomass is treated with strong bases such as NaOH, KOH, and NH_3	Agricultural residues Forest residues Hardwood and softwood Grass OFMSW	Hydrolysis of lignin, hemicellulose and cellulose	Na^+ inhibition of AD Water pollution Expensive because requires chemicals
Acid	Biomass is treated with acids such H_2SO_4, HCL, and HNO_3 at low or high temperatures, depending of the concentration	Agricultural residues Grass MSW	Hydrolysis of hemicellulose	Expensive Corrosion Needs a neutralization step Water pollution Presence of inhibitory compounds and residues
Wet oxidation	Biomass is submitted to oxidation process by addition of water and an oxidizing agent (air or H_2O_2) at high temperature	Agricultural residuals Hardwood Grass MSW	Lignin and hemicellulose hydrolysis	Degradation of sugars Presence of inhibitory compounds and residues
Ozonolysis	Biomass is treated with ozone	MSW	Ligning removal Lower energy demand	Cellulose is not affected Expensive
Oxidation with peroxides	Biomass is treated with peroxides (H_2O_2) at high temperature	Agricultural residues OFMSW Grass	Partially hydrolysis of lignin and hemicellulose	Degradation of sugars Presence of inhibitory compounds and residues
Ionic liquids	Cellulose in biomass is dissolved in solvents like N-methylmorpholine-N-oxide monohydrate (NMMO), 1-n-butyl-3 methylimidazolium chloride (BMIMCl) at mild temperatures	Agricultural residues Grass Softwood	Low energy demand Recovery of solvents.	Expensive Lack of information.
Biological fungal pretreatment	Biomass is degraded by fungal species	Agricultural residues Harwood	Lignin and hemicellulose degradation	High retention times Requires sterilization after treatment

(continued)

Table 13.2 (continued)

Process	Description	Feedstock	Advantages	Disadvantages
Microbial consortium	Biomass is degraded by microbial species	Agricultural residues Manure	Hemicellulose and cellulose degradation No need of sterilization after treatment	High retention times
Enzymatic pretreatment	Enzymes such as cellulose and hemicellulase degrade biomass	Agricultural residues MSW Grass	Hemicellulose and cellulose degradation Low energy demand	Expensive High retention times
Ensilaging	Biomass is chopped to pieces and compressed. Fermentation occurs during storage	Agricultural residues and crops	Enhances digestibility	Generates pollution May not have effect in biogas enhancement High retention times

limitations. Therefore, combination of two or more substrates for AD feedstock could help to overcome individual drawbacks, enhancing biogas yield. Benefits of co-digestion include: dilution of inhibitory compounds, appropriate balance of nutrients (C:N ratio), enhancement of microbial interaction, and adjustment of moisture, which results in process stabilization. Co-digestion also reduces GHG emissions and presents economic benefits since various substrates can be degraded in one AD plant (Hagos et al. 2017). Common co-substrates employed in AD systems include animal manures, energetic crops, agricultural residues, food waste, algae and its residues, and industrial wastewaters (Neshat et al. 2017; Pagés-Díaz et al. 2013).

Several studies have reported increased biogas and methane yields during co-digestion with different substrates at specific conditions. Meneses-Reyes et al. (2017) investigated the co-digestion of oil extracted from microalgae, glycerol and chicken litter to improve the biochemical methane potential. Their results indicated an increase of 15.8% in methane yield, when the three substrates were submitted to co-digestion in the proportions 30:3:67 for oil from microalgae, glycerol and chicken litter, respectively. Similarly, the biochemical methane potential of four food wastes (meatball, chicken, cranberry and ice-cream wastes) in co-digestion with dairy manure was evaluated by Lisboa and Lansing (2013), employing a volumetric proportion of 3.2% of each waste with 96.8% of manure. All combinations registered increases in methane production, being the co-digestion of manure with chicken waste the one with highest increase. On the other hand, Sun et al. (2016) determined the viability of co-digestion of chicken manure and maize silage at different proportions of the former. They concluded that increasing the chicken manure in the feedstock more than 20% inhibited the digestion due to ammonia inhibition. Below that value, a methane content in biogas of ~50% was obtained. (Tasnim et al. 2017)

studied the co-digestion of cow manure, chicken wastes, sewage sludge and water hyacinth in different combinations, under mesophilic conditions. Results indicated that the best mixture was cow manure, sewage sludge and water hyacinth in 1:1:1 proportion, which produced biogas with 65% of methane content.

Regarding crop residues, it has pointed out that their employment as mono-substrates is not always economical feasible due to elevated cultivation costs (Schievano et al. 2009; Sgroi et al. 2015). In the EU, the incentives given to biogas production from crops have helped to sustain their cultivation; however, such subsidies are likely to decrease in the future (Bartoli et al. 2016). Moreover, their environmental impact must be considered. (Agostini et al. 2015) analyzed the GHG emissions of three AD systems fed with dairy manure, sorghum and maize, respectively. They found out that digestion of the two crops have major environmental impacts than digestion of manure. The authors concluded that in order to exploit the benefits of AD systems and decrease the negative environmental impacts, co-digestion of manure and single energy crop would be the ideal choice. Another study involving environmental impact of energy crops co-digestion was carried out by (Lijó et al. 2017). The environmental effects of two biogas plants treating different co-substrates were evaluated by Life Cycle Assessment (LCA) analysis, and it was found out that major impact was achieved in the biogas plant with higher proportion of energy crops. The authors also pointed out that high content of animal manures in feedstock could result in increased production of digestate, which also may have negative impact.

Although the advantages of co-digestion have been demonstrated, this strategy still faces challenges due to the complexity and different composition of substrates, which can result in process failure if the digestion takes place in one reaction tank (one-stage digestion). One solution to avoid this situation is the development of a two-stage system since as it will be exanimated later; it can enhance AD by separating the biochemical and microbial processes into two separate compartments (Hagos et al. 2017; Kabir et al. 2015).

Other issues that must be addressed during the design and implementation of co-digestion plants are the possible pretreatments that some substrates might require (e.g. lignocellulosic biomass) and the evaluation of the resulting digestate, which may cause environmental impact (Lijó et al. 2017; Neshat et al. 2017).

13.3.4 Biogas Production Technology

Although the biochemical and microbial foundations of AD for biogas production remain relatively constant, the set up, technology, and monitoring of AD processes have changed over time in order to treat more waste volume with an optimum biogas production. AD systems are classified according to operational parameters and system configurations such as reactor design, temperature of operation, feeding pattern and feedstock composition (Li et al. 2011).

The design of a biogas plant depends of the composition of the feedstock, specifically the water and dry matter content (Al Seadi et al. 2008). According to this, AD systems can be classified in wet digestion and dry digestion. In the former, solid content of the feedstock is kept below 10% while in the latter; the solid content is increased to 15–25% of total weight of feedstock (Pohl et al. 2013; Weiland 2010). Nowadays, wet digestion is the most common setting for AD reactors treating animal manure and sewage sludge. In contrast, dry digestion is suitable for degradation of lignocellulosic biomass and the solid fraction of organic fraction of municipal wastes. Dry digestion offers several advantages in comparison with wet digestion. Smaller reactors are employed in this setting and energy demand decreases (De Baere 2000). Also, digestate handling is easier because its low water content. Nevertheless, dry digestion is also prone to instability due to the composition of substrate, which requires specialized inoculum, and the possible accumulation of inhibitory compounds. Likewise, longer retention times are needed for an optimum biogas production (Budzianowski 2016; Kusch et al. 2011). In order to overcome such drawbacks, dry digestion systems might implement a pretreatment step for the feedstock before feeding, and operate at thermophilic conditions with the purpose of enhancing the digestibility. The C:N ratio is also a parameter that may be adjusted so as to avoid overload inhibition. Finally, a good monitoring system is recommended for the early monitoring of inhibitory compounds (Li et al. 2011).

As stated in the previous section, two-stage AD may enhance biogas production by physically separating the biochemical and microbial processes. Traditionally, all steps of AD are carried out in the same reactor, however this could result in process failure due to the different characteristics of the microorganisms involved. It has been reported that methanogens are the most susceptible microbial group to inhibitory compounds; therefore, special attention has to be paid to them since the successful operation of the AD systems depends greatly on their activity (Alvarado et al. 2014). The main products of fermentation, volatile fatty acids (VFAs), are the major inhibitory compounds of methanogenesis. Nonetheless, they are necessary substrates for the obligate-hydrogen producing bacteria during the acetogenesis step, which links fermentation and methanogenesis in AD (Almeida et al. 2011). Consequently, a constant monitoring of VFAs content is usually necessary in usually required in AD reactors (Dhanalakshmi Sridevi et al. 2015).

Addressing this situation, in 1971, (Pohland and Ghosh 1971) proposed the separation of the fermentation and methanogenesis steps of AD into two reactors in order to improve the stability of the process. By doing so, hydrolysis and fermentation are carried out in one reactor where high load rate and low pH can be maintained. Once hydrolytic degradation and VFAs production occurred, the liquid effluent from this reactor is fed into a second reactor where acetogenesis and methanogenesis take places (Aslanzadeh et al. 2014). The overall setting allows the optimization of two independent reactors, increasing the efficiency of substrate utilization and energy yield (Massanet-Nicolau et al. 2015; Schievano et al. 2014). Also, this setting is suitable for flexible biogas plants, which is also mentioned in this section. Finally, it must be noted that effectiveness of two-stage AD is not the same for all feed-

stocks (Lindner et al. 2016). Therefore, a carefully evaluation of plant design must be developed for the selected type of feedstock to take advantage of two-stage AD.

One important aspect of AD process is the design of the reactor or digester where it will take place. There are different types of reactors made of materials such as concrete, bricks, plastic, and steel, with diverse shapes and shapes. The selection of a specific type reactor should considerer the composition and volume of the feedstock and the final purpose of the biogas produced (Al Seadi et al. 2008). Reactors can be classified according to the feed regime into batch, semi-continuous and continuous types. They are summarized in Table 13.3.

Reactors that have gained attention recently are the household digesters, which were developed in Asian countries like China and India. These reactors are fed with manure and kitchen wastes once a day, and the biogas produced is used for cooking and illumination. In this manner, GHG emissions from household wastes are prevented and energy is provided to places where electrical infrastructure is not available. Although there are different types of digesters, two of them, the fixed dome and the floating drum digesters, are the most employed in the afore mentioned countries (Surendra et al. 2014).

In Latin America, implementation of household digesters was encouraged in the 1970s. Table 13.4, summarizes the characteristics of household in the Latin American scenario. However, the number of systems installed is far behind the ones found in Asia in the present days. This situation is a result of low social acceptance of rural communities because of deficient education and information, lack of financial support by the government, and absence of monitoring and maintenance practices once the digester has been installed (Garfí et al. 2016). Also, a bad planning, design and implementation of household digesters could result in an increment of GHG emissions rather than a decrement, annulling their benefits to the environment (Bruun et al. 2014). Hence it is important to address and propose solutions that help to overcome these problems so as to encourage the use of biogas from AD to supply energy in rural areas.

The basic design of biogas plants have changed in recent times, responding to the overgrowing energy demand. Some of these changes are associated with the storage of biogas for further use, the integration of the plants within other industrial processes, and the implementation of methods and treatments for biogas cleaning, upgrading, and conversion to other value-added products (Bauer et al. 2013; Budzianowski and Budzianowska 2015; Hahn et al. 2014b; Hengeveld et al. 2016; Lemmer and Krümpel 2017; Mauky et al. 2015; Petersson and Wellinger 2009; Scholz et al. 2013; Singhal et al. 2017; Sun et al. 2015; Yang et al. 2014; Zhu 2014). In this regard, AD systems may contribute to the supply of energy demand of industries like biorefineries or wastewater treatment plants, lowering electricity and heat-associated costs (Budych-Gorzna et al. 2016). Moreover, biogas plants can be configured in a flexible manner, where energy is produced according to the current demand. Such

Table 13.3 Comparison of reactors employed for AD processes (Jeison and Chamy 1999; Kim et al. 2013; Mumme et al. 2010; Mao et al. 2015; Pohl et al. 2012; Poh et al. 2015)

Reactor	Description	Advantages	Limitations
Anaerobic sequencing batch reactor (ASBR)	Single tank for treatment and fermentation of wastewater in batch configuration	Allows variable influent volume Operational simplicity Cheap Low energy input High biogas yield	Poor biomass retention Possible clogging Needs mixing system
Continuous stirred tank reactor (CSTR)	Close reactor in which constant mixing is applied with continuous influent addition and effluent removal Most common reactor system employed in AD	High stability in the steady state High biogas yield Presents several improvements	Washout of microorganisms Cannot be fed with high organic loads Energy demand for mixing
Anaerobic plug-flow reactor (APFR)	Horizontal, long channel constructed above the ground with no internal mixing	Stable process High sludge retention Can treat feedstock with high solid content	Might be expensive when combined with other technologies Might require mixing systems
Anaerobic contact reactor (ACR)	Similar design that activated sludge systems Consists of two tanks: one mixing reactor for AD process and one solids settling tank for recirculation of microbial biomass recycling	For the treatment of wastewater with high-suspended solids content. Efficient Low retention times Limited wash out of microorganisms	Expensive Poor sludge settlement because gas production
Up-flow anaerobic sludge bed reactor (UASB)	Reactor in which a thick sludge bed covers the bottom of reactor. The influent flows from the bottom of the tank and travels toward the top of the tank where it is discharged.	Less volume reactor High biogas yield No effluent recycling Enhances contact between wastewater and microbial biomass Lower retention times	Dependence of granule quality Requires special skills for operation and maintenance Might be initial sludge washout in the first stages of operation Only certain wastes achieve good results
Up-flow anaerobic solid-state reactor (UASS)	Vertical reactor in which particulate solid biomass enters at the bottom and ascends to the top of the reactor, forming a solid-state bed	High efficiency High loading volume rate Low investment costs Simplified operation	Small volume Some feedstock treatment might result in clogging

(continued)

Table 13.3 (continued)

Reactor	Description	Advantages	Limitations
Anaerobic Baffled reactor (ABR)	Reactor divided in a series of compartments in which baffles forces wastewater to pass through a series of blanked sludge	Efficient solid removal Low sludge formation Environmental-friendly High loading rates Physical separation of AD steps	Inefficient mixing Microbial washout Only for the treatment of certain types of wastewater
Internal circulation reactor (ICC)	The system consists in two UASB reactors working together	Improved sludge retention High efficiency Treatment of different wastewaters	N/A
Anaerobic filter reactors (AF)	A biofilm for microbial support is implemented in the reactor	Enhanced performance Elimination of mixing Good adaptation of microorganisms to different substrates	Filter clogging High investment costs
Anaerobic fluidized bed reactor (AFB)	A fluid bed of fine sand or alumina immobilized in a membrane is implemented in the reactor for bacterial attachment	Inhibition resistance High organic load rate Avoids microbial wash out High solid removal	Membrane fouling
Expanded granular sludge blanket reactor (EGSB)	Improved UASB reactor with higher up-flow velocity	Higher efficiency than UASB reactor Enhanced contact between microbial sludge and substrate Good treatment of wastes with inhibitory compounds	Not suitable for wastewater with high suspended solid content

configuration is expected to allow the integration of all renewable energies since electricity produced from biogas could compensate the intermittent provision from solar and wind energies, improving the energy supply and securing a sustainable future regarding energy production (Tafarte et al. 2017).

Mauky et al. (2015) has pointed out that several factors have a great influence in the possible energy flexibility of a biogas plant. These factors are related with the type and equipment for biogas transformation, biogas storage capacity of the plant, type of feedstock employed, and feeding regimen to the reactor. Although, biogas plants with high storage capacities seems to be favored by flexible supply program (Lauer et al. 2017), recent studies are inclined to adjust in order to decrease the storage of biogas (Hahn et al. 2014a; Mauky et al. 2015). By doing so, related costs would decrease along with the dangers associated to gas storage.

In conclusion, there is no doubt that AD systems will continue to suffer changes and improvements in their basic design that will made them be able to meet the

Table 13.4 Characteristics of household digesters employed in Latin America (Garfí et al. 2016)

	Fixed dome digester	Floating drum digester	Tubular digester
Origin	China	India	Adapted from Taiwan
Description	Underground cylindrical closed chamber with feedstock inlet and outlet, and biogas pipeline for collection and reservoir	Underground cylindrical chamber with floating drum, with inlet and outlet and biogas pipeline for collection and reservoir	Buried tubular plastic bag, with inlet and outlet and biogas pipeline for collection and reservoir
Materials of construction	Bricks and concrete Requires specialized knowledge	Concrete and steel PVC or steel for the drum Requires specialized knowledge	PVC and polyethylene Might require additional material for temperature regulation
Volume[a]	10–20 m^3	1.6–10 m^3	6–10 m^3
Mixing and heating systems	No	No	No
Maintenance	Daily feeding Removal of sludge Digestate management Control of biogas leaking and chamber cracking	Daily feeding Removal of sludge Digestate management Control of biogas leaking and drum corrosion	Daily feeding Removal of sludge Digestate management Control of biogas leaking
Life span	20 years	15 years	10 years

requirements of the energetic demand of the world in a sustainable way. However, as Budzianowski (2016) pointed out, such advances must also contemplate their realistic commercialization potential in order to truly impact on the energy market.

13.3.5 Cleaning and Upgrading of Biogas

Biogas composition is a result of the feedstock employed, operational and environmental conditions, and technology used. Although biogas is comprised in its majority of methane, other gases and impurities such as carbon CO_2, O_2, N_2, water vapor, hydrogen sulfide (H_2S), ammonia (NH_3) and siloxanes are present in variable quantities, affecting the quality of biogas. As it is shown in Table 13.5, most of the biogas components and impurities have negative effects in its downstream applications. Hence, they must be removed before its utilization in conversion processes for energy or biofuel production.

Several methods for biogas cleaning have been developed over the years in order to improve its quality and avoid machinery damage (Chaemchuen et al. 2016; Chottier

Table 13.5 Parameters and composition of gases from different origins, impurities and their consequences (Awe et al. 2017; NOM 001-SECRE- 2010; Sun et al. 2015)

Parameters	Unit	Biogas from AD	Landfill gas	Mexican natural gas	Dutch natural gas	Impact on biogas utilization
Lower heating value	MJ/Nm^3	23	16	37.3	31.6	
	KWh/Nm^3	6.5	4.4	10.5	8.8	
Density	kg/Nm^3	1.1	1.3	0.82	0.8	
Relative density	–	0.9	1.1	0.62	0.6	
Wobbe index, upper	MJ/Nm^3	27	18	53.20	43.7	
Methane (CH_4)	vol%	60–70	35–65	84	80–90	
Heavy hydrocarbons	vol%	0	0	11	9	
Water vapour (H_2O)	vol%	1–5	1–5	–	–	Corrosion in compressors, gas storage tanks and engines due to reaction with H_2S, NH_3, CO_2 to form acids
Hydrogen (H_2)	vol%	0	0	0	–	
Carbon dioxide (CO_2)	vol%	30–40	15–40	3	0.2–1.5	Decreasing calorific value, anti-knock properties of engines and corrosion
Nitrogen, range (N_2)	vol%	0–0.5	15	1.5–5.5	15	Decreasing calorific value, anti-knock properties of engines and corrosion
Oxygen (O_2)	vol%	0	1	0.20	–	Corrosion, fooling in cavern storage, risk of explosion
Hydrogen sulfide (H_2S)	Ppm	0–4000	0–100	6	–	Corrosion, catalytic converter poison, emission and health hazards. SO_2, SO_3 are form
Ammonia (NH_3)	ppm	100	5	0	–	Emission, anti-knock properties of engines and corrosion when dissolved

Table 13.6 Methods of removal of undesirable components in biogas (Petersson and Wellinger 2009; Ryckebosch et al. 2011; Yentekakis and Goula 2017)

Element of biogas	Method for removal
Water vapour	Condensation Adsorption in alumina, silica or zeolites Absorption in triethylene glycol or hygroscopic salts
Hydrogen sulfide (H_2S)	Precipitation with Fe^{3+} or Fe^{2+} ions Adsorption on activated carbon, and iron oxide or hydroxide Absorption in $FeCl_2$, and $Fe(OH)_3$ Oxidation by sulfur-oxidizing microorganisms Separation by semi-permeable membranes
Siloxanes	Absorption with in acid, base or in organic solvents Adsorption in silica gel or activated carbon Cryogenic separation
Oxygen/Nitrogen (O_2/N_2)	Adsorption in activated carbon, membranes and molecular sieves Might be removed during pressure swing adsorption (PSA)
Ammonia (NH_3)	Adsorption in activated carbon Elimination in some CO_2-removal processes
Halogenated carbon hydrates	Adsorption in activated carbon

et al. 2014; Paolini et al. 2016; Scholz et al. 2013; Strevett et al. 1995). Most of the methods employed have focused their aim to the removal of hydrogen sulfide (H_2S), as it is known to be one of the major contaminants with high impact on equipment and AD performance (Chen et al. 2008; Ho et al. 2013). Another major impurities in biogas are siloxanes, which are mainly found in landfill gas and are originated from silicone wastes. Siloxanes are undesirable compounds in biogas because their combustion results in accumulation of silicates in gas engines, causing abrasion and decreasing efficiency (Ohannessian et al. 2008). The methods for biogas cleaning can be classified as physical, chemical and biological (Yentekakis and Goula 2017), a summary of them is presented in Table 13.6.

Biogas is usually converted into electric energy in co-generation or in combined heat and power (CHP) systems. However when the methane content is increased to 95–97% by elimination of CO_2, biogas can be employed as an equivalent of natural gas (Kadam and Panwar 2017). In this form, the upgraded biogas can be injected into the gas grid or can be employed as a fuel for stoves and boilers, for engines and turbines, for electricity production in fuel cell, and for vehicles, among other applications (Pöschl et al. 2010; Sun et al. 2015). Moreover, it can also be converted into other less-polluting fuels such as syngas and hydrogen.

Upgrading methods are mostly focused in CO_2 removal, and the most common ones are agent scrubbing, pressure swing adsorption (PSA) and membrane adsorption (Bauer et al. 2013; Scholz et al. 2013; Singhal et al. 2017). Agent scrubbing is usually accomplished with water, organic solvents like methanol, polyethylene glycol and N-Methylpyrrole, or amines such as mono- and diethanolamines (MDEA and DEA). The principle is the same for the three: Biogas is circulated through an

absorption column filled with the agent, and CO_2 is separated by absorption into the agent. In the other hand, PSA consists in the adsorption of CO_2 into porous material such as activated carbon, zeolites, and silica gels. Changes of pressure are used for better adsorption (high pressure) or for regeneration of the material (low pressure). CO_2 adsorption and desorption can be also take place using changes in temperature, but it consumes large amounts of energy compared with the use of pressure. Finally, biogas can be upgraded by employing membranes that retain CH_4 but not CO_2.

Other relative new upgrading methods that have been described recently are cryogenic separation, in situ methane enrichment, and biological methods (Rapport et al. 2016; Riva et al. 2014; Singhal et al. 2017). Cryogenic separation works on the premise of the different condensation temperatures of the gases contained in biogas. This allows methane recuperation in a liquid form, which is easy for transportation. However, the high costs related to the energy demand of this method don't make this method economically feasible. As it name indicates, in situ methane enrichment is a method that can be employed during AD process. In this case, sludge from the digester is circulated towards one desorption column where CO_2 is removed by a current of air or N_2; then, the sludge free of CO_2 is then circulated back to the digester. Biological methods have also been developed. For example, and also briefly mentioned in feedstock section, CO_2 can be employed as a nutrient for algae or growing plants when AD is integrated within biorefineries (Zhu 2014). Furthermore, hydrogenothrophic methanogens have been used in biogas upgrading since they consume CO_2 for energy production and growth (Strevett et al. 1995).

Bauer et al. (2013) indicated that the processes that dominate in the biogas upgrading plants in Europe are water and amine scrubbing, and PSA due to their high efficiency, simplicity and capacity to remove other contaminants. The use of membranes is still limited due to the uncertainty of their lifetime, but their implementation is still growing (Scholz et al. 2013). They also found out that the costs of energy demand usually decrease in upgrading plants with large size. This is understandable since, no matter the technology employed, upgrading of biogas always represents an extra cost (Petersson and Wellinger 2009). In consequence, in the present days, it is still difficult the implementation of small biogas upgrading plants because they are not able to compensate the costs associated with the technology for the upgrade. Nevertheless is expected that the market of biogas becomes more competitive, allowing the optimization of new and different upgrade methods that might made possible the installation of more upgrading plants that meet the individual necessities.

13.4 Contribution of Biogas to the Power Generation

The use of renewable energy is a major objective in many countries. Governments all over the world are joining that consensus, more than 170 countries have established renewable energy targets, nearly 150 have enacted polices to catalyze investments in renewable energy technologies. Technologies for the conversion of renewable energy sources in electricity, heat and steam have undergone substantial progress

over the past two decades: systems efficiency and reliability have improved, while and markets have expanded (Huacuz 2007).

AD can positively impact the energy balance of both chains via its direct contribution in terms of energy in the form of methane, such as a production of heat and steam, electricity production and co-generation, vehicle fuel, substitute for natural gas for domestic and industrial use or in its indirect contribution via the replacement of fertilizers, water and extra energy coming from digestate (Svenskt gastekniskt center 2012).

Most biogas production occurs in the United States, where it is based predominantly on the collection of landfill gas, and in Europe. Production in Europe is focused more on the anaerobic digestion of agricultural wastes. This has led to increase in biogas production plants in Europe from 17,240 with total installed electricity generating capacity of 8339 MW at the end of 2014, a 18% increase compared to 2013 (Awe et al. 2017). Germany leads the growth with 10,786 plants, followed by Italy with 1491, UK 813, and France 733 (EBA Biomethane and Biogas Report 2015). The total amount of electricity produced from biogas is 63.3 TWh and is estimated that the global power generation capacity will increased more than double in biogas production over the next decade, from 14.5 to 29.5 GW (Renewables 2017: Global Status Report 2017). According to the International Renewable Energy Agency (Renewable capacity statistics 2017) and the renewable capacity statistics 2017, the biogas power generation data represent the maximum net generation of power plants.

Europe represents the biggest power generation by biogas accounting 10,883 MW within Germany as country leader in this term (5104 MW). North America follows the second place with 2652 MW, dominated by USA with the 91% of the total power generation. Third place is represented by Asia, followed by South America, Eurasia, Oceania, Middle East, Africa and Central America and Caribbean accounting with 1199, 524, 383, 276, 44, 36 and 18 MW respectively.

13.5 Conclusions

Biomass is one of the most attractive renewable energies sources due to its availability and reduced environmental impacts. Among the processes for energy conversion of biomass, biogas production from AD stands out as the most relevant one in the energetic scenery. Even tough, this process is not new; it has suffered several modifications and improvements related with source of feedstock, operational conditions, technology and final destination of the product with the purpose to enhance its stability, increase its efficiency or expand its further applications. However, there is still a long way for biogas to become a substitute for fossil fuels. Several technological approaches are yet to demonstrate the effectiveness they promise for higher yields of biogas or for improved conversion efficiencies to energy and other fuels at large scale stages. Also, the overall environmental impact of these new processes must be evaluated in order to guarantee their compromise with sustainability. Finally, economical and political impacts must be assessed for each country in order to determine the

true commercial value of the new technologies in biogas production. By doing so, the biogas market will grow along with its share in the energy production market.

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Chapter 14
Microbially Originated Polyhydroxyalkanoate (PHA) Biopolymers: An Insight into the Molecular Mechanism and Biogenesis of PHA Granules

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Abstract Microorganisms especially bacteria and cyanobacteria have the ability to synthesize polyhydroxyalkanoates (PHAs) granules as carbon and energy storage compounds within their cells. Owing to eco-friendly, biodegradability, modifiable mechanical properties, non-toxicity, biocompatibility, hydrophobicity, cellular growth support, piezoelectricity, attachment without carcinogenic effects, optical purity and desired surface modifications, the PHAs have received substantial attention towards research as well as commercial ventures and comparable to non-biodegradable conventional plastics presently in use. Microbial PHA biosynthetic pathways are grouped into four types, where PHA synthases are the main enzymes. The PHA synthases exploit the hydroxyacyl-CoAs as substrates and catalyze the covalent bond formation among the hydroxyl group of one along with the carboxyl group of other hydroxyalkanoate that result into the formation of PHAs. Depending on the specificity of substrate as well as components of subunit, PHA synthases are grouped into four types, i.e., class I synthesizing Short-Chain-Length (SCL) PHAs (represented by the bacterium *Cupriavidus necator*), class II synthesizing Medium-

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Chain-Length (MCL) PHAs (represented by the bacterium *Pseudomonas putida*), class III (represented by bacterial species such as *Allochromatium vinosum*), and class IV PHA synthases (so far represented only by *Bacillus* sp., *B. megaterium*). Interestingly, these PHA synthases have a preserved cysteine residue as a catalytic active site to which the resulting PHA chain is linked through covalent bond. Overall, this chapter gives an overview on the structure and genes of PHA synthases including PHA biosynthetic routes, mechanism of PHAs polymerization together with biogenesis of PHA granules and phasins as major PHA granule-associated proteins.

Keywords Biocompatibility · Biodegradability · PHAs · Phasins · PHA synthases · Piezoelectricity · Polyhydroxyalkanoates · PHA biosynthetic pathways · PHA biogenesis

14.1 Introduction

14.1.1 Conventional Petroleum-Based Plastics

Conventional petroleum-based plastics have exceptional light-weight, stability, durability, economic feasibility and desirable material properties with ability to depict a variety of strengths as well as shapes. These unique properties are responsible for their widespread uses in construction, packaging materials, computer equipments, components of automobiles, medical field, printers, etc. (Bernard 2014; Kumar et al. 2015; Sharma et al. 2016; Singh and Mallick 2017a, b; Singh et al. 2017). In 2013, the global plastic production enhanced up to 299 million tons, which was a 3.9% enhancement than the 2012 (Plastics Europe 2015). Plastic production exhibits a little enhancement in 2016, but is still below pre-crisis level. However, in 2017 plastic production is anticipated to continue on a positive trend (Plastics Europe 2016). These plastics are usually manufactured from polyolefins such as polyethylene (PE), polystyrene (PS), polypropylene (PP), polyvinyl chloride (PVC), etc. that are typically synthesized using fossil fuels, used up as well as cast-off into the environment as non-degradable refuses, where they persist as such for many years as a result of largely xenobiotic nature or recalcitrant to microbial attack. The question of what to do with these conventional plastics waste is turning into a worldwide problematic issue pertaining to environment. The environmental apprehensions like solid waste management, plastic waste incineration and safe disposal posed by these plastics resulted into the synthesis of green polymers/biodegradable plastics (Alexander 1981; Ray and Bousmina 2005; Shah et al. 2008; Kumar et al. 2015; Sharma et al. 2016; Singh et al. 2017). Biodegradable plastics are biologically synthesized materials that are environmentally friendly, degradable and lead to mineralization. Such green plastics are relatively new as well as promising due to their actual use and degradation by microbes like bacteria and cyanobacteria. This green plastic can be divided into four classes viz. photodegradable, semi-biodegradable, chemically synthesized

and polyhydroxyalkanoates (PHAs) (Ray and Bousmina 2005; Singh et al. 2017). Among these, the microbially originated PHAs depicted unique properties such as biodegradability, modifiable mechanical properties, non-toxicity, biocompatibility, hydrophobicity, cellular growth support, piezoelectricity, attachment without carcinogenic effects, optical purity and desired surface modifications, which make them extremely competitive with conventional plastics for various industrial applications. The industrial applications of PHAs are summarized in Table 14.1.

14.1.2 An Overview on Commercialization Trends and Economics of PHAs Production

The biopolymeric PHAs market is differentiated into linear as well as co-polymerized PHAs in which the co-polymerized PHAs emerged as the foremost revenues producing section because of its desirable material properties akin to conventional petroleum-based plastics. PHAs market based on application is classified into various fields like food services, medical, agriculture, packaging (Table 14.1), etc., where packaging field is the most important revenue making segment due to increasing interest of biodegradable packaging materials. Furthermore, PHAs geography market is divided into North America, Europe, Asia Pacific and Rest of the World. Interestingly, Europe followed by Asia Pacific, is the biggest revenue generating sector because of relative increase in the consumer's disposable income together with existence of enormous amount of capital by many industries, which triggering more and more investment in research and development (Global Polyhydroxyalkanoate (PHA) Market 2012–2020). There are many top most companies like Metabolix Inc. (USA), Shenzhen Ecomann Technology Co. Ltd. (China), Tianjin GreenBio Materials Co. Ltd. (China), Meredian Inc. (USA) and Biomer (Germany) that are engaged in the biopolymeric PHAs business. Amongst these companies, USA-based Metabolix Inc. and China-based Shenzhen Ecomann Technology depicted the maximum growth and developments relating to PHAs market. Additionally, they have largest expansion plan with novel PHA product developments (as their ultimate growth policies). Such companies already initiated the research and intensifying efforts for industrial production of many different types of PHA biopolymers (Global Trends and Forecasts 2018). For instance, the *Cupriavidus necator*, a bacterium, is employed by Metabolix, Inc. (USA) for the industrial production of 3-hydroxybutyrate (3HB) and 3-hydroxyvalerate (3HV), i.e., [P(3HB-*co*-3HV)] co-polymer and marketed in the trade name of BIOPOL® (Luzier 1992). In the near future, these companies are anticipating considerable development as well as growth in PHAs market because of exploring the wide-range of feasible uses and forthcoming inclinations of biodegradable plastics. Table 14.2 shows the current global manufacturers of PHA bioplastics.

PHAs are making pace towards commercialization that is attained by decades of research with hard efforts and commitments. Regardless of commercialization, technology is still at the initial stages for economical large-scale PHAs production

Table 14.1 Industrial uses of biopolymeric PHA materials (compiled from Vincenzini and De Philippis 1999; Philip et al. 2007; Chen 2009)

Fields	Applications	References
Pharmaceutical industry	PHA can be used as drug delivery systems and retarded drug release	Vincenzini and De Philippis (1999), Yao et al. (2008); Ali and Jamil (2016)
	PHA monomers could be used as drugs. For instance, 3-hydroxybutyrate (3HB) and its derivatives found to have an inhibitory influence on cell apoptosis	Xiao et al. (2007), Zou et al. (2009)
	PHA monomers, specially 3HB, exhibit therapeutic effects on Alzheimer's as well as Parkinson's diseases, osteoporosis and even memory improvement	Kashiwaya et al. (2000), Massieu et al. (2003), Zou et al. (2009)
Packaging industry	Utensils, cosmetic containers and cup, tray for foods, Packaging films such as containers and paper coatings, feminine hygiene products, tubs for thermoformed articles, bottles like shampoo bottles, compostable bags and lids, diapers, disposable substances like razors, upholstery, carpet, shopping bags and medical surgical garments	Weiner (1997), Vincenzini and De Philippis (1999), Chen (2010), Philip et al. (2007)
Printing and photographic industry	Biopolymeric PHA materials can be effortlessly stained	Chen (2009), Li et al. (2009)
Industrial microbiology	The PHA synthesis operon can be employed as a metabolic regulator or resistance enhancer to increase the performances of industrial microbial strains	Muller et al. (1999), Zhang et al. (2006)
Block copolymerization	PHA materials can be transformed to PHA diols for block co-polymerization with other polymeric materials	Liu et al. (2008), Chen (2009)
Biofuels	PHA polymeric materials can be broken down to form hydroxyalkanoate methyl esters, which are combustible	Zhang et al. (2009)

(continued)

Table 14.1 (continued)

Fields	Applications	References
Textile industry	PHA materials can be processed into fibers similar to nylons	Perepelkin (2005), Chen (2009)
Fine chemical industry	PHA monomeric units are all chiral R-forms and can be used as chiral starting materials for the synthesis of antibiotics	Chen and Wu (2005a)
Medical	PHAs can be used for the development of absorbable sutures, bone plates, surgical pins, films and staples, bone marrow scaffolds, tendon repair devices, spinal fusion cages, ocular cell implants, skin substitutes, heart valves, cardiovascular fabrics, pericardial patches, closure of atrial septal defect, vascular grafts, attractive candidates for nerve guides, tissue engineering applications, osteosynthetic material for stimulating bone growth and effectively repairing damaged nerves etc.	Vincenzini and De Philippis (1999), Chen and Wu (2005b), Wang et al. (2008), Bian et al. (2009), Brigham and Sinskey (2012), Ali and Jamil (2016)
Agriculture	Controlled liberation of substances like herbicides, plant growth regulators pesticides and fertilizers, seed encapsulation and covering foils	Vincenzini and De Philippis (1999), Kyrikou and Briassoulis (2007)
Chiral chromatography	Stationary phase for columns	Vincenzini and De Philippis (1999)
Healthy food additives	PHA oligomers can be used as food supplements for obtaining ketone bodies	Martin et al. (1999)
Protein purification	PHA granule binding proteins phasin or PhaP are used to purify recombinant proteins	Rehm (2007), Wang et al. (2008)
Bulk chemicals	Heat sensitive adhesives, latex, smart gels, PHA matrices can be used to remove facial oils	Lee et al. (1999)

Table 14.2 Worldwide manufacturers of PHA bioplastics (Doug 2010; Ravenstijn 2010)

Name of the company	Country	Brand name	Production/planned capacity (kt/year)
Bio-on	Italy	Minerv	10
Kaneka	Singapore	Mirel	10
Meredian	USA		13.5
Metabolix	USA		50
Mitsubishi gas chemicals	Japan	Biogreen	0.05
PHB industrial S/A	Brazil	Biocycle	0.05
Shenzen O'Bioer	China		–
TEPHA	USA	ThephaFLEX/ThephELAST	–
Tianan biological materials	China	Enmat	2
Tianjin green biosciences	China	Green Bio	10
Tianjin northern food	China		–
Yikeman Shandong	China		3

(Global Trends and Forecasts 2018; Global Polyhydroxyalkanoate (PHA) Market 2012–2020). The most important limitation of PHAs is their high production price. The commercial PHA biopolymeric materials was reported to be 15–17 times expensive over the conventional plastics and 4–6 times costlier than the commercial polylactic acid in 2004 (Castilho et al. 2009). Nevertheless, metabolic engineering, improved fermentation conditions with higher PHAs accumulation capacities were able to diminish the cost up to US$5 kg^{-1} in 2009, which was still three times expensive than the cost of polypropylene (DiGregorio 2009). Hence, PHAs still have a limited market, despite their potential to replace 33% of commercial polymers (Castilho et al. 2009). The final price of PHAs are mostly reliant on the cost of organic substrates that supplemented as a carbon source for microbial cultivation. Remarkably, the cost of organic carbon sources found to contribute about 50% of the overall production cost. In addition, the PHA content and yield on organic carbon substrate, PHA productivity as well as downstream prices are the other important factors responsible towards determining their introduction into global market (Choi and Lee 1999). Analysis and economical assessment established that large-scale PHAs synthesis using octane as carbon source would cost approximately US$5–10 kg^{-1} (Hazenberg and Witholt 1997). It was estimated that the theoretical cost of PHAs accumulated in fed-batch method exploiting waste/cost-effective substances might attain up to 3.51 Eur kg^{-1} PHAs, while synthetic alternatives such as polypropylene as well as polyethylene cost 1.47 and 1.15 Eur kg^{-1}, respectively (Obruca 2010). Overall, the transformation of raw substances to PHAs appears to be a main factor

in the establishment of sustainable biotechnological process and a solution for the price limitations (Możejko-Ciesielska and Kiewisz 2016; Chandel et al. 2018).

As a result of the aforementioned limitations (related to bacterial PHAs production), cyanobacteria are receiving the current attention as alternative hosts for the cost-effective PHAs production because of their little nutrient requirement, their genetic modification is much easier than the higher plants as well as eukaryotic photosynthetic algae, can be stored for long periods of time, ability to transform 'greenhouse gas' into PHA biopolymers by oxygenic photosynthesis and low quality/infertile land for their growth. In spite of these advantages, the prominent bottlenecks associated with the cyanobacterial PHAs production involve the lack of an inexpensive mass cultivation technique, unavailability of efficient biomass harvesting system and significantly lower PHA productivity than the bacteria (Singh et al. 2017; Singh and Mallick 2017b). Therefore, on urgent basis, these bottlenecks need to be resolved in order to push cyanobacterial PHAs production towards commercialization phase.

14.2 Occurrence, Structure and Types of PHAs

The exterior portion of PHAs granules possess a large amount of different protein molecules, which signifying that they represent supramolecular complexes having particular roles rather than being merely simple packets possessing abundant amount of carbon and energy. The term 'carbonosome' was introduced to specify the multifunctionality of PHA granules (Jendrossek 2009). The prokaryotic water insoluble carbon and energy storage compounds PHAs are a kind of naturally occurring biopolymers with diameter in the range of 0.2 ± 0.5 mm, which are produced as granules in the cytoplasm of a huge and varied forms of microorganisms like bacteria and cyanobacteria under different growth and environmental conditions (Lageveen et al. 1988; Huisman et al. 1989; Stal et al. 1990; Zhang et al. 1994; Kato et al. 1996; Miyake et al. 1996; Lama et al. 1996; Braunegg et al. 1998; Nishioka et al. 2001; Thakor et al. 2003; Tajima et al. 2003; Tian et al. 2005a; Sharma and Mallick 2005a, b; Panda et al. 2006; Sharma et al. 2006; Yezza et al. 2006; Mallick et al. 2007; Sharma et al. 2007; Toh et al. 2008; Panda et al. 2008; Singh and Mallick 2008; Chen 2009, 2010; Sankhla et al. 2010; Li et al. 2011; Singh et al. 2013; Hauf et al. 2013; Osanai et al. 2013; Samantaray and Mallick 2014, 2015; Koller and Maršálek 2015; Singh et al. 2015; Reddy and Mohan 2015; Sharma et al. 2016; Gómez Cardozo et al. 2016; Singh and Mallick 2017a, b, c; Singh et al. 2017). The bacterial strains such as *Cupriavidus necator* (previously called as *Wautersia eutropha/Ralstonia eutropha/Alcaligenes eutrophus*) *Rhodopseudomonas palustris*, *Methylobacterium organophilum*, etc. synthesized PHAs under limitations of ammonium, sulphate, phosphate, potassium, magnesium, iron and oxygen (Anderson and Dawes 1990; Singh and Mallick 2008, 2009a, b; Singh et al. 2015; Kumar et al. 2015; Singh and Mallick 2017a, b). The bacterial strains viz. *Alcaligenes latus*, etc. found to produce PHA biopolymers during active cell growth without any nutrient limitation. The occurrence of PHA biopolymers in cyanobacteria was reported in 1966 (Carr 1966; Drosg et al. 2015). Subsequently,

numerous cyanobacterial strains are reported to produce biopolymeric PHAs photoautotrophically, while others under chemoheterotrophic conditions using acetate or other organic carbon substrate (Campbell et al. 1982; Sharma et al. 2006, 2007; Mallick et al. 2007; Bhati and Mallick 2012; Samantaray and Mallick 2012, 2014, 2015). The limitation or deficiency of nutrient such as nitrogen and phosphorus usually results in enhanced accumulation of PHAs in cyanobacteria (Takahashi et al. 1998; Nishioka et al. 2001; Bhati and Mallick 2012; Samantaray and Mallick 2012). Table 14.3 summarized the accumulation of PHA biopolymers by different bacterial and cyanobacterial strains/species under diverse environmental conditions.

The first studies on bacterial PHA granules were carried out by Williamson and Wilkinson (1958) followed by Griebel et al. (1968). Interestingly, the extracted PHA granules found to possess nearly 97.5% PHA biopolymer, 2% proteins as well as 0.5% phospholipid molecules (Griebel et al. 1968). The electron microscopic investigations depicted that the exterior portion of PHA granules of *B. megaterium* as well as *B. cereus* is coated with a membrane having a thickness of about 15–20 nm (Lundgren et al. 1964). De Koning and Maxwell (1993) suggested the involvement of a single layer of phospholipid covering the PHA granules based on in vitro data of isolated PHA granules. When accumulated by natural PHA producers, an interesting phenomenon occurs, where soluble or membrane-bound PHA synthases catalyze the transformation of hydrophilic monomeric units into hydrophobic polymers (Grage et al. 2009; Nobes et al. 2000). This accountable for the synthesis of intracellular granules made up of a hydrophobic, amorphous polymeric core enclosed by a single layer of phospholipids as well as protein molecules (Jurasek and Marchessault 2004). These proteins involve the PHA synthase, intracellular PHA depolymerases responsible for the depolymerization of PHA polymers that resulting into remobilization of the carbon source (PhaZ family), a regulatory protein (PhaR) and small proteins called phasins, which regulate the size and morphology of granules (York et al. 2002). Various investigations have confirmed the existence of a phospholipid layer in PHA preparations (Parlane et al. 2016). Nevertheless, in vivo occurrence of a phospholipid coat has never been established. Several data have put into question the real occurrence of the lipid layer in vivo (Pötter and Steinbüchel 2006; Beeby et al. 2012; Jendrossek and Pfeiffer 2014), specifically from the studies of electron cryotomography (Wahl et al. 2012) as well as fluorescence microscopy (Bresan et al. 2016), according to which the existence of the lipid coat might develop from an experimental artefact on PHA extraction and preparation.

Merrick and co-workers were initially observed that extracted polyhydroxybutyrate (PHB) granules showed sensitivity towards denaturation phenomenon processes and coined the term native PHB granules (nPHB) so as to specify that such homopolymeric PHB granule preparations relatively similar to the native in vivo condition of PHB granules (Merrick and Doudoroff 1964). Interestingly, merely nPHB granules contain active PHB synthase that are vulnerable to depolymerization with active PHB depolymerases. Once nPHB granules are subjected to chemical treatment like alkali or solvents, physical such as freezing/pelleting through centrifugation or biochemical stresses viz. treatment with enzymes, bioactive compounds etc., the granules quickly denature and remain unaffected towards the action of native PHB

Table 14.3 Overview of biopolymeric PHAs accumulation in different bacteria and cyanobacteria

Organism	Type of organism	Culture/growth environment	% PHA (dcw)	Category of PHA	PHA composition	References
Spirulina platensis	Cyanobacterium	Photoautotrophic	6	SCL-PHA	PHB	Campbell et al. (1982)
Alcaligenes eutrophus H16	Bacterium	Propionate	56	SCL-PHA	P(3HB-*co*-3HV)	Doi et al. (1987)
Gloeothece sp. PCC 6909	Cyanobacterium	Acetate	9	SCL-PHA	PHB	Stal et al. (1990)
Ralstonia eutropha	Bacterium	Glucose + Propionate	70–80	SCL-PHA	P(3HB-*co*-3HV)	Byrom (1992)
Aeromonas caviae	Bacterium	Dodecanoate	36	SCL-MCL-PHA	P(3HB-*co*-3HHx)	Doi et al. (1995)
Pseudomonas sp. 61-3	Bacterium	Glucose	26	SCL-MCL-PHA + SCL-PHA	P(3HB-*co*-3HO-*co*-3HD) + PHB	Kato et al. (1996)
		Fructose	17	SCL-MCL-PHA	P(3HB-*co*-3HHx-*co*-3HO-*co*-3HD-*co*-3HDD)	
Anabaena cylindrica 10 C	Cyanobacterium	Propionate	2	SCL-PHA	P(3HB-*co*-3HV)	Lama et al. (1996)
		Photoautotrophic + N-starved	0.2		PHB	
Synechococcus sp. MA19	Cyanobacterium	Photoautotrophy nitrogen-starved	27	SCL-PHA	PHB	Miyake et al. (1996)
Recombinant Synechococcus sp. PCC7942	Cyanobacterium	Acetate + N-deficiency	26	SCL-PHA	PHB	Takahashi et al. (1998)

(continued)

Table 14.3 (continued)

Organism	Type of organism	Culture/growth environment	% PHA (dcw)	Category of PHA	PHA composition	References
Bacillus cereus UW85	Bacterium	ε- caprolactone + Glucose	9	SCL-MCL-PHA	P(3HB-*co*-3HV-*co*-6HHx)	Labuzek and Radecka (2001)
Synechococcus sp. MA19	Cyanobacterium	Phosphate-deficiency	55	SCL-PHA	PHB	Nishioka et al. (2001)
Bacillus megaterium	Bacterium	Sugarcane molasses	46	SCL-PHA	PHB	Gouda et al. (2001)
Bacillus mycoides RLJ B-017	Bacterium	Sucrose	69	SCL-PHA	PHB	Borah et al. (2002)
Comamonas testosteroni	Bacterium	Naphthalene	85	SCL-PHA	PHB	Thakor et al. (2003)
Ralstonia eutropha H16	Bacterium	Soybean oil	76	SCL-PHA	PHB	Kahar et al. (2004)
Recombinant Ralstonia eutropha H16 strain	Bacterium	Soybean oil	74	SCL-MCL-PHA	P(3HB-*co*-3HHx)	
Nostoc muscorum	Cyanobacterium	Dark + Acetate	43	SCL-PHA	PHB	Sharma and Mallick (2005a)
Recombinant *Aeromonas hydrophila* 4AK4	Bacterium	Lauric acid	53	SCL-MCL-PHA	P(3HB-*co*-3HHx)	Tian et al. (2005a)
Methylobacterium sp. GW2	Bacterium	Methanol	30	SCL-PHA	P(3HB-*co*-3HV)	Yezza et al. (2006)
Nostoc muscorum	Cyanobacterium	Acetate + Glucose + Dark	46	SCL-PHA	PHB	Sharma et al. (2007)

(continued)

Table 14.3 (continued)

Organism	Kind of organism	Culture/growth environment	% PHA (dcw)	Category of PHA	PHA composition	References
Synechocystis sp. PCC 6803	Cyanobacterium	P-deficiency + gas-exchange limitation + Acetate	38	SCL-PHA	PHB	Panda and Mallick (2007)
Pseudomonas aeruginosa MTCC 7925	Bacterium	Ethanol	69	SCL-LCL-PHA	P(3HB-*co*-3HV-*co*-3HHD-*co*-3HOD)	Singh and Mallick (2008)
Spirulina platensis UMACC 161	Cyanobacterium	Acetate + CO_2	10	SCL-PHA	PHB	Toh et al. (2008)
Pseudomonas aeruginosa MTCC 7925	Bacterium	Palm oil + Extract of palm oil cakes	75	SCL-LCL-PHA	P(3HB-*co*-3HV-*co*-3HHD-*co*-3HOD)	Singh and Mallick (2009a)
Pseudomonas aeruginosa MTCC 7925	Bacterium	Ethanol + Glucose	78	SCL-LCL-PHA	P(3HB-*co*-3HV-*co*-3HHD-*co*-3HOD)	Singh and Mallick (2009b)
Arthrospira subsalsa	Cyanobacterium	Photoautotrophic + N-limitation + Increased salinity	15	SCL-PHA	PHB	Shrivastav et al. (2010)
Brevibacillus invocatus MTCC 9039	Cyanobacterium	Glucose + Acetate + Propionate	65	SCL-PHA	P(3HB-*co*-3HV)	Sankhla et al. (2010)
Aulosira fertilissima	Cyanobacterium	Photoautotrophy	7	SCL-PHA	PHB	Bhati et al. (2010)
Recombinant *E. coli* DH5α	Bacterium	Decanoate + Glucose	7	SCL-MCL-PHA	P(3HB-*co*-3HHx-*co*-3HO-*co*-3HD)	Li et al. (2011)

(continued)

Table 14.3 (continued)

Organism	Kind of organism	Culture/growth environment	% PHA (dcw)	Category of PHA	PHA composition	References
Nostoc muscorum	Cyanobacterium	CO_2 + P-deficiency	22	SCL-PHA	PHB	Haase et al. (2012)
Pseudomonas aeruginosa MTCC 7925	Bacterium	Palm oil + Extract of palm oil cakes	77	SCL-LCL-PHA	P(3HB-*co*-3HV-*co*-3HHD-*co*-3HOD)	Singh et al. (2013)
Aulosira fertilissima CCC 444	Cyanobacterium	Fructose + Valerate	77	SCL-PHA	P(3HB-*co*-3HV)	Samantaray and Mallick (2014)
Recombinant *E. coli* XL1	Bacterium	Glucose	62	SCL-PHA	P(3HB-*co*-3HV)	Yang et al. (2014)
Nostoc muscorum Agardh	Cyanobacterium	Optimized condition + N-deficiency	78	SCL-PHA	P(3HB-*co*-3HV)	Bhati and Mallick (2015)
Serratia ureilytica	Bacterium	Volatile fatty acids	51	SCL-PHA	P(3HB-*co*-3HV)	Reddy and Mohan (2015)
Bacillus megaterium	Bacterium	Residual glycerol	52	SCL-PHA	PHB	Gómez Cardozo et al. (2016)
Synechococcus elongates	Cyanobacterium	Sucrose + N-deficiency	17	SCL-PHA	PHB	Mendhulkar and Shetye (2017)
Cupriavidus sp. USMAA1020	Bacterium	1-pentanol + Oleic acid	76	SCL-PHA	P(3HB-*co*-3HV)	Huong et al. (2017)

N Nitrogen, *P* phosphorus/phosphate, *3HB* 3-hydroxybutyrate, *3HV* 3-hydroxyvalerate, *3HHx* 3-hydroxyhexanoate, *3HO* 3-hydroxyoctanoate, *3HD* 3-hydroxydecanoate, *3HDD* 3-hydroxydodecanoate, *3HHD* 3-hydroxyhexadecanoic acid, *3HOD* 3-hydroxyoctadecanoic acid

depolymerases (Merrick 1965; Merrick et al. 1965; Griebel and Merrick 1971). The polymeric chains are usually in an amorphous form in nPHB, while the denatured PHB associate with a substantially crystalline part.

PHAs are a class of linear polyesters of hydroxyalkanoic acids (HA), which are linked together by ester bonds. Figure 14.1 displayed the reaction catalyzed by the PHA synthases, where 'N' can gain a value of 35,000. The structure of PHAs synthesized by bacteria/cyanobacteria can be manipulated through physiological or genetic strategies (Steinbüchel 1991). The organic carbon sources are transformed to hydroxyacyl-CoA thioesters within the bacterial/cyanobacterial cell metabolism. In PHAs biosynthesis, the carboxylate group of one monomeric unit establish an ester bond with the hydroxyl group of the adjacent monomeric unit (Philip et al. 2007), where the reaction is catalyzed by the PHA synthase of host. Each PHA monomer involves a side chain R group with ability to vary from methyl (C_1) to tridecyl (C_{13}) (Madison and Huisiman 1999; Lu et al. 2009). This alkyl side chain, generally a saturated alkyl group, can also hold the uncommon chemical structure like halogenated, aromatic, unsaturated, branched, epoxidized and substituted alkyl groups (Lageveen et al. 1988; Abe et al. 1990; Doi and Abe 1990; Fritzsche et al. 1990a, b, c; Kim et al. 1991, 1992, 1995; Choi and Yoon 1994; Hazer et al. 1994; Curley et al. 1996; Song and Yoon 1996). In the side chains of biopolymeric PHAs, substituents can be transformed chemically by cross-linking of unsaturated bonds (de Koning et al. 1994; Gagnon et al. 1994a, b). The modifications in the side chains and the ability to change their substituents are responsible for the variation of PHAs family (Madison and Huisiman 1999). Furthermore, the molecular weight of PHA polymeric materials varies from 2×10^5 to 3×10^6 Da that reliant on the microorganism in which the polymer is synthesized and the growth conditions maintained (Byrom 1994; Lee 1995). In these PHA biopolymeric materials, the HA monomeric units are all in the *R*(-) configuration because of the sterospecificity of the PHA synthase (Philip et al. 2007; Verlinden et al. 2007). Therefore, biosynthesis of PHAs in microorganism assurances sterospecific integration of the *R*(-) monomeric unit that is important towards the biocompatibility as well as biodegradability of PHAs (Zinn and Hany 2005). The length of the side chain together with its functional group significantly affects the properties of the PHA polymer viz. crystallinity, melting point and glass transition temperature that in turn governs its final uses. The PHAs producers are hopeful and still see potential in this biopolymeric materials claiming that PHAs are new generation of biopolymers and their market requires time to develop. It is projected that demand for PHAs will grow tenfold by 2020 (Aeschelmann et al. 2015).

More than 300 species of microorganisms are recognized so far, which are capable to synthesize PHA biopolymers (Lee et al. 1999). The PHA polymers broadly classified into three types (Table 14.4) depending upon the number of carbon atoms present in the monomeric units, i.e., (a) short-chain-length-PHAs (SCL-PHAs), (b) medium-chain-length-PHAs (MCL-PHAs) and (c) long-chain-length-PHAs (LCL-PHAs) (Anderson and Dawes 1990; Steinbüchel et al. 1992; Vincenzini and De Philippis 1999; Singh and Mallick 2008). Among the various groups of PHA biopolymers, homopolymeric PHB is the most common as well as widespread in different bacteria and cyanobacteria. Interestingly, bacteria have the ability to synthesize SCL-MCL-

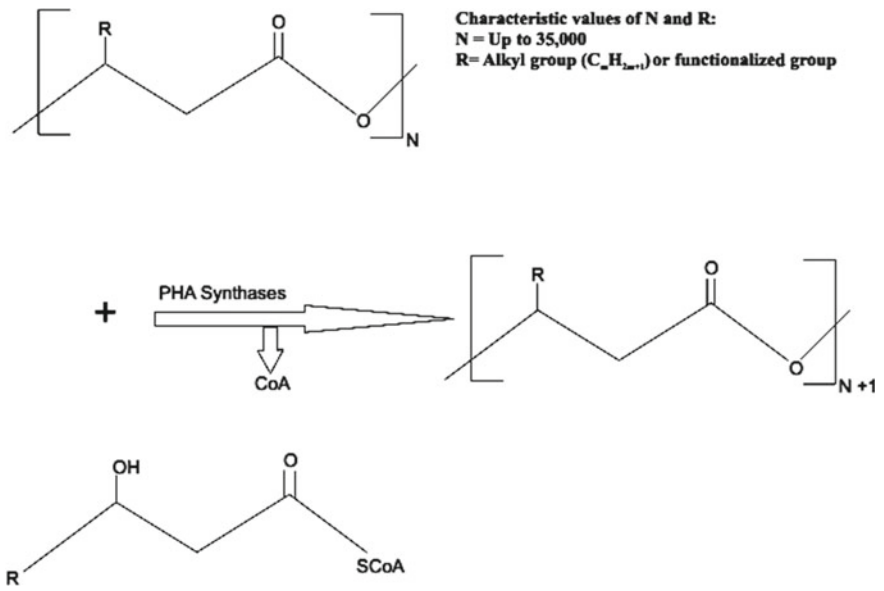

Fig. 14.1 Reaction catalyzed by PHA synthases (compiled from Rehm 2007)

PHA or SCL-LCL-PHA co-polymers apart from SCL-, MCL- or LCL-PHA polymers (Matsusaki et al. 2000; Lee and Park 2002; Singh and Mallick 2008). Majority of the heterotrophic bacterial species produce either SCL- or MCL- or rarely LCL-PHAs, however, usually failed to synthesize co-polymers of SCL-MCL- or SCL-LCL-PHAs because of PHA synthases substrate specificity that can recognize HAs of a specific range of carbon length (Kato et al. 1996; Singh 2009; Ashby et al. 2002; Singh and Mallick 2017a). Presently, there is increasing attention for investigation and commercial ventures on suitable bacterial species towards the accumulation of PHA co-polymers due to their exceptional physical and mechanical properties (Chen et al. 2001; Lee and Park 2002). A perusal of the literature depicted that except PHB and P(3HB-*co*-3HV) co-polymers, there is no report traceable towards the synthesis of MCL-/LCL-PHAs or SCL-MCL-PHAs/SCL-LCL-PHAs co-polymers in cyanobacteria. Therefore, intensifying and innovative research is needed for a wide screening of new as well as extremely prolific cyanobacterial strains from diverse environmental conditions and examination for the accumulation of MCL-/LCL-PHAs or SCL-MCL-PHAs/SCL-LCL-PHAs co-polymers, similar to bacteria (Singh et al. 2017).

Table 14.4 Categorization of biopolymeric PHAs

Types of PHAs	Hydroxyalkanoic acids (PHAs monomeric units) with number of carbon atoms	Examples	Examples of PHAs accumulation by microorganism	References
(a) SCL-PHAs	3–5	PHB	*Brevibacillus invocatus* MTCC 9039 (Bacterium)	Sankhla et al. (2010)
			Nostoc muscorum (Cyanobacterium)	Bhati et al. (2010)
			Aeromonas sp. (Bacterium)	Sangkharak and Prasertsan (2012)
			Recombinant *Synechocystis* sp. PCC 6803 (Cyanobacterium)	Wang et al. (2013)
			Arthrospira subsalsa (Cyanobacterium)	De Morais et al. (2015)
			Bacillus megaterium (Bacterium)	Gómez Cardozo et al. (2016)
			Synechococcus elongates (Cyanobacterium)	Mendhulkar and Shetye (2017)
		[P(3HB-co-3HV)]	*Bacillus* sp. (Bacterium)	Shamala et al. (2012)
			Aulosira fertilissima CCC 444 (Cyanobacterium)	Samantaray and Mallick (2014)
			Recombinant *E. coli* XL1 (Bacterium)	Yang et al. (2014)
			Nostoc muscorum Agardh (Cyanobacterium)	Bhati and Mallick (2015)

(continued)

Table 14.4 (continued)

Types of PHAs	Hydroxyalkanoic acids (PHAs monomeric units) with number of carbon atoms	Examples	Examples of PHAs accumulation by microorganism	References
			Nostoc muscorum Agardh (Cyanobacterium)	Bhati and Mallick (2016)
			Cupriavidus sp. USMAA1020 (Bacterium)	Huong et al. (2017)
(b) MCL-PHAs	6–14	P(3HB-*co*-3HHx-*co*-3HO-*co*-3HD-*co*-3HDD)	Recombinant *Ralstonia eutropha* PHB^{-}4	Luo et al. (2006)
		P(3HB-*co*-3HO)	Recombinant *E. coli* LS521	Gao et al. (2012)
		[P(3HB-*co*-3HHx-*co*-3HO-*co*-3HD)]	Recombinant *E. coli* DH5α	Li et al. (2011)
		[P(3HB-*co*-3HHx)]	Recombinant *E. coli*-$ABC_{Re}J1_{Pp}$	Phithakrotchanakoon et al. (2013)
		P(3HB-*co*-3HHx-*co*-3HO)	Recombinant *E. coli*-$ABC_{Re}J4_{Pp}$	
(c) LCL-PHAs	15 or more than 15	[P(3HB-*co*-3HV-*co*-3HHD-*co*-3HOD)]	*Pseudomonas aeruginosa* MTCC 7925	Singh et al. (2015)

3HB 3-hydroxybutyrate, *3HV* 3-hydroxyvalerate, *3HHx* 3-hydroxyhexanoate, *3HO* 3-hydroxyoctanoate, *3HD* 3-hydroxydecanoate, *3HDD* 3-hydroxydodecanoate, *3HHD* 3-hydroxyhexadecanoic acid, *3HOD* 3-hydroxyoctadecanoic acid

14.3 Microbial PHA Synthases: Main Enzymes of PHA Biosynthetic Pathways

Microbial PHA synthases of PHA biosynthetic pathways are the key enzymes, where they perform a remarkable role in PHA polymerization process by exploiting the coenzyme A (CoA) thioesters of HAs, i.e., (*R*)-3-hydroxyacyl-CoA as substrates (Fig. 14.1). The percent yield and the physicochemical properties of PHAs are primarily reliant on the catalytic performance of PHA synthases, i.e., the polymeriza-

tion of 3-hydroxyacyl-CoA monomeric units into PHA with concomitant liberation of acetyl CoA (Fig. 14.1). Approximately 88 diverse kinds of PHA synthase genes were characterized as well as sequenced from 68 diverse bacterial species (Rehm 2007). During polymerization reactions, they catalyze the covalent bond formation among the hydroxyl group of one and the carboxyl group of another HA (Steinbüchel and Hein 2001). So far, four different groups of PHA synthases (I, II, III and IV) have been reported based on the structure/subunits, amino acid sequence and substrate specificity. The PHA synthases belonging to group I, III and IV polymerize SCL monomers, i.e., C3 to C5, while group II PHA synthase uses MCL monomers ranging from C6 to C14 or more than C_{14}, i.e., LCL monomers. The group I, II, III and IV PHA synthases are signified by *C. necator* including phototrophic non-sulfur purple bacterial species/strains as well as in majority of heterotrophic bacteria, but excluding the pseudomonads from rRNA homology group I, *Pseudomonas putida* together with all pseudomonads of rRNA homology group I, *Allochromatium vinosum* and *Bacillus* sp. (such as *Bacillus megaterium*), respectively (Steinbüchel and Hein 2001; Singh and Mallick 2008; Możejko-Ciesielska and Kiewisz 2016; Sharma et al. 2016). Nevertheless, there are few exceptions to this classification as some PHA synthases depict wide-range substrate specificity. For example, PHA synthases of *Thiocapsa pfenningii* that categorized as group III showed the broad substrate specificity towards CoA thioesters of SCL as well as MCL 3-HA. Similarly, PHA synthases belonging to *Aeromonas caviae* depict high resemblance to group I with capability to produce a 3-hydroxybutyrate and 3-hydroxyhexanoate co-polymer. Moreover, *Pseudomonas* sp. 61-3 synthases of group II depict competence to synthesize a co-polymer of 3-hydroxybutyrate and MCL 3-HA. Remarkably, PHA synthase of *C. necator* belonging to group I capable to produce MCL 3-HA CoA thioesters (Rehm 2003). The group I as well as group II PHA synthases composed of single subunit (PhaC) depicting molecular weights within the range of 61 and 73 kDa (Qi and Rehm 2001). However, group III (e.g. *Allochromatium vinosum*) as well as group IV PHA synthases (e.g. *Bacillus megaterium*) need two kinds of subunits, i.e., PhaC (40.3 kDa) and PhaE (20 or 40 kDa), PhaC (41.5 kDa) and PhaR (22 kDa) for their catalytic activities, respectively. The diversity among the group I PHAs synthases was studied signifying that this group depicts more diverse enzymological features. Nevertheless, the data showed that PHA synthases of all groups revealed an identical topology. Interestingly, group I and II PHAs synthases varied at locations 100–130 or 80–110 (Rehm 2003). It was established that PhaE as well as PhaR are participated in the polymerization of PHAs, but their exact function is still not known. Moreover, PhaCR subunits of group IV can be classified into two types, i.e., *Bacillus cereus* and *Bacillus megaterium* groups. Recently, it was found that group IV PHA synthases belonging to *Bacillus cereus* decrease the molecular weights of PHB (Tomizawa et al. 2011). While structures of PhaC are still not clear, but most recent report advocating that the active site of group III PHA synthases could be more polar compared to group I PHA synthases and both are sensitive to the alterations in the alkyl side chain (Jia et al. 2016). Interestingly, these various kinds of PHA synthase found to depict one stringently preserved cysteine residue

that is potentially the active site engaged in the polymerization reaction (Griebel et al. 1968).

However, biosynthesis of PHA granules in cyanobacteria has received the consideration since the report of PHB accumulation in N_2 fixing cyanobacterium, *Chlorogloea fritschii*, but identification, cloning, as well as molecular characterization of cyanobacterial PHA synthase has been reported for the first time in the *Synechocystis* sp. PCC6803, a cyanobacterium (Carr 1966; Hein et al. 1998). It was shown by Hein et al. (1998) that PHA synthase belonging to *Synechocystis* sp. PCC6803 is a two-component enzyme that represented by two open reading frames (ORFs), i.e., slr1830 as well as slr1829. The ORF slr1830 that encoding a protein having 378 amino acids was designated as *phaC* (*phaC*Syn) whereas the other ORF slr1829, located colinear and upstream of *phaC* was designated to as *phaE* (*phaE*Syn). The multialignment of the *phaE* and *phaC* gene products showed remarkable sequence resemblance with the two subunits viz. PhaE and PhaC of group III PHA synthase belonging to three γ-Proteobacteria namely *Chromatium vinosum, Thiocystis violacea and Thiocapsa pfennigii* (Hein et al. 1998; Liebergesell et al. 2000; Ansari et al. 2016). Similarly, the cyanobacterium, *Synechococcus* sp. MA19 also found to possess type III PHA synthases (Steinbüchel and Hein 2001).

14.4 Molecular Basis of PHA Biosynthesis Enzymes

The PHA synthesis genes are usually bunched in the genomes of bacteria. *C. necator* has been investigated in detail in which the genes encoding PHA synthase (*phaC*), β-ketothiolase (*phaA*) and NADPH-linked acetoacetyl-CoA reductase (*phaB*) form the *phaCAB* operon (Peoples and Sinskey 1989a, b; Schubert et al. 1988; Slater et al. 1988). In addition, the generally found genetic organization of *C. necator* amongst PHB producing bacteria, many bacteria depict a diverse gene order, nevertheless, at least the PHB synthase gene is co-localized with other PHB biosynthesis genes. Several bacterial species like *P. denitriicans* possess next to the PHA synthase additional genes such as *phaP* (encoding phasin) and *phaR* (encoding regulator protein) linked to PHA biosynthesis. An operonic organization of PHA biosynthesis genes, linked to the SCL-PHA production (group I PHA synthase gene) was found amongst the β-proteobacteria like *C. necator*, *Burkholderia* sp., *Delftia acidovorans* and *Alcaligenes latus*.

The pseudomonads that produce MCL-PHAs involve two different genes encoding group II synthases that are parted by the structural gene *phaZ* encoding a putative intracellular PHA depolymerase (Timm and Steinbüchel 1992; Hoffmann and Rehm 2004, 2005). Merely one of these PHA synthase genes is needed towards the biosynthesis of MCL-PHA (Langenbach et al. 1997; Pham et al. 2004). The *phaD* gene is positioned directly downstream of the second synthase gene, but upstream of the genes *phaI* and *phaF* that are transcribed in opposite direction. PhaI along with PhaF are recognized as structural and regulatory proteins (Prieto et al. 1999; Hoffmann and Rehm 2004, 2005). Remarkably, the genes encoding enzymes like transacylase

(PhaG) or the enoyl-CoA hydratase (PhaJ) that are directly involved in the provision of substrate of PHA synthase are not co-localized in pseudomonads (Rehm et al. 1998; Tsuge et al. 2000). The group III PHA synthase genes *phaC* and *phaE* are co-localized in the corresponding genomes comprising presumably a single operon (Liebergesell et al. 1992, 1993). The group IV synthase genes are present in the bacterial species belonging to genus *Bacillus* and consist of *phaR* and *phaC* that are separated by *phaB* (McCool and Cannon 1999, 2001).

The cyanobacterial PHA synthase with its activity was reported in the membrane fractions of a cyanobacterium, *Spirulina* sp. MA19 for the first time under nitrogen-deficient condition (Miyake et al. 1997; Asada et al. 1999). The genome analysis of *Synechocystis* sp. PCC6803 that accumulated about 0.1 g PHB per g dry cell weight (dcw) under acetate supplemented condition depicted synthase genes corresponding to the open reading frame (ORF) *slr1830* (designated as *phaC*) and to the collinear ORF *slr1829* upstream of *phaC* (designated as *phaE*). Interestingly, *phaE* and *phaC* genes showed much greater sequence resemblance with the corresponding group III PHA synthase subunits present in the anoxygenic purple sulfur bacterial species viz. *Thiocystis violacea*, *Chromatium vinosum* and *Thiocapsa pfennigii*, which are typical PHA accumulators containing group III PHA synthases. In addition, expression of these genes in *Escherichia coli* as well as *Alcaligenes eutrophus* evidently exhibited that co-expression of both *phaC* and *phaE* is required to attain an active *Spirulina* sp. PCC6803 PHA synthase (Hein et al. 1998). Later, it was revealed that *Spirulina* sp. PCC6803 contains a PHA specific β-ketothiolase encoded by *phaASyn* and an acetoacetyl-CoA reductase encoded by *phaBSyn*. Resemblance investigation of the whole genome sequence of this strain showed a cluster of two putative ORFs for these genes, *slr1993* (409 amino acids; *phaASyn*) and *slr1994* (240 amino acids; *phaBSyn*). These ORFs are collinear and co-expressed. The transformation of *E. coli* cells with *phaASyn* and *phaBSyn* along with the PHA synthase of *Spirulina* sp. PCC6803 led to production of 0.123 g PHA per g dcw under glucose supplemented condition. Furthermore, phylogenetic analysis was carried out to group the origin of *phaASyn* and *phaBSyn* genes in the γ-subdivision of Proteobacteria (Taroncher-Oldenburg et al. 2000).

The wide range and generic incidence of cyanobacterial group III PHA synthases was later supported by Hai and colleagues, together with the molecular characterization of PHA synthases of the thermophilic *Chlorogloeopsis fritschii* PCC 6912 as well as *Synechococcus* sp. strain MA19. The PHA accumulation potential and the kind of accountable PHA synthases belonging to eleven various cyanobacterial strains were studied by Southern blot analysis with *phaC* specific probes, Western blot analysis using specific polyclonal anti*PhaE* antibodies, sequence analysis of PCR products with *phaC*-specific oligonucleotide primers, cloning techniques, and finally sequence analysis of the PHA synthase structural genes. The presence of group III PHA synthase was evidenced in *S.* ssp. MA19 and PCC 6715, *Chlorogloeopsis fritschii* PCC 6912, *Anabaena cylindrica* SAG 1403-2, *Cyanothece* ssp. PCC 7424, PCC 8303 and PCC 8801, and *Gloeocapsa* sp. strain PCC 7428. As a positive control, the screening was compared with crude protein extracts and DNA of *Synechocystis* sp. strain PCC 6803. No group III PHA synthase was observed in *Stanieria* sp. strain

PCC 7437, *Cyanothece* sp. strain PCC 8955, and *Gloeothece* sp. strain PCC 6501 (Hai et al. 2001). Most recently, Numata et al. (2015) reported the specific activity of PHA synthase of the *Synechocystis* sp. PCC 6803, where both genes, *phaC* and *phaE* were co-expressed in a cell-free synthesis system. Specific activity of *phaCE* was analogous to group I PHA synthases, which usually occurring in most common PHA producers such as *C. necator* and therefore, contradicting earlier assumptions that inadequate synthase activity could be the cause for modest PHA productivity in cyanobacteria.

14.5 Biosynthetic Routes Involved in the Production of PHAs from Different Carbon Substrates

One of the parameters, which govern the kind of PHA components, is the organic carbon substrates. Microbial world is proficient in accumulating PHAs from different organic carbon substrates varying from cost-effective, complex waste effluents (such as beet and cane molasses) to plant oils and its fatty acids, alkanes and simple carbohydrates (Lageveen et al. 1988; Hängii 1990; Page 1992; Eggink et al. 1993, 1995; Tan et al. 1997; Fukui and Doi 1998). The detections of various PHA monomer units apart from 3-hydroxybutyrate (3HB) more than three decades ago clearly indicated that the PHA synthases depict a wide-range substrate specificity and thus, a wide diversity of PHA monomers can be polymerized (Sudesh et al. 2000). Furthermore, only the existence of a PHA synthase is not enough to allow the accumulation of PHA polymers. PHA production will not take place if the genes that encode enzymes needed towards the production of hydroxyacyl-CoA thioesters are not present or if the routes established through these enzymes are for any kind of reason not functionally active. This is supported by the fact that as soon as there is expression of PHA synthase gene in wild type or ordinary laboratory *E. coli* strains, even if a functionally active PHA synthase is synthesized, no or merely traces of PHAs are synthesized (Steinbüchel and Hein 2001).

So far, PHA biosynthetic routes present in microorganisms widely grouped into four kinds (Kumar et al. 2015). The PHB biosynthetic route is best studied in a number of bacterial strains/species such as *Azotobacter beijerinckii*, *C. necator* etc. In these groups of bacterial species, the PHB polymer synthesis begin from acetyl-CoA when they supplemented with carbohydrates or acetyl-CoA providing organic carbon substrates. This PHB route includes three enzyme assisted reactions sequences viz. 3-ketothiolase, acetoacetyl-CoA reductase as well as lastly PHB synthase (Oeding and Schlegel 1973; Senior and Dawes 1973). The initial stage of this route includes a reversible reaction catalysed by β-ketothiolase that transformed two acetyl-CoA molecules into acetoacetyl-CoA intermediates (Masamune et al. 1989; Moskowitz and Merrick 1969). The acetoacetyl-CoA molecules so generated are then converted into monomer *R*-(−)-3-hydroxybutyryl-CoA in the presence of acetoacetyl-CoA reductase and NAD(P)H as reducing power. The last stage includes the PHB

synthase, which integrates the monomeric unit, i.e., *R*-(−)-3-hydroxybutyryl-CoA into the growing polymeric chain of PHB. One general observation regarding the kinds of PHAs synthesize by *C. necator* is that the integrated monomeric units always consist of merely C3–C5. However, the nature and the ratio of these monomeric units are affected by the kind as well as the relative amount of organic substrates added in the cultivation media (Steinbüchel 1991; Steinbüchel et al. 1993). It has been observed that addition of propionate or valerate with glucose into the growth media of *C. necator* facilitates the biosynthesis of PHA co-polymer, i.e., [P(3HB-*co*-3HV)]. This signified that the PHA synthase of *C. necator* is only active towards polymerizing the monomeric units belonging to SCL HA. Nevertheless, the position of the oxidized carbon in the monomeric unit is seemingly not a critical parameter that supported by the integration of 4- and 5-HA units apart from the more general 3HA units (Doi et al. 1987). *C. necator* is proficient in producing PHAs from specialized organic carbon substrates viz. 4-hydroxybutyric acid, γ-butyrolactone and 1,4-butanediol, where synthesis of 4HB monomeric units occur together with 3HB (Doi et al. 1989, 1990). The reports show that the polymerizing enzyme of *C. necator* could really possess a wider range of substrate specificity. This was comprehended when the PHA synthase gene belonging to *C. necator* was expressed in a heterologous condition that can supply towards a broader variety of HA monomeric units. It was reported that the *C. necator* PHA synthase can integrate minor quantities of 3-hydroxyhexanoate (3HHx,) 3-hydroxyoctanoate (3HO) and 3-hydroxydodecanoate (3HDD) units (Dennis et al. 1998; Antonio et al. 2000).

Interestingly, a slight modification of aforementioned PHB biosynthetic route has been detected and represented by *Rhodospirillum rubrum*, where two additional enzymes (enoyl-CoA hydratases) are involved as NADH dependent acetoacetyl-CoA reductase catalyzed the reduction of acetoacetyl-CoA into *S*-(+)-3-hydroxybutyryl-CoA. These two enoyl-CoA hydratases then transform the resulting *S*-(+)-3-hydroxybutyryl-CoA isomer into *R*-(−)-3-hydroxybutyryl-CoA isomer (Moskowitz and Merrick 1969). This constitutes second type of PHA biosynthetic route.

The third kind of PHA biosynthetic route that found to exist in bacterial species/strains like *Pseudomonas oleovorans*, *Pseudomonas aeruginosa* as well as the most of pseudomonads from the rRNA homology group I contains the β-oxidation and thiolytic cleavage of fatty acids, i.e., 3-hydroxyacyl-CoA and intermediates of the β-oxidation pathways (Doi 1990; Punrattanasin 2001; Singh and Mallick 2009a). These bacterial species accumulate MCL-PHAs and hardly LCL-PHAs under supplementation of alcohols, alkanes or alkanoates. Also, they are usually not capable of accumulating SCL-PHAs similar to *Cupriavidus necator*. Under growth environments favouring PHA synthesis, the intermediates of the β-oxidation cycle could be transformed into *R*-(−)-3-hydroxyacyl-CoA by enoyl-CoA hydratases, epimerases or ketoacyl-CoA reductases and are then polymerized through the catalytic action of PHA synthase (Huisman et al. 1991).

The fourth PHA biosynthetic route occurs in *Pseudomonas aeruginosa* and PHA is synthesized from acetyl-CoA using fatty acid biosynthetic route (Huijberts et al. 1995; Steinbüchel 1996; Singh and Mallick 2008, 2009b). Some pseudomonads from the rRNA homology group I also synthesize MCL-PHAs including SCL-LCL-PHA

co-polymer through this route (Singh et al. 2013). MCL-PHA/SCL-LCL-PHA co-polymer produce through this route includes unrelated organic carbon substrates such as glucose, ethanol, gluconate or acetate. Such organic carbon sources are initially employed towards the synthesis of fatty acids that then produce precursors for PHA polymerase for synthesizing MCL-PHA/SCL-LCL-PHA co-polymer (Singh and Mallick 2017a, b).

Nevertheless, similar to bacteria, cyanobacteria also possess PHA biosynthetic routes for PHAs production. Vincenzini and De Philippis (1999) have widely documented the presence of PHAs in almost ninety strains from four different phylogenetic subdivisions of cyanobacteria. The PHA biosynthetic routes of cyanobacterial species so far only reported to synthesize SCL-PHAs, i.e., PHB homopolymer and P(3HB-*co*-3HV) co-polymer (Singh et al. 2017). Apart from this, their PHA biosynthetic routes showed similarity with the bacteria (Wang et al. 2013).

14.6 Mechanism of PHAs Polymerization Reaction

The PhaC fits in the α/β-hydrolase family of enzymes, where it catalyzes the stereoselective transformation of the activated precursor (*R*)-3-hydroxyacyl-CoA into polyoxoesters along with the concomitant liberation of CoA (Peoples and Sinskey 1989b; Schubert et al. 1988; Slater et al. 1988). Commencement of PHA formation needs activation of the thiol group of the cysteine residue of the PhaC active site through the preserved histidine in the same active site, allowing a nucleophilic attack on the thioester bond of the (*R*)-3-hydroxyacyl-CoA (HA-CoA) substrate, concomitantly liberating CoA and producing a covalent enzyme-substrate intermediate (Fig. 14.2) (Wodzinska et al. 1996; Tian et al. 2005b). PHB synthase of *C. necator* occurs in aqueous solution as an equilibrium among monomeric and homodimeric enzyme molecules (Gerngross et al. 1994; Zhang et al. 2000). The earlier investigations exhibited that synthase activity was reversibly lost by dilution and the dimer depicts a considerably more specific activity compared to the monomeric form (Zhang et al. 2000). Besides, the experiential relationship between molecular weights of PHB and substrate: enzyme ratios is constant with a single polymeric chain being synthesized by the homodimer synthase that again suggests dimer is the active form of the enzyme (Zhang et al. 2000).

The active-site model for PHA synthase was initially put forwarded by Ballard et al. (1987), where two thiol groups were proposed to be participated in locating the HA units. This model was later modified by Kawaguchi and Doi (1992) by suggesting that water might function as a chain transfer agent. The present theory of the reaction mechanism of PHA synthases is based on a model in which two thiolates participate towards the covalent catalysis of PHA polymer biosynthesis (Fig. 14.2) (Griebel et al. 1968). Biosynthesis of PHAs commences most possibly at thiolate groups [S_1H and S_2H] furnished by the PHA synthases [E], where one thiol group acts for a loading site and the second thiol group acts as a priming and elongation. Based upon this present model (Müh et al. 1999; Ballard et al. 1987;

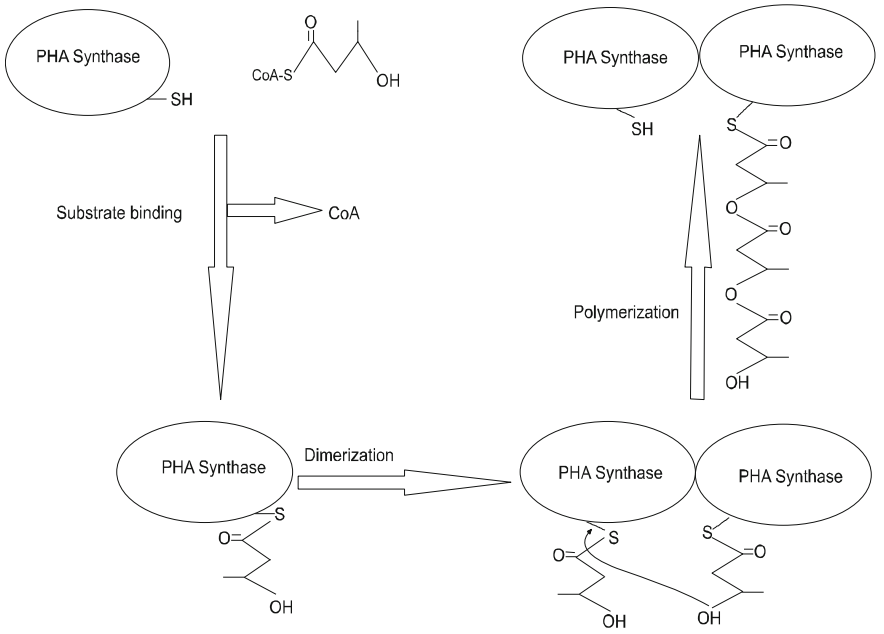

Fig. 14.2 Model depicting the catalytic mechanism of PHA synthase (Rehm 2007)

Kawaguchi and Doi 1992), the reaction mechanism involves the following steps: one thiol group [S_1H] accepts a hydroxyalkanoic acid from CoA thioester with the latter becoming covalently linked to this thiol group [E-S_1-CO-Alkyl-OH] and the CoA being liberated, while the developing polymer chain is linked with the second thiol group [E-S_2-poly(HA)-OH]. The latter is then transferred to the free hydroxyl group upon a nucleophilic attack of the hydroxyl oxygen atom on the carbonyl carbon atom that give rise to [E-S_1-poly$(HA)_{n+1}$-OH]. A successive transesterification of the lengthened polymer chain from S_1 to S_2 give rise to [E-S_2-poly$(HA)_{n+1}$-OH] and [E-S_1H] and the latter can now receive the next hydroxyalkanoic acid from a CoA thioester. This catalytic reaction cycle essentially is repeated for many thousand times as otherwise the extremely high molecular masses of the synthesized polymers cannot be explained. Furthermore, the catalytic reaction cycle might be interrupted if the developing polymer chain is liberated from [E-S_2-poly(HA)-OH] by the nucleophilic attack of a hydroxyl group of a molecule that is not linked to [E] like water. For instance, there is report that hydroxy-compounds such as polyethylene glycol, glycerol or 1,3-propanediol also furnish such a chain terminating hydroxyl group that results in a PHA molecule, which possesses the corresponding hydroxy-compounds covalently connected with the PHAs (Madden et al. 1999; Shah et al. 2000). Report also depicted an enhanced PhaC copy number with a reduction in PHAs chain length, which signifying that the quantity of PhaC in a host cell has a function in regulating chain length of PHA (Sim et al. 1997). This general mechanism

illustrated more clearly with the polymerization reaction of PHB, where the histidine (His) deprotonates the cysteine (Cys) to produce the active site thiolate that reacts with (*R*)-3-hydroxybutyryl-CoA (HB-CoA). This reaction resulted into the acylation of PHB synthase with hydroxybutyrate (HB). A second HB-CoA then binds and the Asp acts as a common base to trigger its hydroxyl group for attack on the HB-PHB synthase thioester, liberating the developing $(HB)_n$-SCoA chain inside the active site. This noncovalent intermediate then quickly reacylates the active site Cys, where the polymerization lasts till the polymer achieves a comparatively uniform molecular mass that varies with organism (Liebergesell and Steinbüchel 1992; Wodzinska et al. 1996; Jia et al. 2000, 2001; Rehm et al. 2002). Experimental evidence showed that chain termination taking place with the transfer of majority of the polymer chain to a second, surface-exposed amino acid that breaks the chain (Tian et al. 2005b).

The preserved residues Cys-319, aspartate (Asp)-480 and His-508 of the group I PHA synthase from *C. necator* were the first be studied with site-specific mutagenesis that furnished evidence of direct participation in covalent catalysis (Gerngross et al. 1994; Jia et al. 2001). Furthermore, the extremely preserved tryptophan (Trp)-425 was exchanged with alanine strongly decreasing the enzyme activity. Trp-425 has been proposed to be participated in protein-protein interaction through producing a hydrophobic surface towards dimerization of PhaC subunits (Gerngross et al. 1994). Likewise, the homologous amino acid residues Cys-149, His-331 and Asp-302 of PHA synthase from *A. vinosum* were subjected to site-specific mutagenesis that almost inhibited enzymatic activity. These reports strongly indicated an involvement in covalent catalysis (Jia et al. 2000). Furthermore, the preserved catalytic triad residues of group II PHA synthase of *P. aeruginosa* were investigated with site-specific mutagenesis in which Cys-296 as well as Asp-452 were reported to be important for the enzyme activity as was observed with the other groups of PHA synthases (Amara and Rehm 2003). Remarkably, substitution of the putative general base catalyst His-480 resulted in strongly impairment of enzymatic activity. However, exchange of the preserved cysteine as well as aspartic acid deactivated the enzyme. Thus, it is anticipated that this cysteine residue supplies one of the thiolate groups towards covalent catalysis. It is the merely cysteine residue that is preserved in any PHA synthase. As the present reaction mechanism of PHA synthase is based on the thoroughly characterized fatty acid synthase, therefore, it is postulated that two thiol groups are essential, where the second thiol was supposed to be available following post-translational modification by a phosphopantethine moiety (Wakil 1989; Chang and Hammes 1990; Gerngross et al. 1994). A likely candidate for this modification is the preserved serine residue at location 260 of the PHA synthase structural gene of *C. necator*. Nevertheless, existing findings have failed to support this theory for a specific post-translational modification (Rehm and Steinbüchel 1999). The suggested step including chain-transfer was confronted based on the hypothesis that the PHA synthase is not proficient of transferring to a new chain (Kawaguchi and Doi 1992; Gerngross and Martin 1995; Su et al. 2000). However, investigation utilizing the purified *C. vinosum* PHA synthase indicated a mechanism concerning a chain-transfer step (Liebergesell et al. 1994). Based on kinetic investigations studying the lag phase of the enzyme reaction, it is now suggested that in group I- and group II-

PHA synthases, the two thiol groups are furnished by two Cys-319 residues situated on two subunits and that a dimer of PhaC is, hence, the smallest size of these PHA synthases. In this model, Cys-319 alternates between the function of the loading thiol as well as the role of the elongation thiol as aforementioned (Müh et al. 1999). This could certainly also be realistic for the group III PHA synthases. Nevertheless, investigation of the primary structures of all so far sequenced group III PHA synthases depicted a second extremely preserved cysteine residue that is for instance, Cys-130 in PhaC of *C. vinosum* (Rehm and Steinbüchel 1999). Sinskey and co-workers substituted this cysteine with an alanine in which they achieved an enzyme, which showed merely 0.003% of the activity of the wild-type enzyme (Müh et al. 1999). Overall, the α/β-hydrolase-based catalytic mechanism, mainly considering lipases and cysteine proteases, furnishes a good model for groups I-III of PHA synthases as strengthened by mutational study of the *C. necator* group I PHA synthase, the *A. vinosum* group III PHA synthase and the *P. aeruginosa* group II PHA synthase (Rehm 2003). Currently, no clear final inference can be drawn concerning the provision of the second thiol group towards the catalytic cycle of PHA synthases.

14.7 Mechanism of Biogenesis of PHA Granules

Interestingly, the biosynthesis of PHA granules is somewhat differ over the synthesis of a prokaryotic neutral lipid inclusion such as wax esters or triacylglycerols granules (Wältermann et al. 2005; Wältermann and Steinbüchel 2005). PHA synthase facilitated the template independent polymerization reaction from the corresponding HA-CoA. The PHA synthase is present in the cytoplasm of exponentially cultivated cells that have not yet synthesized PHAs (Haywood et al. 1989). On the other hand, *C. necator* and *Allochromatium vinosum* grown cells under environments appropriate for PHA biosynthesis, the PHA synthase is accompanying with the PHA granules as the developing PHA material is covalently associated with the enzymes throughout polymerization reaction, conferring amphiphilic property to the enzyme-polymer complex (Haywood et al. 1989; Gerngross et al. 1993; Liebergesell et al. 1994). Three models namely micelle formation of PHB synthases, budding of PHB from the cytoplasm membrane and scaffold model so far have been proposed for in vivo PHA granule formation (Stubbe and Tian 2003; Tian et al. 2005a, b). They are based on hypothetical considerations as well as experimental data of *C. necator*. The Micelle Model based on the fact that soluble (cytoplasmic) PHB synthase molecules (PhaC1 dimers) begin to form the hydrophobic PHB polymer if the substrate (HB-CoA) quantity is adequately high (Ellar et al. 1968; Griebel et al. 1968; Gerngross et al. 1993). Owing to the hydrophobic behavior along with lower dissolution of PHB in an aqueous environment, nascent polymeric chains assembled and produce micelle-like structures within the cytoplasm with partly hydrophilic PhaC molecules residing on the polymer surface (Stubbe and Tian 2003; Stubbe et al. 2005). Later, phasins together with other PHB granule-associated proteins (PGAPs) attach with the developing granules. An importance of this model is that commencement of PHB granules

should occur at any place within the cytoplasm and these granules usually should be essentially randomly localized within the cell. In budding model, the synthases would be peripheral membrane bound proteins, attached to the inner face of the plasma membrane. Their attachment to the membrane could be assisted by a primer molecule mainly a long chain fatty acid or oligomers of 3-HB covalently bonded to the active site cysteine of the synthase. As the PHB chains grow and the PhaP phasin is formed, budding of a vesicle with a monolayer or partial monolayer of lipid could result, leading to granule formation. At least, the initial step of PHB granules should localize in/at the cytoplasmic membrane if the budding model is correct. This model similar to the model for the biosynthesis of eukaryotic neutral lipid bodies (Wältermann et al. 2005). Nevertheless, this budding model neglects the fact that PhaC as well as phasins are never situated at the cytoplasmic membrane. Tian et al. (2005a, b) proposed a third model for granule formation called scaffold model. This model is based on the fact that PHB synthase of nascent PHB granules is or becomes linked to a yet unknown scaffold molecule within the cell. Also, the subcellular localization of PHB granules in this case would depend on the nature and localization of the scaffold of the PHB producing cell. Jendrossek and Pfeiffer (2014) suggested substituting the earlier micelle mode of PHB granule formation with the scaffold model. This is presently the most accepted model to explain the existing experimental data on PHB carbonosome synthesis in *C. necator*. For instance, the TEM investigations depicted that PHB granules in the cells, which were cultivated under environments permissive for PHB formation but not for growth (high carbon content, low or no nitrogen source) were often situated relatively at the centre of the cells in close neighbourhood to dark-stained known as mediation elements (Tian et al. 2005a, b). These findings were in agreement with the scaffold model, nevertheless, the nature of the scaffold (mediation element) remained ambiguous. Furthermore, the same group studied the PHB formation in *C. necator* with cryotomography (Beeby et al. 2012), where it was in agreement with their earlier TEM investigations. The budding model of PHB granule synthesis was excluded in this study owing to the high resolution of cryotomography. Nevertheless, a clear differentiation between the scaffold as well as micelle model was not probable. As the nucleoid area was not evident in Beeby's cryotomograms, the data do not exclude the probability that the nucleoid signifies the scaffold to which PHB granules are attached. Further studies should be carried out to specify the areas of PHB granule attachment and whether there is a specificity of the DNA locus to which the granules become attached (Jendrossek and Pfeiffer 2014).

14.8 Phasins: Major PHA Granule-Associated Proteins

In PHA synthesizing bacteria, PHA chains are aggregated and form PHA granules within the cytoplasm. These granules are surrounded by different protein molecules known as PHA granule-associated proteins (PGAPs) in which phasins (PhaPs) are composed of small size amphiphilic protein molecules and found to be foremost

PGAPs commonly dispersed in different PHA accumulating organisms (Wieczorek et al. 1995, 1996; Pötter and Steinbüchel 2005; Jendrossek and Pfeiffer 2014). The roles of PhaPs are believed to regulate the properties of PHA granules surface apart from the effect upon PHA biosynthesis. The PhaP1$_{Re}$ is the most plentiful phasin of *R. eutropha* H16 (Wieczorek et al. 1995). The expression level is extremely high because of the strong promoter, i.e., *phaP1* through which transcription is controlled as a result of PHA granule binding transcriptional factor PhaR$_{Re}$ (Pötter et al. 2002; Pötter and Steinbüchel 2005; Brigham et al. 2010; Shimizu et al. 2013). The PhaP1$_{Re}$ contributed about 5% of the overall protein molecules of the crude extract of the cells grown on fructose as a carbon source and was anticipated to surround 27–54% of exterior portion of the polymer granules (Wieczorek et al. 1995; Tian et al. 2005c). It was established that PhaP1$_{Re}$ played important role in regulating the size as well as number of polymer granules within the cells including resulting quantity of PHAs (Wieczorek et al. 1995; York et al. 2001b). Remarkably, PhaP1$_{Re}$ failed to bind PhaC1$_{Re}$ straightforwardly in two hybrid assays, nevertheless, produced a high molecular mass complex in the company of PhaC1$_{Re}$ and soluble PHB oligomer in *R. eutropha*. The PhaC1$_{Re}$-PhaP1$_{Re}$-PHB complex exhibited no lag phase in PhaC activity assay that signified that PhaC$_{Re}$ had an active form in the complex (Pfeiffer and Jendrossek 2011; Cho et al. 2012). A comparable reduction in activity was also observed for the synthase belonging to *D. acidovorans* (PhaC$_{Da}$) (Ushimaru et al. 2014). In contrast, PhaP1$_{Re}$ enhances the activity for the synthases belonging to *A. caviae* and *P. aeruginosa* by decreasing the enzymatic lag phase, though it does not influence the activity of PhaC belonging to *C. vinosum* (Jossek et al. 1998; Qi et al. 2000; Ushimaru et al. 2014). In addition, various other phasin proteins have been reported in other microorganisms like *Sinorhizobium meliloti*, *Haloferax mediterranii* or *Herbaspirillum seropedicae*. However, there is little information about them apart from their function towards polymer accumulation (Wang et al. 2007; Cai et al. 2012; Tirapelle et al. 2013; Alves et al. 2016). Furthermore, the investigation regarding the secondary structure of the PhaP1$_{Re}$ sequence forecasts a highly α-helical conformation, which is characteristic of phasins. The phasin has been revealed to attain a planar, triangular shaped homotrimeric conformation as depicted by small angle X-ray scattering study. First sequence studies did not reveal a clear, predicted PHA binding motif like long hydrophobic patches (Neumann et al. 2008).

Currently, PHA granule accompanying proteins have received more attention as various research laboratories have revealed that defective or deficient phasins depict considerable impacts on PHA polymer formation. For instance, Tn5-induced *phaP1* mutants having defective phasin production are even then capable to form PHB polymer, nevertheless, they produce polymer at a substantially lower rate and nearly the whole PHB is exist in merely one single big granule within the cell (Wieczorek et al. 1995). Likewise, studies conducted with the gene deletion of *phaP1* established that the PHB quantity under specific culture cultivation condition was decreased by 50% compared to the wild-type *C. necator* (York et al. 2001b). However, overexpression of PhaP1$_{Re}$ resulting into enhance granule numbers (Pötter et al. 2002). Furthermore, investigations were also carried out with the *phaR* deletion mutant strain of *C. necator*, where PhaP was constitutively expressed at high levels. Nevertheless,

no PhaP is formed if *phaC* is deleted (Wieczorek et al. 1995; York et al. 2001a, b). Notably, deletion of together *phaR* as well as *phaC* found to trigger high levels of PhaP expression, even though the organism fails to synthesize PHB polymer.

The *R. eutropha* phasins also play a role in the stability as well as mobilization of PHB inclusions. Absence of $PhaP1_{Re}$ in a single deletion mutant results a certain degree of PHB autodegradation in vivo, a phenomenon, which is dramatically increased when integrated with the multiple deletion of other phasins, signifying that phasins are important towards stabilization of the granule (Kuchta et al. 2007). Surprisingly, phasins are also essential towards the PHB mobilization triggered by CoA thiolysis that catalyzed by the PhaZ depolymerase. However, PHB lacking phasins is fail to undergo depolymerization by PhaZ, the $PhaP1_{Re}$ alone is enough to help the depolymerase in the process of PHB degradation (Uchino et al. 2007; Eggers and Steinbuchel 2013). In contrast, in the lack of $PhaP1_{Re}$, the other minor phasins could also play role in PHB mobilization to a variable level (Kuchta et al. 2007; Uchino et al. 2007; Eggers and Steinbuchel 2013). The expression of PhaP1 is stringently controlled at the transcription level by PhaR, therefore, confirming that the phasin is formed merely when environments are permissive towards PHB formation and PhaC is present and in sufficient amount to surround all the surface of biopolymer, but without triggering a protein stock in the cytoplasm (York et al. 2001a; Pötter et al. 2002; York et al. 2002; Wieczorek et al. 1995).

In order to presume a model concerning the regulation of phasin expression, further investigations were carried out with *C. necator*. It was established that PhaR might attach with artificial PHB granules (Pötter et al. 2002). Moreover, Western immunoblotting as well as immunoelectron microscopic localization with antibodies produced against PhaR undoubtedly revealed that PhaR was attached with the PHB granules. Hence, PhaR has the capability to attach at least to four diverse receptors of the *C. necator* viz. the promoter regions of *phaP1* (1) *phaP3* (2) and *phaR* (3) and the surface of PHB granules (4). These studies support the simple and elegant model towards the regulation of expression of PhaP in *C. necator*, where PhaR playing the role as a repressor of transcription. Under cultivation environments not appropriate towards PHAs formation or in mutants faulty for PHAs accumulation, PhaR attaches with the *phaP* promoter region and represses transcription of this gene. The concentration of PhaR in cytoplasm is adequately high to repress transcription of *phaP1* and *phaP3*. However, under physiological environments that are apposite towards PHB accumulation, the constitutively expressed PHA synthase begins to produce PHB polymers that remain covalently associated with this enzyme. At the commencement, tiny micelles are synthesized that become bigger and form the nascent PHB granules. Proteins with a binding capability with the hydrophobic surfaces such as PhaR attach with the granules. However, the concentration of PhaR in cytoplasm from a certain point turn out to be sufficiently low, where it no longer repressed *phaP1* as well as *phaP3* transcription. Thus, PhaP1 as well as PhaP3 were formed, which then attached with the exterior portion of PHB granules. The PHB granules turn out to be larger and achieved their maximum size. Concurrently, PhaP1 was found to produce uninterruptedly in adequate quantities. Furthermore, little quantities of PhaP3 were also produced. Under to the physiological conditions when the PHB

granules have attained the maximum probable size and when majority of the surface of PHB granule is surrounded with PhaP1, no more space will be accessible towards the attachment of additional PhaR or PhaR may even be displaced with PhaP1 (and PhaP3). Therefore, the amount of PhaR in cytoplasm will increase and surpass the threshold amount needed to repress again *phaP1* as well as *phaP3* transcription. As a result, PhaP1 as well as PhaP3 protein molecules are as no longer formed and these phasins are consequently, not produced too much and therefore, do not surpass the quantity needed to surround the exterior part of PHB granules (Pötter et al. 2005).

Phasins offer a diverse opportunity in the field of Biotechnology (Maestro and Sanz 2017). For instance, the amphiphilic property of phasins makes them appropriate to be exploited as natural biosurfactants. In this regard, pure recombinant $PhaP_{Ah}$ belonging to *A. hydrophila* 4AK4 depicts a strong effect to form emulsions with lubricating oil, soybean oil and diesel when compared to sodium dodecylsulfate, bovine serum albumin, Tween 20 or sodium oleate, even maintaining its activity after heat treatment of the protein or the emulsions themselves (Wei et al. 2011). The most extensively studied application of phasins results from their PHA binding capability. In this sense, the *N*-terminal, PHA binding domain of PhaF of *P. putida* GPo1 known as BioF sequence has revealed to be extremely efficient as an affinity tag to immobilize in vivo fusion proteins exploiting mcl-PHA as support (Moldes et al. 2004, 2006). PHA granules having BioF tagged fusion proteins can be simply isolated through centrifugation and utilized directly or if needed, the purification of the adsorbed protein can be attained via gentle elution with detergents, keeping their overall activity in both cases (Moldes et al. 2004). This approach has been shown to be an environment friendly for delivering active proteins to the environment like the Cry1Ab toxin with insecticidal activity (Moldes et al. 2006). Analogous in vivo immobilization approaches have also been established for $PhaP1_{Re}$ exploiting *E. coli* as heterologous host towards the PHA synthesis (Chen et al. 2014). Here, the gene coding for the D-hydantoinase (DHDT) (enzyme participated in the production of D-amino acids of commercial values like one of the precursors needed towards the formation of semi-synthetic antibiotics) was fused with *pha*P1. The recombinant fusion protein, $PhaP1_{Re}$-HDT, resulted to be efficiently attached with the granules and the enzyme depicted to be active and stable (Chen et al. 2014). Apart from this, the specific immobilization of fusion proteins to PHA through phasins is initiated to be used in medicine, both in diagnostic as well as drug delivery uses (Backstrom et al. 2007; Yao et al. 2008; Dong et al. 2010). For instance, two hybrid genes responsible for coding either the mouse interleukin-2 or the myelinoligodendrocyte glycoprotein fused to $PhaP1_{Re}$ were constructed and expressed in a recombinant PHAs producing *E. coli* strain. The PHA beads achieved from this approach exhibited the eukaryotic proteins appropriately folded and they were then employed for specific and sensitive antibody detection by the fluorescence-activated cell sorting technology (Backstrom et al. 2007). On the other hand, Yao et al. (2008) carried out investigation in which two recombinant fusion proteins with $PhaP1_{Re}$ were produced to attain specific delivery: mannosylated human α1-acid glycoprotein (hAGP), which is capable to bind with the mannose receptor of macrophages and a human epidermal growth factor (hEGF), capable to identify EGF receptors on carcinoma cells. The

resulting proteins (rhAGP-PhaP1$_{Re}$ and rhEGF-PhaP1$_{Re}$) were self-assembled upon the co-polymer of 3-hydroxybutyrate and 3-hydroxyhexanoate [P(3HB-*co*-3HHx)] nanoparticles, attaining the specific delivery of the payload both in vitro and in vivo. Furthermore, the sequence coding of a peptide having the amino acids Arg-Gly-Asp, the most effective peptide sequence employed to improve cell adhesion on artificial surfaces, was fused to PhaP (Dong et al. 2010). Diverse polyesters like P(3HB-co-3HHx) or 3-hydroxybutyrate and 3-hydroxyvalerate [P(3HB-*co*-3HV)] co-polymers were coated with purified PhaP-RGD hybrid protein and the complex showed efficient in adhesion as well as improvement of cell growth on two diverse fibroblast cellular lines, signifying feasible uses on implant biomaterials (Dong et al. 2010).

14.9 Conclusion

Polyhydroxyalkanoates (PHAs) signify a complex group of storage polymeric materials, which are produced as insoluble inclusions in the cytoplasm of bacteria and cyanobacteria. Usually, PHA polymer accumulation is stimulated under the supplementation of excess organic carbon substrate together with nutrient limitation/deficiency like phosphorus, nitrogen etc. The key enzymes of PHAs biosynthesis are the PHA synthases that catalyze the enantio-selective polymerization of (*R*)-hydroxyacyl-CoA thioesters into polyesters. Different metabolic pathways have been identified in bacteria and cyanobacteria to furnish substrate for PHA synthases. PHAs are surrounded with a proteinaceous surface coat known as PHA granule associated proteins (PGAPs), which conforming a network like surface of structural, metabolic as well as regulatory polypeptides including configuring the PHA granules as complex and well organized subcellular structures called 'carbonosomes'. PGAPs consist of many enzymes like PHA synthases and PHA depolymerases that associated with PHA metabolism. In addition, they also involve small size proteins devoid of catalytic functions called phasins, which surround most of the PHA granule. Phasins play an important role in the physical stabilization of the PHA granule within the cell, ensure the correct distribution of the polymer upon cell division and assist other proteins (synthases and depolymerases) in PHA metabolism. Nevertheless, their specific role is highly dependent both on the microbial strain and on the metabolic state of the cell. Structurally, phasins are amphiphilic proteins which protect the hydrophobic polymer from the cytoplasm. Phasins receiving consideration interest currently as they open up new possibilities of applications such as biosurfactants, diagnostic and drug delivery uses. The most recent investigations revealed that the PHA granules have no phospholipids in vivo and hypothesized that the PHB or PHA granule surface coats in natural producers normally are devoid of phospholipids and merely composed of proteins. In order to draw final conclusion regarding the provision of the second thiol group for the catalytic cycle of PHA synthases, further investigations need to be carried out. The scaffold model, where PHB synthase-PhaM complexes are associated with the bacterial nucleoid and form the PHB granule initi-

ation complex, is presently more accepted model to explain the existing experimental data on PHB carbonosome production in *C. necator*. However, future studies should be conducted to specify the areas of PHB granule association and whether there is a specificity of the DNA locus to which the granules become associated.

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Chapter 15
Biopolymer Synthesis and Biodegradation

Sudhakar Muniyasamy, Özgür Seydibeyoğlu, Boopalan Thulasinathan and A. Arun

Abstract The present chapter is focussed on development of sustainable biobased biodegradable polymers for various end use applications. Biobased polymers are promising green materials since they are completely made from renewable agricultural resources, they also provide significant energy savings in their production compare to petroleum based plastics. However, it is necessary to address the environmental biodegradability of biobased materials in order to meet various commercial and environmental needs for their sustainable growth. In this chapter we discussed biobased polymers with a controlled onset of biodegradation induced by triggered system from natural fillers such as cellulose, hemicellulose and lignin, and evaluating their biodegradability in natural environments.

Keywords Biopolymers · Bioplastics · Biocomposites · Biodegradation

15.1 Introduction

Polymeric materials have considerably improved our everyday life. Polymeric materials have replaced metals and glass materials because of their excellent mechanical properties, low density, durability and low cost. They are widely used in the daily

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needs of contemporary society, ranging from simple packaging to heavy construction. The use of these materials continues to grow: for instance approximately 30–40% of plastics are used worldwide for packaging applications. This utilization is still expanding at 12% growth rate per year. There is increasing concern over the negative environmental impact generated by plastic waste disposal and litter control. It has been estimated that plastic wastes accumulate in the environment at a rate of 25 million tons per year. This creates serious environmental problems to the terrestrial and aquatic environments (Jambeck et al. 2015; Barnes et al. 2009). The increasing economic growth of plastics resulted in the amount of plastic waste generated during the past decade and plastic waste disposal problems have become a major issue in the world. It is expected that there will be two to three fold increases in plastics consumption, particularly in the developing nations (PlasticsEurope 2014). However, plastic materials are recalcitrant and resistant to biodegradation as such its accumulation has a deleterious effect on the environment as an obvious contributor to pollution.

The worldwide increase in plastics waste has evolved within the global vision of environmental protection and sustainability. A great deal of actions and strategies aimed at minimizing the negative impact of the increasing production and consumption of polymer materials has been put in place. In general, waste management strategies employed in different regions of the world are similar and are based on the prevention and recycling of waste (Fig. 15.1). For example, Japan has extensive legislation related to waste and other sustainable production and consumption policies under the "3R-reducing, reusing and recycling" umbrella.

- prevent waste in the first place;
- recycle waste;
- optimize the final disposal of waste.

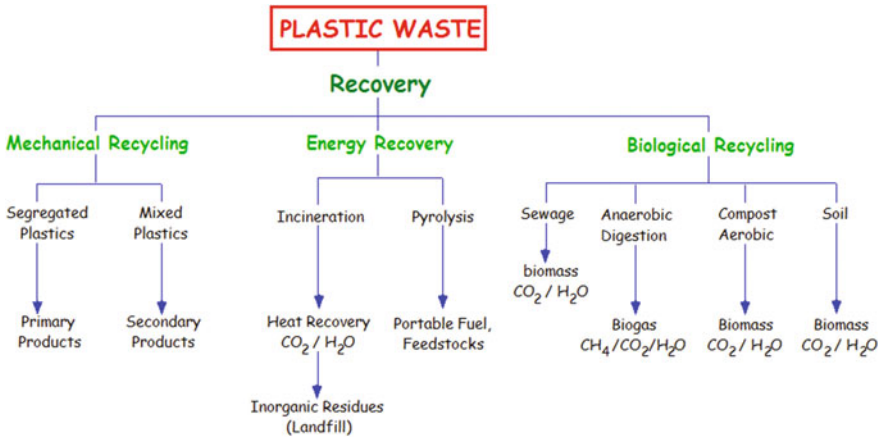

Fig. 15.1 Options of plastic waste management. Figure redrawn after reference from (Wiles and Scott 2006)

In response to the growing challenges of waste production and management, the European Parliament and the Council have adopted a number of Directives to ensure that waste is recovered or disposed of without impairing the environment and human health. According to the EU Directive on Packaging and Packaging Waste (Prendergast and Pitt 1996; Bailey 2017; Avella et al. 2001), the management of packaging and packaging waste should include as a first priority the prevention of packaging waste and as additional fundamental principles, reuse of packaging, recycling and other forms of recovering packaging waste, hence reduction of the final disposal of such waste.

Prevention means the reduction of the quantity and harmfulness to the environment of materials and substances contained in packaging and packaging waste; packaging and packaging waste at production process level and at marketing, distribution, utilization and elimination stages, in particular by developing "clean" production methods and technology.

Reuse is defined as any operation by which packaging, which has been conceived and designed to accomplish within its life cycle a minimum number of trips or rotations, is refilled or used for the same purpose for which it was conceived, with or without the support of the auxiliary products present on the market enabling the packaging to be refilled.

Recovery includes operations provided for in Annex II.B to Directive 75/442/EEC on waste (Prendergast and Pitt 1996; Bailey 2017; Avella et al. 2001), for example use as a fuel or other means to generate energy, recycling/reclamation of organic substances which are not used as solvents (including composting and other biological transformation processes).

Energy recovery means the use of combustible packaging waste as a means to generate energy through direct incineration with or without other waste but with recovery of the heat. Recycling is defined as the reprocessing in a production process of the waste materials for the original purpose or for other purposes including organic recycling but excluding energy recovery. Disposal operations include deposit into or onto land (e.g. landfilling), incineration, etc. The use of compostable plastics is one valuable recovery option (biological or organic recycling).

According to the EU Directive on Packaging and Packaging Waste (Prendergast and Pitt 1996; Bailey 2017; Avella et al. 2001) organic recycling means the aerobic (composting) or anaerobic (biomethanization) treatment, under controlled conditions and using microorganisms, of the biodegradable parts of packaging waste, which produces stabilized organic residues or methane. Landfill is not considered as a form of organic recycling.

The Waste Management Hierarchy, i.e. minimization, recovery and transformation, and land disposal have been adopted by most developed countries with strategies used depending on such factors as population density, transportation infrastructure, socioeconomic and environmental regulations.

15.1.1 Situation in Europe

From 2009 to 2010 the global production of plastic materials increased from 250 million tons to 265 million tons considering the long term growth of plastic production at 5% per year over the past 20 years. The total production of plastics in Europe reached 57 million tons i.e. 21.5% of the global production and china overtook Europe as the biggest production region at 23.5%. In Europe, generation of 24.7 million tons of post-consumer plastic waste concentrated in the packaging, construction, automotive and electrical and electronic equipment sectors (Villanueva and Eder 2014). Out of these, 10.4 million tons were disposed of in landfills, and 14.3 million tons were recovered as energy (Villanueva and Eder 2014; Datta and Kopczyńska 2016). Recycling quantities from the packaging collection schemes and recycling companies increased by 8.7%. The post-consumer plastic waste, used for energy recovery increased by 9.8% due to increased use of post-consumer plastic waste as a complementary fuel in power plants and cements kilns (Villanueva and Eder 2014; Datta and Kopczyńska 2016) (Fig. 15.2).

At present, intensive efforts taken to reduce the amounts of waste, has resulted in a significant decrease in the quantity of solid waste within the European Union. As a result, landfilled waste has decreased by 2.5% in post-consumer plastic waste in 2013. Composting contributes considerably to waste management in several countries such as Belgium, Denmark, Germany, Spain, France, Italy and the Netherlands. Between 13% and 24% of municipal waste is treated by composting in these countries, the composted amounts ranging between 71 kg/person in France and 147 kg/person in the Netherlands.

The U.S. Environmental Protection Agency and Community of European Norms recommend composting as one of the most promising methods of waste management 2013 (Villanueva and Eder 2014; Datta and Kopczyńska 2016; Rochman et al.

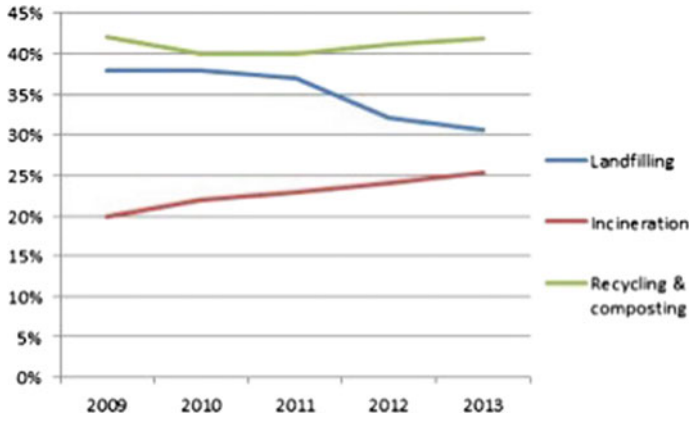

Fig. 15.2 Total plastic waste management system from 2009–2013 (Villanueva and Eder 2014; Datta and Kopczyńska 2016; Rochman et al. 2013)

2013). Since biodegradable polymers are suitable for composting, there is an increasing interest in polymers that can be biologically be recycled into biomass. This type of polymers may either be based on renewable resources or on petrochemicals. The biological recycling of polymers should be considered as an alternative to the more traditional recycling procedures. This has stimulated research around the world to modify existing polymers or to synthesise new polymers that can be returned to the biological cycle after use (Narayan 2006; Davis and Song 2006; Ikada and Tsuji 2000). Taking into account all these routes for waste reduction, it seems inevitable that environmental biodegradable polymers will have an increasing role in the management of waste and litter in the future.

15.1.2 Situation in the United States

From 1980 to 2003, the total annual generation of municipal solid waste (MSW) increased by more than 50% to 236.2 million tons per year. Organic materials are the largest components of MSW in the USA. Paper and paperboard products account for 35% of the waste stream, followed by yard trimmings and food scraps with about 24%. Plastics comprise 11%, representing 26.7 million tons, at third place in municipal solid waste composition. It is noteworthy that containers and packaging made up the largest portion of waste generated, about 75 million tons. Nearly 9% of plastic containers and packaging was recycled, compared with 22% of glass containers and 15% of wood packaging recovered for recycling (Jambeck et al. 2015; Subramanian 2000; Kang and Schoenung 2005).

15.1.3 Situation in Other Regions of the World

The significant increase in plastics consumption is also observed in other regions of the world. For example, rapid industrialization and economic development in Singapore have caused a tremendous increase in solid waste generation. The annual amount of disposed solid waste increased from 0.74 million tons in 1972 to 2.80 million tons in 2000. It is estimated that solid waste generation in Singapore amounted to about 4.5–4.8 million tons per year. Plastics account for 5.8% of the total solid waste, in third position after food waste (38.3%) and paper/cardboard (20.60%). Taking into account that plastic bags and bottles have become one of the major solid waste streams, using plastic wastes to manufacture polymer concrete and developing biodegradable plastics has received much attention in recent years. In Australia, the annual plastics consumption has increased from 1336386 tons in 1997 to 1521394 tons in 2003, whereas the total recycling rate of plastics increased from 7.0% to 12.4% (Jambeck et al. 2015; Subramanian 2000; Kang and Schoenung 2005). It is noteworthy that plastics packaging recycling in 2003 were 134905 tons, which is 20.5% of packaging consumption during a year. In China, the production of municipal

solid waste and sewage sludge is changing rapidly along with economic development (Zhang et al. 2010; Hicks et al. 2005). The amount of solid waste produced in China is large and is increasing rapidly. The average amount of MSW produced by each person daily increased from 1.12 kg to 1.59 kg from 1986 to 1995 (Zhang et al. 2010; Hicks et al. 2005). In 1995, China produced 644.74 million tons of industrial solid waste and 237 million tons of MSW. At present it is estimated that the amount of municipal refuse produced by each person annually is between 204.4 and 440 kg, and the total solid waste produced in China is about 27.15% of that in Asia and 15.07% of that globally 1995 (Zhang et al. 2010; Hicks et al. 2005). About 85% of the total amount of MSW production in China is in cities, about 60% of the total amount of MSW produced in China is in 52 cities, whose population is over 0.5 billion. MSW in China is mainly treated by landfilling and composting, and a smaller amount of MSW is treated by incineration. The amount of MSW treated by landfilling and composting is over 70% and 20% of the total amount of MSW disposed of, respectively. Composting has emerged as a potentially viable alternative by local governments because of lower investment and operation costs. However, despite the efforts that have been made, overall waste volumes are growing. Management of plastics waste remains a problem. The observed increased in plastic consumption throughout the world makes the development of more recyclable and/or biodegradable plastics necessary to reduce the amount of plastic wastes to landfills. According to an amendment to the European Directive on Packaging and Packaging Waste (Prendergast and Pitt 1996; Bailey 2017; Avella et al. 2001), recovery and recycling of packaging waste should be further increased to reduce its environmental impact.

Compostable polymers have been designed to be disposed of after their useful life to undergo organic recycling. Composting is one of the strategic options available for the management of plastics waste. Composting is an attractive alternative for reducing solid waste and is especially suitable for those segments of conventional plastics in which recycling is difficult or economically not feasible.

The growing environmental awareness and new rules and regulations, as well as new trends in solid waste management, have led scientists and researcher to increase activities in the design of compostable polymeric materials that can easily degrade under well-defined environmental conditions.

15.2 Biodegradable and Compostable Polymers from Renewable Resources

15.2.1 Classification of Bioplastics

Bioplastics are plastics derived from nature (biopolymers) and/or can go back to nature (biodegrade). These bioplastics are classified into three major categories (Babu et al. 2013) as shown in the Fig. 15.3.

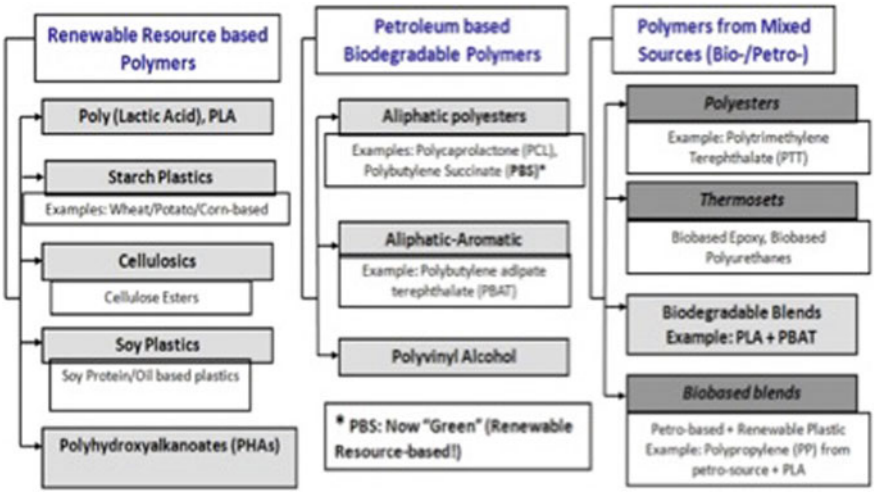

Fig. 15.3 Classification of bioplastics based on the resources

15.2.1.1 Bioplastics from Renewable Resources

Bioplastics derived entirely from renewable resources and those synthesized naturally by plants and animals belong to this category. Starch, cellulose, proteins, lignin, Chitosan; poly lactic acid (PLA), and polyhydroxyalkanoates/polyhydroxybutyrates (PHAs) are included in this category.

15.2.1.2 Biodegradable Plastics from Petroleum Resources

Petroleum based monomers are used to synthesize these polymers and they biodegrade at the end of their useful life. Some examples in this category are polycaprolactone (PCL) and polybutylneadipateterpthalate (PBAT).

15.2.1.3 Mixed Sources (Bio-/Petro) Based Bioplastics

Monomers used to produce these polymers are from a combination of biobased and petroleum sources. The biodegradability of these polymers depends on their structure. Poly trimethylene terephthalate (PTT), bio-thermosets and biobased blends are some examples in this category. PTT is manufactured using biologically derived 1,3-propanediol and petroleum derived terephthalic acid.

The following section provides a detailed discussion about the important bioplastics that are commercialized for various applications.

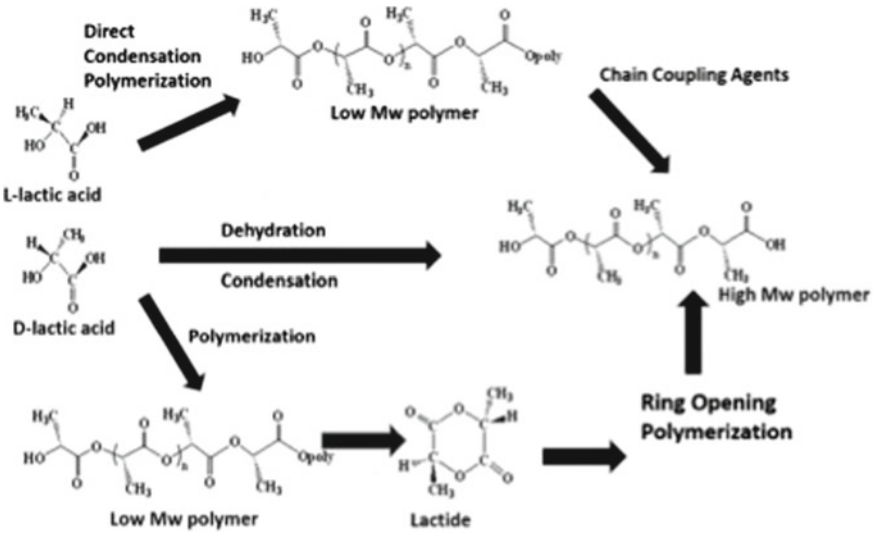

Fig. 15.4 Processing scheme for PLA production, redrawn after reference from (Gupta et al. 2007)

15.2.2 Polylactic Acid (PLA)

Poly lactic acid (PLA) is a widely used bioplastic and is obtained from polymerization of lactic acid/lactide which is obtained from fermentation of carbohydrate feedstock like corn, wheat, and maize. PLA is prepared by polycondensation, ring-opening polymerization and other methods (chain extension, grafting). High molecular weight PLA is generally produced by the ring-opening polymerization of the lactide monomer. The conversion of lactide to high molecular weight polylactide is achieved commercially by two routes. Three separate processing and distinct steps as shown in Fig. 15.4 leading to the production of lactic acid, lactide, and PLA high polymer from (Gupta et al. 2007).

The material properties of PLA including degradation depend on its chain stereochemistry. The L only mesoform based PLA will have a very high melting point and high crystallinity. The isotactic poly (L-lactide) (PLLA) is a semicrystalline polymer with a melting transition near 180 °C, while atactic poly-(rac-LA) (rac-LA is a 1:1mixture of L-lactide and D-lactide) and poly (meso-lactide) are amorphous polymers (Gupta et al. 2007; Lasprilla et al. 2012). It is also known that 90/10% D/L copolymer gives a material which can be polymerized in the melt, oriented above its Tg and is easy processable showing very high potential of meeting the requirements of food packaging. This material is commercialized by different companies and most known among them is Natureworks.

The main advantages of PLA are that it is 100% renewable resource based, compostable and also recyclable with very good mechanical properties.

PLA has water, CO_2, O_2 permeability coefficients lower than those of PS and higher than those of PET. The glass transition changes with time and shrinks when PLA films are sealed near its melting temperature. The migration of lactic acid and its derivatives from PLA packaging is lower than values set by government agencies (Gupta et al. 2007; Lasprilla et al. 2012).Thus, PLA is safe for use in fabricating articles for contact with food. PLA is fully biodegradable under composting conditions at temperatures of 60 °C and above.

15.2.3 Polyhydroxyalkanoates (PHAs)

Polyhydroxyalkanoates (PHAs) are biopolyesters of various hydroxyalkanoates that are accumulated and synthesized by at least 75 different bacteria as energy and carbon reserves. PHAs have a structure with the same three carbon backbones and differing alkyl group at the β, or 3, position. Based on culture conditions two main categories of bacteria are used for the synthesis of PHAs, the first category requires the limitation of nutrients while the other category doesn't require the nutrient limitation (Bhatt et al. 2008; Witt et al. 2001; Anderson and Dawes 1990; Lee 1996; Sudesh et al. 2000). *Alcaligeneseutrophus*, *Protomonasextorquens*and *Protomonasoleovorans* requires limitation of nutrients, while *Alcaligenes latus*, a mutant strain of *Azotobactervinelandii*, and recombinant *Escherichia coli* accumulate polymer during their growth (Anderson and Dawes 1990; Lee 1996; Sudesh et al. 2000). Fermentation technologies are used for the large scale production of PHAs. The standard PHA production process by bacterial fermentation consists of three basic steps: fermentation, isolation and purification, and blending and pelletizing. PHAs are produced from a wide variety of substrates from renewable resources such as sucrose, starch, cellulose, triacylglycerols and from fossil resources (methane, mineral oil, lignite, hard coal) as well as by-products (molasses, whey, glycerol) and chemicals (propionic acid, 4-hydroxybutyric acid) and carbon dioxide.

The properties of PHA depend on its structure, homopolymers, random copolymers, and block copolymers of PHA can be produced depending on the bacterial species and growth conditions. Material properties of PHA can be tailored by varying HV content. There is a linear relationship between HV content and impact strength, while thermal properties, crystallinity, water permeability and tensile strength decrease with HV content. Also PHA copolymers biodegrade faster in compost environment than homopolymers and therefore are very attractive for many industrial applications (Anderson and Dawes 1990; Lee 1996; Sudesh et al. 2000).

The homopolymer poly (3-hydroxybutyrate) (PHB) is a brittle, crystalline thermoplastic and undergoes thermal decomposition just at its melting point, thus making its processing window very narrow thereby limiting its commercial usefulness. There are many efforts directed towards synthesis of copolymers that can have better properties than that of PHB. The ratio of HB to HV monomer can be varied by changing the glucose to propionic acid ratio. By increasing the ratio of HV to HB, the melting temperatures are lowered and mechanical properties are improved. Also,

PHBV properties can be modified by using plasticizers, citrate ester (triacetin) is a well-known plasticizer used for PHAs.

Applications that have been developed from PHA and related materials (e.g. Biopol) can be found in very different areas and cover packaging, hygienic, agricultural, and biomedical products. Technically, the prospects for PHAs are very promising. Since this is still more expensive than synthetic polymers, different strategies including the use of better bacterial strains and low-cost renewable resources have been attempted to improve its physical properties and to bring down its cost.

15.2.4 Thermoplastic Starch (TPS)

Starch is one of the most widely used biopolymer for packaging applications; it is also one of the most abundant natural polymers. Perhaps, this most researched biobased material for various applications have become reality especially for food packaging and compostable materials. However, not much success has been seen for beverage packaging applications. Starch is composed of two major components, amylose and amylopectin. In corn starch, nearly 30% is amylose which is linear and the rest is branched amylopectin. The molecular weight of amylose ranges from 200,000–700,000 while that of amylopectin varies anywhere between 100 and 200 million. Starch can be converted into thermoplastic by processing it in the presence of heat and plasticizer (Xie et al. 2014; Liu et al. 2009). During this process starch is destructurized, the destructurization of starch is defined as a partial fragmentation of the crystalline structure within the polysaccharides. In the presence of plasticizers and high shear rates, starch can be destructured. Water, glycerol, sorbitol, glycol, poly (ethylene glycol), urea, glucose and maltose are used as plasticizers for starch.

The glass transition temperature of thermoplastic starch depends on plasticizer content. Depending on the plasticizer content, processing conditions, type of processing and other contents gives a complex starch plastic material. This is composed of residual swollen granular starch, partially melted, deformed and disrupted granules, completely molten starch, and recrystallized starch. The degree of disruption and melting of the various granular starches is regulated by the plasticizer content and by the processing parameters (shear stress, melt viscosity and temperature). Native starch can be transformed into highly amorphous thermoplastics by preformulation processes. This compounded thermoplastic starch (TPS) formulation can be remelted through extrusion or injection molding techniques by renewing the energy input. A schematic representation of micro-structural changes of starch under processing with different plasticizers is shown in Fig. 15.5. Native starches can be destructurized within co-rotating twin screw extruder systems by a controlled feeding of suitable destructurization additives (water, glycerol) in combination with defined operating parameters.

The use of thermoplastic starch in the production of biodegradable plastics has gained momentum since the last decade. The main drawbacks of thermoplastic starch are its water affinity and poor mechanical properties (Xie et al. 2014; Liu et al. 2009).

These problems can be overcome by blending TPS with synthetic polymers and modified by cross-linking agents such as Ca and Zr salts. Waxes and lignin are also tested to decrease the water uptake of starch-based materials. Mechanical properties are being improved by addition of fillers and blending with synthetic polymers.

Blends or composites materials have been produced by the processing of starch with biodegradable polymers such as poly(ε-caprolactone), poly(lactic acid), poly(vinyl alcohol), poly(hydroxybutyrate-co-valerate), and polyesteramide. The most common are Mater-Bi from Novamont (Bastioli 1998).

Mater-Bi is one of the commercially available thermoplastic starch mixtures which is shaped by pressing or extrusion techniques (Bastioli 1998). This TPS is based on starch, plasticizers, and hydrophilic biologically degradable substances from synthetic polymers. This biopolymer is degradable, either in the presence of oxygen or in its absence. Another popular TPS biopolymer material is BIOPLAST, useful for Bioflex materials. Depending on processing methods it may be formed by film blowing, thermoforming, or injection molding.

TPS is useful to make commercial articles either by injection molding or film blowing. TPS can be used in thermoform of biodegradable foams widely employed as cushioning materials for the protection of fragile products during transportation and handling. TPS films display low oxygen permeability and so become attractive materials for food packaging.

15.2.5 Cellulose

Cellulose is the most abundant natural polymer available on earth. It is the major structural component of plant cell wall. It is different from other polysaccharides produced by plants because its molecular chains forming are very long and are made up of single repeating unit. Cellulose can be obtained from many agricultural residues such as bagasse, stalks and straws. It is also the major components of cotton (95%), flax (80%), jute (60–70%) and wood (40–50%) (Moon et al. 2011; Pandey et al. 2011; Siró and Plackett 2010).

Chemically cellulose is a polydisperse linear polysaccharide consisting of β-1, 4-glycosidic linked D-glucose units (so-called anhydroglucose unit). This supramolecular structure of cellulose results in its insolubility in water as well as many

Fig. 15.5 Schematic representation of thermoplastic starch preparation (TPS) (Liu et al. 2009)

organic solvents. Cellulose is converted into cellulose esters due to its insolubility in many common organic solvents and also cellulose is not melt processable (its decomposition temperature is much lower than softening temperature).

Cellulose esters have been commercially important polymers for nearly a century, and have found a variety of applications, including solvent-borne coatings, separation, medical and controlled release applications as well as composites, laminates and plastics. Cellulose acetate (CA), cellulose acetate propionate (CAP), and cellulose acetate butyrate (CAB) are most common cellulose esters produced. Cellulose esters are thermoplastics produced by esterification of cellulose using cellulose from various resources including cotton, wood, recycled paper and bagasse. Cellulose acetate is produced through an esterification process of cellulose with acetic anhydride. CAP and CAB are mixed esters produced by treating cellulose with appropriate acids and anhydrides in the presence of sulphuric acid.

15.2.6 Chitin and Chitosan

Chitin is the second most abundant natural polymer, it is found to be the supporting material in many invertebrate animals such as insects and crustaceans. Wastes from seafood processing industries are used for commercial production of chitin. Chitin is obtained as an almost colourless to off-white powdery material. The process of obtaining chitin from the shells of crab or shrimp involves removal of protein and dissolution of calcium carbonate. The chitin obtained from this process is usually deacetylated in 40% sodium hydroxide at 120 °C for 1–3 h to obtain 70% deacetylated chitosan (Kumar 2000; Rinaudo 2006). Chitosan is produced by deacetylating chitin using 40–50% aqueous alkali at 100–160 °C for a few hours. The resultant chitosan has a degree of deacetylation up to 0.9. The molecular weight of chitosan depends on the source and it varies from 50 to 1000 kDa. Chitin has a similar structure to that of cellulose with 2-acetamido-2-deoxy-β-D-glucose through a β $(1 \rightarrow 4)$ linkage and degrades by chitinase. Chitosan is the deacetylated chitin with more than 50%. Chitosan is receiving more attention as a possible polysaccharide resource for biomedical applications. Deacetylation in commercial chitosan varies from 50 to 90% and has been explored for films and fibres. Fibres from these polymers are very useful as wound dressing materials and absorbable sutures. They have generated interest in biomedical applications (Kumar 2000; Rinaudo 2006).

15.2.7 Petroleum-Based Biodegradable Polyesters

Petroleum based monomers are used to synthesis a wide range of biodegradable polyesters, polycaprolactone, aliphatic and aromatic copolyesters are the main classes in these materials. Polycondensation of diols and a diacid are used to produce

these polyesters, also ring open polymerization (ROP) is also used to produce many biopolyesters (Ikada and Tsuji 2000; Zhao et al. 2005).

15.2.8 Polycaprolactone(PCL)

Polycaprolactone (PCL) is linear polyester obtained by ROP of Ɛ-caprolactone in the presence of metal alkoxides (Labet and Thielemans 2009). It is widely used in various biomedical (drugs controlled release) and packaging (compostable packaging) applications. PCL shows a very low glass transition temperature of −61 °C and melting point of 56 °C leading to it limited applications. To overcome this, PCL is blended with other polymers and fillers. Blends of PCL with other biodegradable polymers such as PHB, PLA, and starch have been prepared. Addition of starch to PCL matrix significantly increases the biodegradation rate (Fukushima et al. 2010; Mezzanotte et al. 2005; Råberg and Hafrén 2008; Yagi et al. 2009).

15.2.9 Aliphatic and Aromatic Copolyesters

Petroleum base biodegradable aliphatic polyesters are obtained by the combination of diols and diacids. 1,2-ethanediol, 1,3-propanediol or 1,4-butadenediol and adipic, sebacic or succinic acids are used in the synthesis of aliphatic copolyesters (Witt et al. 2001; Fukushima et al. 2010). The chemical structures of common aliphatic copolyesters are shown in Fig. 15.6. The properties of these copolyesters depend on their structure and the combination of raw materials used in its synthesis. This combination also influences the biodegradability.

Poly (butylene succinate) (PBS) is a biodegradable synthetic aliphatic polyester obtained from condensation of succinic acid and 1,4-butendiol. It is a fully biodegradable polymer, easily processable and currently made from petroleum sources; however, it is possible to produce this polymer from renewable resources making it biobased. This polymer is of great interest as it has similar properties to that of PET. It has a wide range of applications including mulch film, packaging film, bags and many other applications. Showa High Polymers produces Bionolle series PBS (1000 series) and PBSA (3000 series) polymers through polycondensation of ethylene glycol, 1,4 butanediol and succinic acid, adipic acid as principal raw materials (Witt et al. 2001; Fukushima et al. 2010).

Aliphatic-aromatic polyesters are designed to combine biological susceptibility of aliphatic groups and good material performance of aromatic groups. It is well known that aliphatic polyesters are susceptible to biological degradation while aromatic polyesters are not. These polyesters are produced by standard polycondensation reaction techniques and so far best results are achieved with a combination of terephthalic acid, adipic acids and 1,4-butanediol. Most of these polyesters are based on terephthalic acid and this modifies melting temperature and biodegradation rate.

15.3 Biodegradation

Since biodegradation depend on both polymer structure and environmental factors, wide scopes of tests are required to fully quantify the biodegradability of a given plastic (Chiellini et al. 1999; Corti et al. 2012; Krzan et al. 2006). To ensure a global uniformity in determining the biodegradability of plastics, standardized testing procedures have been defined by international organizations, such as the International Organization for Standardization (ISO), Organization for Economic Co-operation and Development (OECD) and ASTM International. These organizations define and regulate a number of standard test methods that are used to properly assess biodegradable polymers, taking into consideration a variety of environmental factors. The definition of these biodegradability tests was established by the ISO. For biodegradable plastics, these factors include humidity, temperature, acidity, presence of oxygen and most importantly, the presence of microorganisms. Each factor affects the rate of degradation, and altering them will affect the result of the experiment.

As a result of the exponential growth of global plastic production, plastic pollution in the environment has increased dramatically over the last century. If not

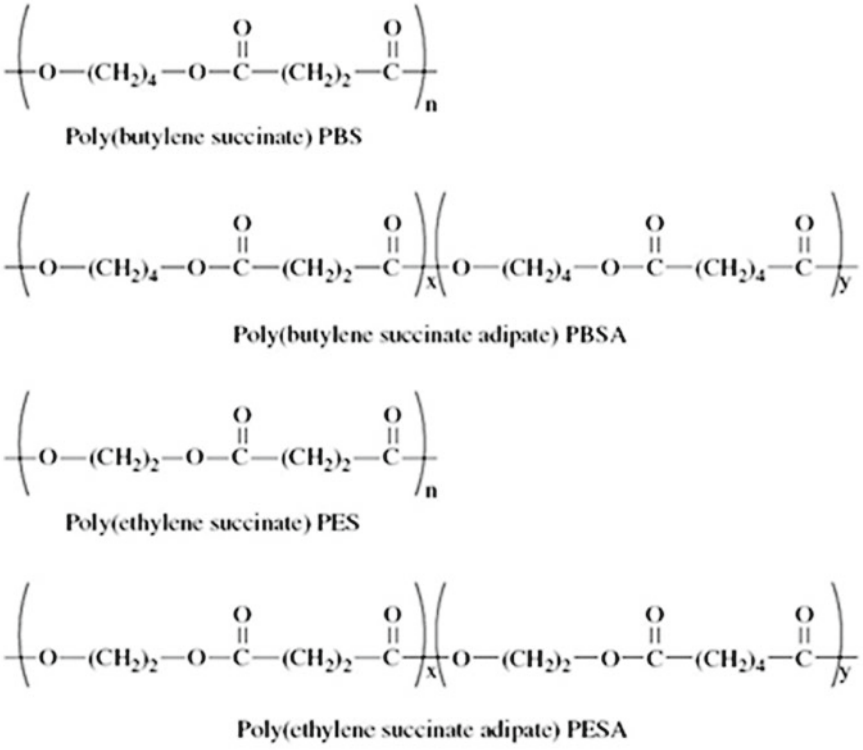

Fig. 15.6 Chemical structures of common biodegradable aliphatic polyesters

controlled, plastic will continue to cause damage to the environment, this is because it takes an incredibly long time for plastic degrade in a natural environment (Krzan et al. 2006; Barone and Arikan 2007; Pagga 1995). However, because plastics have become so ingrained in modern industry and consumer practices, it is not feasible to stop using it entirely. Instead, it is possible to engineer plastics that are biodegradable, and will decompose after they have been used and disposed off. The process of plastic biodegradation is complex, and it depends on a host of factors, including polymer structure, temperature, humidity, pH level, and the presence of microorganism. Biodegradation can also be promoted by the addition of natural fibres to the polymer matrix as well as modified material properties. Thorough testing and analysis is necessary both to optimize the properties of the polymers, and also to verify the true biodegradability of the polymer. In order to ensure consistency throughout the biopolymer industry, standardized testing methods have been developed and are regulated by organizations such as the ISO and ASTM International (ASTM 1992; Kale et al. 2006; Standard 2009; Muniyasamy et al. 2013a). These tests cover a broad scope of conditions in order to accommodate all types of plastics, and the various environmental conditions they could be exposed to. Thus, the continuous development and innovation by the bioplastic industry is essential to the future of plastics industry, and will be necessary to reduce the global issue of plastic waste pollution.

Definition

Biodegradation is defined as a process in which plastic materials undergo chemical change through the action of enzymes that are secreted by living microorganisms such as bacteria, fungi and algae. The process of biodegradation comprises of two phases; the initial phases (primary biodegradation) and the secondary phase (ultimate biodegradation). During the initial phase, the material undergoes disintegration which is significantly associated with the deterioration in physical properties such as discoloration, embrittlement and fragmentation. The second phase is assumed to be the ultimate conversion of plastic fragments, after being broken down to molecular sizes, to CO_2, water, cell biomass when the plastic material is exposed to aerobic conditions and CH_4, CO_2 and cell biomass in the case of anaerobic conditions. The primary and ultimate biodegradation must occur within a specific time at specific rate to avoid accumulation of plastics in the environments.

The American Society for Testing and Materials (ASTM) and the International Organization for Standardization (ISO) define:

(i) ***Degradation*** as *"An irreversible process leading to a significant change of the structure of a material, typically characterized by a loss of properties(e.g. integrity, molecular weight, structure or mechanical strength) and/or fragmentation. Degradation is affected by environmental conditions and proceeds over a period of time comprising one or more steps"*. According to the ASTM definition,

(ii) ***Biodegradable plastic*** is *"A degradable plastic in which the degradation results from the action of naturally occurring microorganisms such as bacteria, fungi and algae"*

(iii) ***Compostable plastic*** is *"a plastic that undergoes biological degradation during composting to yield carbon dioxide, water, inorganic compounds and biomass at a rate consistent with other known compostable materials and leaves no visually distinguishable or toxic residues"*.

15.3.1 Factors Affecting the Biodegradability of Plastics

In natural conditions, the rate of (bio)degradation depend on various environmental factors such as microbial population, temperature, humidity, oxygen, pH, UV radiation and polymer properties. The properties of plastics are associated with their biodegradability. Both the chemical and physical properties of plastics influence the mechanism of biodegradation. The surface conditions (surface area, hydrophilic, and hydrophobic properties), the first order structures (chemical structure, molecular weight and molecular weight distribution) and the high order structures (glass transition temperature, melting temperature, modulus of elasticity, crystallinity and crystal structure) of polymers play important roles in the biodegradation processes.

15.3.2 Biodegradation Mechanism of Polymer

All polymers contain either hydrolysable or oxydisable bonds (Fig. 15.6) but degradation process of all polymers undergo two processes: primary degradation which is influenced by various environmental abiotic conditions (chemical, thermal, photo) and the secondary degradation processes which is ultimate biodegradation (enzyme and microbes). The most important degradation mechanism of hydro-biodegradable polymers is chemical degradation via hydrolysis or enzyme-catalysed hydrolysis (Muniyasamy et al. 2013a) where rate of biodegradation varies in different natural environments such as soil, compost and aqueous media because of the high variability in biologically mediated processes as well as physical-chemical properties of polymers. A high rate of biodegradation is effectively available for naturally occurring biopolymers like polysaccharides, proteins (gelatine and collagen) and poly (b-hydroxyl acids), where appropriate enzymes are available. For most biodegradable materials, especially artificial polymers, passive hydrolysis is the most important kind of degradation. There are several factors that influence the rate of this reaction and the most important being type of chemical bond, pH, copolymer composition, water uptake and morphology. In the case of carbon-chain polymers such as polyolefins and other natural polymers such natural rubber and lignin, in the primary degradation step, degradation undergoes by oxidation process mediated by photo, thermal and enzymatic process followed by mineralization process (ultimate biodegradation) as depicted in Fig. 15.7.

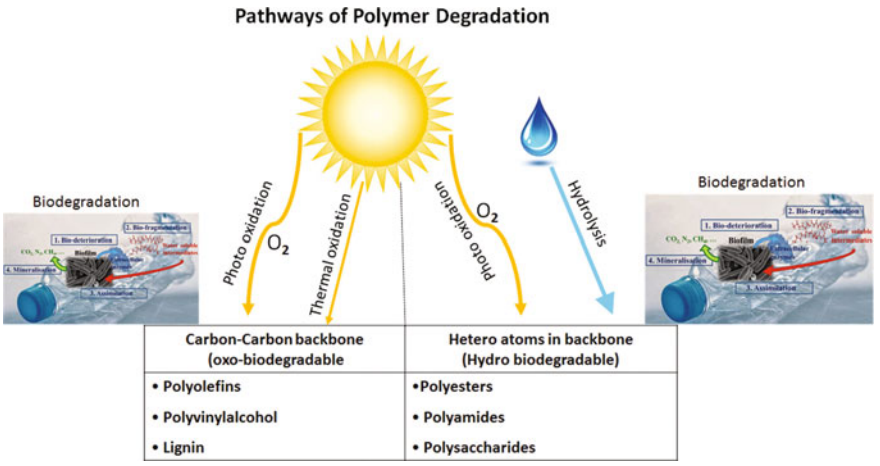

Fig. 15.7 Polymer degradation pathways of polymers

15.3.2.1 Hydrolytic Biodegradation of Polymer

Hydrolytic degradation takes place when polymers containing hydrolysable groups such as polysaccharides, polyesters, polyamides are exposed to moisture with biotic environmental conditions. The biodegradation of aliphatic polyester is similar to the biodegradation of cellulose and chitin by enzymatic hydrolytic degradation. An important group of esterase enzymes are responsible for the hydrolytic degradation of aliphatic polyesters (Muniyasamy et al. 2013a, b). These enzymes are known to hydrolyse triacylglycerol (fat) to fatty acid and glycerol. Esterase group enzymes such as lipase has been well studied and are able to hydrolyse aliphatic polyesters in contrast with aromatic polyesters (Muniyasamy et al. 2013b). The main reason is that in aliphatic polyesters the flexibility of the main carbon chain and the high hydrophilicity of the aliphatic polyester allow for intimate contact between the polyester chain and the active site of the lipase in marked contrast to the rigid main chain and hydrophobicity of the aromatic polyester. As these enzymatic reactions are heterogeneous, the hydrolytic enzymes first adsorb onto the surface of the polymer substrate through the binding site of enzyme molecules. The active site of the enzyme then comes into direct contact with the ester bond of the substrate molecule. Among all the hydrolytic enzymes, each group has specific active binding site for the substrate molecules based on the binding capacities to the substrate. The enzymes excreted from microorganisms may hydrolyze polymers into low molecular weight compounds which then undergo bioassimilation by the naturally occurring microorganisms. The bioassimilation process results in the production of carbon dioxide methane and water as well as new microbial biomass. The biodegradability of some biobased polymers such as starch, cellulose, PLA, PHB, chitin and protein as well as petro based polyester (PCL, PBAT) has been extensively studied the hydrolytic biodegradation process (Fig. 15.8). Some industrial composite materials

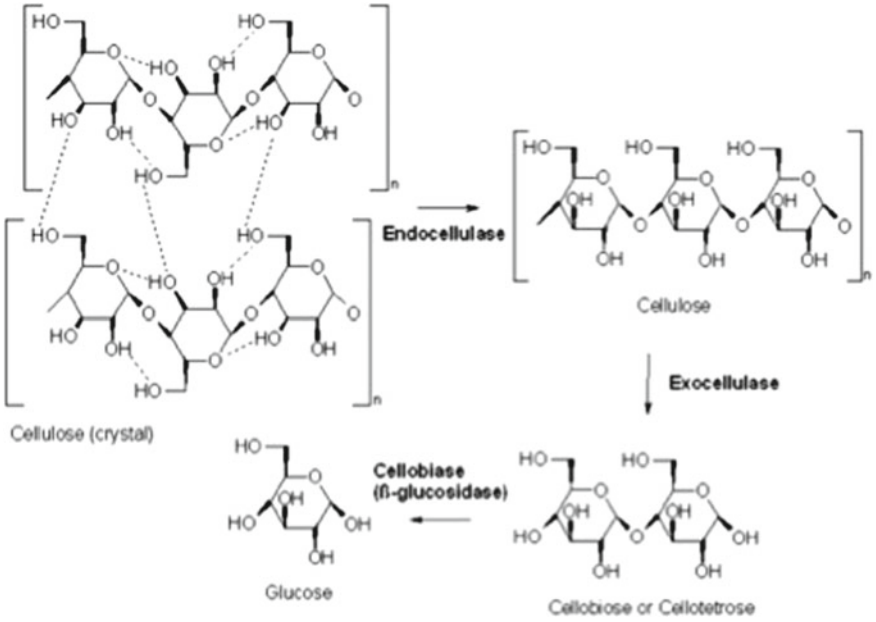

Fig. 15.8 Enzymatic hydrolysis mechanism of cellulose

based on polyester blended with starch matrix can readily undergo biodegradation under composting conditions (Krzan et al. 2006; Kale et al. 2006; Fukushima et al. 2009; Itävaara et al. 2002; Kunioka et al. 2006; Lucas et al. 2008).

15.3.2.2 Oxidative Biodegradation of Polymer

When polymers containing oxidizable groups such as lignin, natural rubber, polyvinyl alcohol and polyolefins are exposed to either abiotic or biotic conditions, oxidative degradation takes place which leads to breakdown of polymer chains into smaller oligomers and dimers (smaller molecules). The oxidized smaller molecules are further assimilated by microorganisms (Chiellini et al. 1999, 2006, 2007) to produce the final end products like CO_2, CH_4 and water as well as new microbial cell biomass. Lignin is another example of natural polymer like natural rubber, *cis*-poly(isoprene), cannot biodegrade by a hydrolytic process, but biodegrades slowly by the oxidative attack due to extracellular peroxidases produced by fungi and *actinomycetes* (Muniyasamy et al. 2013a). Lignin is a cross-linked polymer containing benzene rings. It is formed in chemical association with cellulose (lignocellulose) and constitutes the tough cell wall structure of plants. The aromatic structures contain alkoxy and hydrocarbon substituent that link the basic unit into a macromolecular structure through C-C and C-O bonds. Lignin, due to its hydrophobic nature and chemical inertness, does not readily degrade in abiotic or biotic conditions and when it does

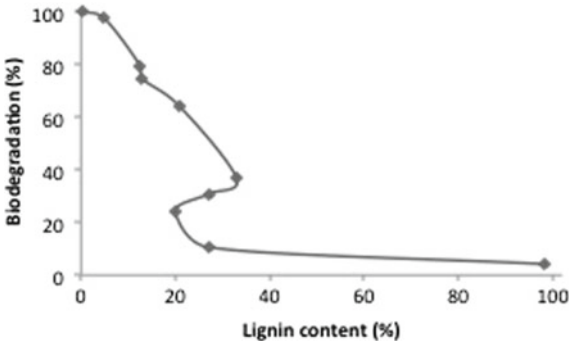

Fig. 15.9 Biodegradability of lignin content under composting conditions at 58 °C (Redrawn figure after reference from (Tuomela et al. 2001)

Table 15.1 Various crop waste products of lingocellulosics of each components (Muniyasamy et al. 2013a, b)

Crops product	Components (%)				
	Cellulose	Hemi cellulose	Lignin	Ash	Others
Wheat straw	32.1	29.2	16.4	4.8	17.5
Corn stovers	44.0	28.0	5.0	8.6	14.4
Sun flover stovers	33.0	27.5	10.9	12.7	12.9
Baggase	40.0	25.0	20.0	11.5	3.5
Pine nut shells	13.2	2.0	75.0	7.8	3.0

occur the lignin tends to accumulate. However, lignin does biodegrade slowly under composting conditions and the biodegradation behaviour is dependent on the lignin content (Fig. 15.9) and the microbial population. in the environment systems. Lignin content of various crop waste product has been extensively studied and found to differ depending on the plant origin (Table 15.1).

Lignin content in grass and straw were found to biodegrade to the extent of 17–53% in 100 days. In laboratory studies of thermophilic composting, grass straw showed 45% biodegradation in 45 days but the process is slowed down considerably at more extended times.

Oxo-biodegradation of full carbon backbone polymers such as polyethylene is increasing attention to re-engineered thermoplastic formulations inorder to satisfy in terms of environmental friendliness and used as commodity plastics in many short-life applications. The major strategies to facilitate polyethylene (PE) disintegration and subsequent biodegradation, were focused on the introduction of functional groups within the backbone or on their in situ generation by bio-based pro-oxidant/pro-degradant additives (Fig. 15.10). Extensive studies from authors (Chiellini et al. 1999, 2003, 2006; Krzan et al. 2006; Corti et al. 2010, 2012), it was clearly showed that the pro-degradant systems (carboxyl groups, aliphatic salts,

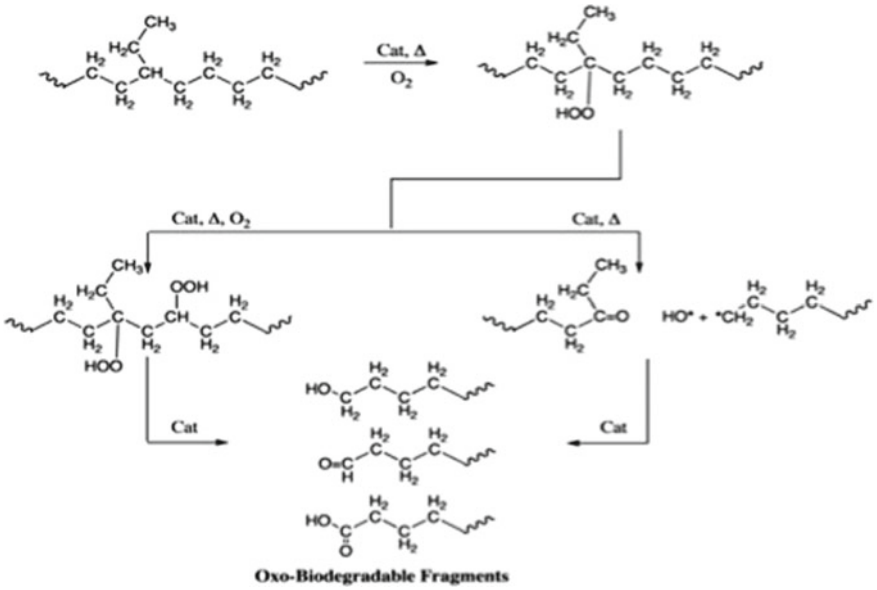

Fig. 15.10 Oxidative degradation and biodegradable functional groups of polyethylene containing bio-based pro-oxidant/pro-degradant additives

manganese stearate and other bio-based oxidizable group) used in the formulation of polythylene film samples were effective to inducing the oxidative degradation of the polymer backbone. A substantial drop in the molecular weight were identified by the production of low molar mass, oxidized fractions, which due to their wettability and functionality, become vulnerable to microorganisms (Chiellini et al. 1999, 2003, 2006, 2007, Krzan et al. 2006; Corti et al. 2010, 2012). Based on these concept and degradation path way, there are some wrong claims of biodegradable materials produced without its scientific evidences of ultimate biodegradability. Therefore, various ISO, ASTM and European standards have regulated for plastic packaging and its biodegradable claims.

15.4 Concluding Remarks

As the consumption of the human beings increase, the need for containers will be more and more important. Plastics are crucial as they are light, easily processable, colorable, and affordable thus plastics are the first choice for many different applications. However, lack of sufficient recycling and lack of awareness of the plastic separation (even in developed countries) create huge plastic wastes that are not reused. The quantity of non-recycled plastic containers is the major issue in many landfill areas in every country, and thereby the number of countries that ban plastic usage

increases significantly. Degradation of these bioplastics is one of the big hurdles in today's environmental problems as it takes at least 1000 years to degrade. Moreover, one of the big troubles with plastics is the formation of "microplastics" after certain degradation level that can mix with the soil and water thus mixing with food that is critical for the health of animals and human beings.

Scientists in the area of polymers have been searching for bioplastics that can be degradable/compostable after certain time. This chapter outlines all the efforts that have been taken so far. The oxodegradable polymers have been used since 2000s but that brings another problem of heavy metals used as degradation catalysts. Moreover, fast degrading polymer is a real urgent need for the humanity. We continue our efforts to make the plastics more environment friendly.

Microbial polymers and biobased polymers are growing with a certain momentum but it will not be very soon to replace the petroleum based plastics. Some recent study on new microorganisms that degrade the plastics is important for the plastic materials and plastic world.

We hope that this chapter will be valuable for the biotechnology area and the polymer/chemical industry.

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Chapter 16
The Application of Microbial Consortia in a Biorefinery Context: Understanding the Importance of Artificial Lichens

Cristiano E. Rodrigues Reis, Aravindan Rajendran, Messias B. Silva, Bo Hu and Heizir F. de Castro

Abstract The exchange of nutrients among different species is one of the most complex, intriguing, and important factors that favored the development of certain microorganisms in evolutionary systems. As a direct result of this, microorganisms in natural system often co-exist with many others in an organized manner. From an engineering approach, the benefits of having multiple microorganisms in a bioprocess may be beneficial to a number of reasons, including favoring the preferred metabolic pathways of one organism, simultaneously by promoting other additional effects by another. Among the many examples of microbial communities or consortia, one of the most studied from a biological point of view are known as lichens—a combination of fungi and algae and/or cyanobacteria. Adding to the fact that over one fifth of all fungal species today known to be in its lichenized form, it is crucial to understand the hidden benefits that such combination can offer to biosystems engineers. Fungal-algal symbiotic relationships often offer significant and efficient exchange of gases, promoting a healthy growth, and can be directly applied towards the solution of a wide range of issues faced today by biorefineries. This chapter summarizes some of the applications of this novel, yet promising, concept, including algal harvesting, lipid accumulation, and bioremediation of wastewater, presenting also a brief history and perspectives on the concept.

Keywords Microalgae · Fungi · Co-culture · Mycoalgae · Lichen

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16.1 Introduction

The interaction between different species is a key driver for evolutionary purposes (Shong et al. 2012). The exchange of nutrientes among symbiotic organisms is one of the most important and most studied factors in microbial communities (Sabra et al. 2010). Despite innumerous advances over the past centuries, understanding how a species collaborates with another has been one of the hardest tasks in the biological realm of studies, and many reasons and mechanisms remain nuclear. Among the many types of mutual relationship between species, one of the most intriguing and omnipresente is found in what we know today as lichens. Lichens exist as a dynamic system between fungi and algae or cyanobacteria, and are widespread on the most diverse habitats on the Earth crust mainly for reasons of nutrient exchange, and therefore, for mutual survival (Rajendran and Hu 2016). Today, about 25% of all fungi species are known to be in their lichen form (Lutzoni et al. 2001), and not only they represent part of the geographic history of the world on their own, but they may be part of the next wave of discoveries in biological engineering.

The proper mechanism of lichenization is species-dependent (Chen et al. 2000; Lutzoni et al. 2001). The association of cyanobacteria or algae with fungi represent a successful nutritional mode in which a heterotrophic fungal species partner with a carbon dioxide-consuming and oxygen-emitting algae (Rajendran and Hu 2016). Such association allows both species to co-exist without the need to rely on "external" sources of energy, e.g., sunlight and other carbon sources. The overall evoluationary success of lichens, today estimated to cover roughly 10% of the terrestrial ecosystem, is hypothesized to play an important role in early development of terrestrial ecosystems, and have been reported to correlate properly with paleological events, such as global cooling (Quirk et al. 2015). Due to evolutionary and adaptation to different habitats, lichens have adapted themselves to a wide range of growth forms and habitat preferences. Lichens can be found as small crusts adhered to leaf surfaces in the tropics to large turfs in the Arctic (Park et al. 2015). Ascomycota, the group to which most lichenized fungi belong to, has been reported to have gained and lost lichenization preferences over the geological eras (Blanco et al. 2006). With recent developments in microalgal and fungal research and their potential applications in bioprocessing, the field of artificial, or laboratory-made lichens has become reality.

The recent boom in microalgae research over the past two decades has answered many questions over the many promising properties of microalgae in bioprocessing. A common bottleneck that still represents an enormous task for their use relies on the economic feasibility of the harvesting step (Reis et al. 2014). Though microalgae brings the potential to become a key driver to a bio-renewable economy, the operation facilities of microalgae production are still hampered by the energy-intensive or by the application of hazardous and recalcitrant chemicals in the harvesting process (Mata et al. 2010). Significant challenges to be overcome are also related to the water and nutrient requirements, and the ecological implications of massive invasion to the local biota. The harvesting process of microalgae remains a challenging task mainly due to the morphological properties of a microalgae cell. Differently from multi-cellular

organisms, as fungi, or even large unicellular yeast cells, microalgae cells are small and have specific gravity values close to those of their culture medium (Pragya et al. 2013). Microalgae cells are usually cultured in suspension. The naturally occurring negative charge of a microalgae cell surface usually brings stability for the cells (Pragya et al. 2013). Microalgae cells are usually cultivated in dilute conditions, which generally yields a poor ratio of biomass to liquid, increasing not only the water footprint of such process, but also the downstream requirements. Conventional algae harvesting operations are dependent on centrifugation, flocculation, flotation, or filtration methods (Mata et al. 2010). Though there have been tremendous advances in the field of algae harvesting, this chapter will focus only on the processes related to the combination of algae and fungi, which will be better described in the further sections. As previously mentioned, other species, as fungi, are relatively easier to harvest than microalgae. With the proper combination of fungi and microalgae, proper cell attachment can occur, leaving a fungal biomass rich in microalgae cells on its surface, and a clear culture medium (Gultom and Hu 2013). The application of such concept is relatively new and requires further understanding in order to fully optimize the process. As of today, the process of co-culture, or co-harvesting, is limited to a few microalgal species (Zamalloa et al. 2017). As the mechanism of attachment is still unclear, research has pointed difficulties in obtaining totality of cell aggregation and in scaling up such process.

In summary, the application of microbial consortia, as is the case of artificial lichens, compared to monocultures can be advantageous from different perspectives from a biological engineering standpoint. Microbial consortia allow different species to share different synthetic functions, i.e., it does not necessarily exploit the potential of a single species to its maximum, but rather, divide the issues among different species. In addition, microbial consortia can aid the fine-tuning of different functionalities of a biorefinery. It does poses additional difficulties, however, as many interactions are not predictable from single-strain experiments. Thus, in order to fully understand the potential of microbial consortia in a biorefinery setting, one must understand the benefits and challenges of a given species and how such species collaborates with another one. This chapter introduces some of the applications of artificial lichens—one of the many groups defined as microbial consortia, and their potential in bioprocessing.

16.2 Characteristics and Formation of Fungal-Algal Systems

Naturally occuring algal biofilms are composed of three-dimensional, multispecies and multilayered structures, involving a complex consortia of heterotrophic and autotrophic organisms (Gauslaa 2014). The photoautotrophic microorganisms may often include unicellular macro- and microalgae in natural systems, and these organisms are characterizaed by their pigmentation, i.e., chlorophyll presence in their cells,

which is able to capture solar energy and promote cellular functions (Campbell et al. 1998). The heterotrophic portion of these complex bodies can often include protoza, bacteria, and fungi, which colonize different zones within the biofilm structure aiming at the most suitable conditions for cell growth (McCune et al. 1997). The microorganisms present in these complex ecosystems are usually held together by matrices of EPS, which functionalize adhesion, retention of water and nutrients, diffusion, protection, and aggregation (Rajendran and Hu 2016). EPS can account for up to 90% of the dry biofilm mass and is mainly constituted by linear or branched heteropolysaccharides, which monomers include hexoses, pentoses, uronic acids, and deoxy-sugars (Nithyanand et al. 2015).

The cell surface of a biofilm material, such as the case of artificial lichens, is usually soft and covered with hydrated flexible macromolecules that facilitate adhesion between cells and from cells to surfaces (Gultom and Hu 2013). Algal and fungal cells produce surface macromolecules, which are often bridged. It has been proposed that specifically in the case of a fungal-algal biofilm, the formation of such complex structure is given by the following steps: (i) adhesion of fungal spores or germinating fungal spores and algae to a matrix, (ii) proliferation of mononucleated cells over the surface or the matrix, (iii) induction of fungal hyphal formation, inducing algal attachment on mycelia (Rajendran and Hu 2016). Another important morphological aspect is that fungal spores present crystalline-like layers, which prevent aggregation within themselves and with other strains, in such a way that the growing hyphae acts as the major promoter of bridge interactions between germinating spores and other species. Microscopically, lectin-carbohydrate is an important factor in the binding of algae and fungi. Particularly involving the role of lectin-carbohydrate is the presence of calcium ions, which act as a link between negatively-charged cell and extracellular nucleic acids, which enable cell-to-cell communication through electrostatic interactions (Zamalloa et al. 2017). Through unclear mechanisms to date, the weak binding of lectin-carbohydrate forms stable cell arrangements and increases the shear resistance.

The use of artificial matrices has been tested in the formation of lichen-type biofilms. Using a polypropylene spun and tape matrix, (Rajendran and Hu 2016) described the four major steps in the formation of such biofilm on a *M. circinelloides* and *C. vulgaris* setting: (i) preferential attachment of strains: fungal spores are known to be highly hydrophobic, thus, the preferential attachment is found on the tape yarn of the matrix within a few hours of the innoculation. It has also been observed that algae cells attach to fungal cells within moments after the spore germination. Within such system, it has been observed that the polypropylene fraction acts more as a mechanical support than a growing support. The surface characteristics of the hydrophobic material also have effects on the rate and on the extent of attachment by fungal spores, on a sense that rougher and more hydrophobic materials will likely develop biofilms more rapidly. (ii) germination of fungal spores: in which the fungal spores attached to the polypropylene matrix propagate along its length, and algae cells attach to their surface. Since *M. circinelloides* is a dimorphic fungus, it can either grow as yeast or filamentous. It is unclear, though, what type of dimorphism *M. circinelloides* presents at the initial stages of growth. Microscopic evidence

shows that algal cells are covered by fungal cells, with little algal growth. (iii) elongation and branching: after the fungal cells have filled in the space in-between the perpendicular rows of polypropylene spun and tape yarn by its hyphae form, algal cells start to autotrophically develop in the liquid phase. It is hypothesized that the lichen formation of *M. circinelloides* is similar to the one reported by *Candida albicans*, in which yeast and hyphal cells comprise the fungal network (Finkel and Mitchell 2011). (iv) mature lichen biofilm: as the thickness of the biofilm increases, algal cells attach to the fungal mycelia. EPS concentrations increase and as pointed above, may significantly contribute to the cell cohesion to the surface.

16.3 Applications in Microalgae Harvesting

The cultivation of autotrophic algae is highly promising towards the development of novel bioprocesses (Reis et al. 2014). The ability to utilize carbon dioxide as carbon source is renowned as a 'win-win' situation, in which effluent gases, e.g. flue gas, can be directly used as feedstock for growing microalgae, at the same time reducing the treatment costs for such effluent. However, in order to fully establish an autotrophic microalgae biorefinery, the cell harvesting step remains as the most significant obstacle on the downstream processing. Autotrophic microalgae cells usually have low density, small size, and quasi-colloidal properties (Zhang et al. 2010). A number of different harvesting techniques have been applied, such as gravity sedimentation, centrifugation, flocculation and flotation, ultrasonic aggregation, and filtration, and combinations of the aforementioned operations (Reis et al. 2014). It has been reported that the utilization of most of these approaches are feasible when it comes to the extraction of high-value chemicals from microalgal biomass, however, for the production of commodity and fuel chemicals, harvesting hinders the economic feasibility. Harvesting fungi, on the other hand, is an easier operation when compared to microalgae. Filamentous fungi grow from a hyphae structure, i.e., a polarized pattern in the form of filaments (Xia et al. 2011). The elements present in a fungal hyphae outgrow of single cells or spores as multinucleate tubes, which moves within a hypha towards the hyphal tip (Balmant et al. 2015). The hyphal tip is the only place where the hyphae grows. From the hyphal tip, which is place responsible for additional growth, diversification of hyphal growth is given, forming a network of hyphae, or also known as mycelia. Depending on the conditions applied to a given submerged fungal growth, mycelia can form pellets, which are spherical or elipsoidal masses of hyphae. Such pellets can vary significantly from loose to compact structures, which form a clear phase separated from the submerged growth, easily harvested after the fermentation process (Xia et al. 2011).

A method to harvest microalgae has been disclosed based on the utilization of fungal pellets as adsorbent matrix and has been reproduced within different studies (Gultom et al. 2014). The fungal-algal co-pelletization process can be considered as a type of co-culture, in which the fungi co-exist with microalgae cells. In the cases involving autotrophic microalgae, without any external carbon source to the system,

the symbiotic relationship of algae and fungi has been reported to be similar to the one found in natural lichens (Gultom et al. 2014). The fungal-algal co-pelletization is reported to be similar to a flocculation process, in which the hyphae acts as flocculants to the microalgae cells (Ummalyma et al. 2017). Though the mechanism of such attraction remains unclear to date, it can possibly be explained through the understanding of zeta potential at each cell surface (Zamalloa et al. 2017). It is known that the surface cell charge and size of microalgae are the main factors for its suspension stability. Under neutral pH, microalgae is usually characterized to have a negative charge due to the presence of carboxylic, phosphoric, and hydroxyl groups on the proteic cell surface (Zamalloa et al. 2017). The zeta potential is usually within the range of −10 to −35 mV (Henderson et al. 2008). Fungi, on the other hand, if grown on acid conditions, usually possess positive zeta potentials (Holder et al. 2007). It has been reported for *Aspergillus flavus* measurements of +46.1 mV, and for *Beauveria bassiana*, +22 mV (Holder et al. 2007). Therefore, it is hypothesized that an electrostatic attraction between fungi and algae is a major factor for this phenomenon to happen. Such phenomena is similar to the one found in bioflocculation, a concept studied for decades, using bacteria and algae. The principles of bioflocculation are linked to the production of bioflocculant chemicals on the cell surface of different bacteria species.

It has been reported for a system of *A. niger* and *C. vulgaris* that within the best culturing conditions, more than 90% of microalgal cells can easily be harvested by using pelletized fungal cells (Gultom et al. 2014). In such system, an organic carbon source was necessary in order to sustain the fungal growth, and it has been observed that such concept can be applied to *C. vulgaris* under hetero- or photoautotrophic modes. Regarding the external carbon source, glycerol and acetate were reported to be successfully used, both which were functional, but with lower results in terms of harvesting efficiency than glucose. Competition was clearly observed regarding the nutrients in heterotrophic conditions, but the symbiotic results indicated greater biomass accumulations than the axenic cultures (Gao et al. 2015; Gultom et al. 2014). Yields no higher than 75% of attachment have been reported on large scale operations, and the most successful application of such concept in the literature is related to a biofilm membrane photobioreactor, utilizing a submerged membrane, which was able to harvest *Chlorella vulgaris* at an efficiency of 72.4% (Gao et al. 2015).

16.4 Lipid Accumulation

The production of lipids by oleaginous fungi is a promising route to reduce the need of oil feedstock for fuel and chemical applications (Rivaldi et al. 2017). Similarly to oleaginous fungi, several algae strains are also known to accumulate high contents of lipids, which have been reported to be successfully used as feedstock for biodiesel and other applications (Reis et al. 2014). Both fungi and algae can accumulate lipids with high content of fatty acids, thus, being considered a substitute for

plant-based oils for biodiesel production. Fungi and algae have the ability to grow rapidly in bioreactors, and are able to utilize a wide range of lignocellulosic waste material as nutrient source, as nitrogen and phosphorus. Though the cell wall of fungi can represent additional challenges to the extraction of lipids, especially due to their extensively cross-linked chitin composition, the purpose of this chapter remains within the scope of presenting the opportunities from utilizing lichens, and technical challenges of single-species cultures will not be thoroughly discussed. Therefore, the beneficial physical and physiological interactions among different microbial communities can represent a promising strategy for next-generation lipid production. It may seem counter-intuitive to try to consolidate multiple microorganisms into a single processing if one thinks about consolidated bioprocessing (CBP) approaches. CBP is often described as the engineering behind the consolidation of multiple pathways and operations to the least amount of processing possible, usually into a single organism (Olson et al. 2012). Among the drawbacks CBP is known for, the metabolic bottlenecks stands as the most critical ones (Banerjee et al. 2010). Consequently, in applications like lipid production, a mixed culture may be a solution for the selection of microalgae and fungi species and strains that are more suitable to perform one task of the overall process, instead of many. Therefore, specially in lipid producing and accumulating strains, the application of microbial consortia can therefore deviate the burden of introducing new functionalities to strains, and rather simply improving naturally-occurring metabolic pathways.

Lipid accumulation in microorganisms is regulated by starvation of a nutrient, usually N (Ratledge and Wynn 2002). Experiments have been performed to demonstrate that nitrogen depletion started earlier in a co-culture system when compared to axenic cultures. In such case, various other metabolites have also been enhanced, such as peptides, amino acids, and nitric oxide. The strategy of early nitrogen depletion can also be thought of being an engineering approach, as it shortens the culture time. Systems like these tend to increase the complexity of a given study, and understanding the competition effects may be necessary. In a competitive system of *Monoraphidium* sp. and *Chlorella* sp., the microalgae cells produced more C18 fatty acids that inhibited the growth of *Monoraphidium* cells (Zhao et al. 2014). The 'survival' strategy adopted by *Chlorella* cells is in accordance to other studies that claim that *C. vulgaris* is able to release inhibitory compounds when co-cultivated with other microorganisms (Cheirsilp et al. 2011). In addition, the utilization of mixed cultures may be beneficial in systems with nutrient sources derived from lignocellulosic feedstock. As an example, as *R. toruloides* is known to be a poor producer of inulases and amylases, it was grown with *Saccharomycopsis fibuligera*, a known producer of both enzymes, in a cassava starch medium (Dostálek and Häggström 1983). The result was an efficient and low-cost method of producing single cell oil, achieving 64.9% of dry cell weight as lipids and cell concentrations as high as 20.1 kg m^{-3}. Similarly, the use of *Rhodotorula mucilaginosa* and *P. guilliermondii* was tested in a 2.0%-inulin medium, achieving lipid concentrations as high as 53.16% on a weight basis (Zhao et al. 2011).

In order to establish a lipid producing system based on a microbial consortia, one must take in consideration that the strains selected should have high lipid pro-

ductivities, with similar nutrient profiles, preferably obtained from complementary niches, which may help to reduce the competition between species. In a study with *A. fumigatus* and different microalgae strains, it was concluded that lipid production in the fungal-algal pellets showed complex profiles, to which three main factors are attributed: (i) total biomass production, (ii) lipid concentrations in fungal and algal cells before and during cultivation process, and (iii) harvesting efficiency of *A. fumigatus* pellets (Wrede et al. 2014). It has also been reported that after 48 h of co-culturing *A. fumigatus* with oleaginous microalgae, which were defined with lipid concentrations greater than 10% of biomass dry weight, lipid concentration in the formed pellets were lower than in mono-cultured algae, but similar or greater than those observed in mono-cultured *A. fumigatus*. Regarding fatty acid composition, the fungal contribution led to high concentrations of C16:0, C18:0, C18:1, and C18:2 (Wrede et al. 2014).

It is important to understand the feasibility of using fungal-algal based lipids in the production chain of biofuels. In this case, considering biodiesel as the main product from microbial lipids, the fungal-algal biomass can be used as feedstock usually through extraction of lipids and followed by transesterification, or by in situ transesterification followed by extraction of biodiesel from biomass (Li et al. 2008). Extraction processes are generally energy intensive and costly, since they involve the use of solvents in traditional processes and significant consumption of electricity. The Department of Energy (US DOE) proposed the following benchmark for biofuel production from algae, which could be extrapolated to the use of fungal-algal biomass: the extraction should consume no more than 10% of the total energy produced on a daily basis (Liu et al. 2012). Some microalgal species have additional morphological barriers to energy-efficient extraction processes, as is the case of *Nannochloropsis occulata*, which have very tough cell walls (Borges et al. 2011). Similarly to *N. occulata*, the production of fungal-algal platforms involves an increase in EPS, which as discussed in other parts of this chapter, increase significantly the toughness of the overall structure, posing additional difficulties to the extraction process. The transesterification process per se can be considered less challenging than the extraction steps, as conversion yields in the literature are usually close to totality in acid-, base-, and enzyme-catalyzed systems (Meng et al. 2009).

16.5 Bioremediation Applications

The use of algal-based systems in wastewater treatment has been extensively studied over the past decades (Mallick 2002), and has been successfully applied in a number of large industrial scale operations (Craggs et al. 2012). Despite the advances, there are technical limitations, which limit the application of algal-based systems. As of today, applications as reverse osmosis are being used worldwide in order to meet demand for water reclamation in intensive urban systems. Reverse osmosis, however, generates saline concentrate streams, which contain almost all of the nutrients and contaminants at elevated concentrations (Dialynas et al. 2008). The growing need

to explore cost-effective treatment operations is an opportunity for biological-based systems to be fully explored in order to reduce environmental and health risks that traditional methods, as reverse osmosis, can often bring. The concept of utilizing wastewater treatment methods based on algae-based biofilms is that they offer a simple, yet energy-efficient technology for sorption of key nutrients, as N and P, and offer an easy and robust separation from the wastewater bulk (Mehta and Gaur 2005). The use of co-culture to treat various types of wastewater is a developing field of study. As mentioned in the previous sections, the combination of different microorganisms in a complex media may significantly increase the biotechnological effect of a given culture. Recently, the use of fungal-algal pellets has been used on reducing the toxicity of different effluents. Fungal-algal pellets of *Aspergillus sp.* and *C. vulgaris* have been used to treat centrate (Wrede et al. 2014), i.e. a liquid stream generated through centrifugation of activated sludge, and diluted swine manure. The first one contained about 50 mg L^{-1} of ammonia and phosphates, while the later, a higher concentration of ammonia (89 mg L^{-1}) and lower concentration of phosphates (1.8 mg L^{-1}). The researchers observed that in both conditions, the pellets were able to significantly decrease N and P, as well as color, producing a practically colorless suspension in about 24 h (Wrede et al. 2014). Similar results were observed by the utilization of *A. fumigatus* with *C. protothecoides* and *Tetraselmis suecica* using digestate from swine manure, with concentration uptakes of as high as 73.9% of ammonia and 55.6% of phosphates. It has also been observed that the uptake of key nutrients led up to 1.7 fold increase in biomass production after 48 h of treatment. The use of *A. fumigatus* with *T. chuii* has also been tested with diluted swine wastewater, diverted from a lagoon system, in which up to 86% of ammonia and 69% of phosphate were uptaken in just 48 h of treatment (Wrede et al. 2014). In addition, pellets from *A. fumigatus* and *Synechocystis* sp. were grown on digestate wastewater from a municipal treatment facility (Miranda et al. 2015). For the latter study, it has been reported that in relatively concentrated suspensions (25% of original wastewater concentration), pellets were able to reduce ammonia-N to levels close to 90% (164.3 to 18.2 mg L^{-1}) and phosphate-P to levels as high as 75% (38.7 to 9.8 mg L^{-1}). Interestingly, the synergystic effect was higher than in the axenic cultures, which achieved results no higher than 52% for ammonia-N, and 45% for phosphate-P. Still regarding this study, biomass yields were increased to 2.3-fold compared to the control system, and lipids were increased by 1.5-fold (Miranda et al. 2015).

An experimental setup was also established using corn to ethanol co-products as culture medium (Rajendran et al. 2017). The major difference between corn to ethanol co-products, in particular, stillage water, and agricultural effluents is the concentration of nutrients (Reis et al. 2017). Particularly speaking, Condensed Distillers Solubles (CDS) has at least one order of magnitude on concentrations of P and N than diluted swine manure and other hydrolysates used by (Miranda et al. 2015). In a study with diluted CDS, a biofilm composed by *Mucor sp.* and *C. vulgaris* was tested, and results have shown that the microbial biomass achieved concentrations as high as 9.358 g L^{-1} at a 12-day culture, being mostly composed by fungal cells (97.2% of the mature biofilm) (Rajendran et al. 2017). Bioremediation of viscous materials like CDS should also take in consideration the presence of solids attached

to the biofilm. Particularly in the aforementioned study, the attached solids decreased from 6.304 to 3.349 g L^{-1} at 4 days and 12 days of culture respectively. Nutrient wise, the biofilm was able to retain 55.7 and 74% of the original concentrations of P (818 mg L^{-1}) and N (924 mg L^{-1}), respectively. Results also show a decrease in Chemical Oxygen Demand (COD) levels up to 65.6% (Rajendran et al. 2017). As other studies have also shown, the combination of different cultures achieved higher biomass concentrations than the axenic cultures under all the conditions tested.

It is important to note that most of these applications of co-culturing microorganisms are relatively new, though the concept of applying lignocellulosic waste as nutrient source to fungi and algae has been a key topic of research for decades (Sánchez 2009). Fungal treatments are known for their ability to digest lignocellulosic biomass, especially by the production of active enzymes including cellulases and hemicellulases, pectinases, phytases, laccases and manganese peroxidases. Understanding the fungal role in producing such enzymes may be an important factor when upscaling bioremediation and processes involving accumulation of commodity biomass. For example, in order to potentially decrease the costs of fungal-assisted bioflocculation using *A. fumigatus* as the fungal species, rice straw could succesfully be used as partial replacement of nutrients for the microorganisms involved due to the extensive production of endo- and exo-glucanases, beta-glucosidases, laminarinases, lichenases, xylanases, and pectin lyases, which are able to hydrolyze rice straw and provide a healthy system for microbial growth (Zhang et al. 2011). It has been observed in the cited study that in a media containing wheat straw as the sole source of carbon, fungal pellets showed similar growth rates and sizes to the control experiments, i.e., those grown on glucose (Muradov et al. 2015). One reported drawback, however, was that fungal pellets when grown on wheat straw biomass release chemicals that are inhibitory for algal growth (Muradov et al. 2015). Therefore, the application of lignocellulosic materials for fungal-assisted algal flocculation needs to be further studied in order to reduce the production of anti-algal chemicals.

16.6 Perspectives on the Development of Lichen-Based Bioprocesses

Though the application of microbial consortia in the reported literature results mostly considers the results of a 'single' organism, i.e., as if the mutualistic organisms acted as they were one, it is important to understand that the characteristics of a given biomass material generated from artificial lichens can be differed according to the composing species. It is true that mixed cultures generate a single biomass type, which is close to being homogeneously distributed across its volume, except in cases with gradient conditions of growth, e.g. light and nutrient. The resulting biomass can be roughly estimated as the weighted average of the composing organisms contribution (Wrede et al. 2014). It has been reported that particularly in lichens, the fungal weight contribution exceeds greatly the algal composition (Rajendran et al. 2017;

Rajendran and Hu 2016); therefore, one must take in account that the fungal composition may largely exceed the algal contribution to the overall biomass. Understanding this concept is particularly of interest in cases in which a compound is either fungal or algal-based, and a co-culture is used to produce it. Another important factor to be taken in consideration is that co-culture operations lead to more robust systems, which may reduce the energy required for sterilization (Muradov et al. 2015). Though single-cell cultures can be preferred due to their reproducibility, an ideal co-culture system should be able to provide support to the development of natural biological characteristics of each organism in order to enhance their cellular interactions.

Innovative ways of co-cultivating microorganisms can lead to outcomes that are close to unachievable in non-gene modified single-cell cultures. Examples regarding lipid accumulation are: (i) increase in release of free fatty acids into the extracellular medium; (ii) production of enzymes that are beneficial to the other species, such as inulases and amylases in a *Saccharomycopsis fibuligera* and *R. toruloides* in a cassava starch medium (Gen et al. 2014). In this regard, the choice of a consortium of lipid-producing strains may also pose challenges on the rheology of submerged cultures and lipogenic capacities. From an engineering perspective, the enhancement of mass transfer coefficients is a major challenge improving production yields (Boopathy 2000). Though it may seem counter-intuitive to think that aeration may be necessary in systems with algae, usually fungal consumption of oxygen is higher than production by algae, therefore, oxygenation and oxygen mass transfer is necessary for an efficient growth and production of desired metabolites. In high-cell density cultures, growth is dependent on the mass transfer rate. In such sense, diffusion limitations results in lower productivities. Therefore, an adequate reactor system that can provide high mass transfer rates is usually necessary in order to establish a bioprocess like the one discussed (Boopathy 2000). Even though high mass transfer rates are needed, one also must take in account the shear and the stress suffered by the cells inside a bioreactor. As a bottomline, these and other issues should be taken in consideration when addressing the commercial viability of a fungal-algal medium.

As is the case with many other emerging technologies, many questions are still unanswered. Usually experiments in co-culture systems are carried out by having two or more controls using monocultures in a pure culture system, in which the strains are submitted to identical conditions, e.g. operating conditions, substrate concentration, medium type, etc., in order to mimic the biological environment and their reactions to the growth. Co-culture assays, however, may impose competitiveness among different cultures, and results may not be easy to interpret and obtain 'logical' data from. Competition may benefit one species in detriment of the other, or may harm both. A clear example is found in the case of lipid-producing strains, in which competition can clearly impact the lipogenesis process. In order to overcome such difficult comprehension, a handful of techniques can be applied. One of them is molecular biology and genetics, in order to establish the metabolic guidelines and function of genes involved in microbial lipid assimilation, accumulation, and processing. Therefore, thoroughly understanding the strain abilities, kinetics, and biological mechanisms has become a focus for researchers, not only to fundamentally develop mechanisms for enhancement of processes, but also to avoid

the misinterpretation of results. Among the techniques and mechanisms researchers have used to better understand the cell-to-cell communication are cross feeding and metabolite exchange. Metagenomic sequencing is another proeminent tool for steering the functionalities of microbial communities, especially by describing cellular regulations at metabolic levels and by drawing a simplified microenvironment of a given mixed culture (Morfopoulou and Plagnol 2015).

Finally, additional challenges are often found not only in the fermentation itself, but also in the downstream process. Downstream processes are usually considered high-cost and challenging depending on the cultivation process. For example, continuous centrifugation to separate lipid-producing cells from the fermentation broth is one of the most considered alternatives to the separation of biomass from the fermentation broth. However, such process is highly intensive and often present technical challenges. Other options, as is the case of flocculation and filtration may also present cost inhibition to an overall biotechnological process (Milledge and Heaven 2013). In the case of intracellular metabolites, as is usually the case for lipid bodies, following the cell separation steps, cell lysis and solvent extraction are also a suggested route. Both processes are well understood and reviewed in the literature, however as it is pointed out, chemical treatment can also pose environmental risks. In addition, many extraction processes rely on dry biomass, which pose additional energy requirements for drying the cells prior to extraction.

16.7 Conclusion and Future Perspectives

This chapter comes in the context that in a world with growing population, increasing urban densities, and increased footprint on natural resources, alternatives must be developed in order to ensure a sustainable future. Artificial lichens can be a promising alternative to some of the challenging tasks of the nearby future. The extensive use of plantable lands and the growing consumption of freshwater by energy crops have been an incentive for next generation bioenergy and a bio-based economy. As pointed by (Langeveld et al. 2010), the selection criteria for suitable alternatives in a bio-based economy must be as follows: (i) strains with high growth rates and increased biomass production, (ii) strains that are able to produce high concentrations of energy-dense molecules, e.g., lipids, (iii) high harvesting index and ease of harvesting, (iv) capacity to grow on marginal and low-value lands, with lack of competition with existing agricultural crops, (v) low dependence on freshwater, (vi) low costs for growth and harvest, (vii) production of value-added products. Generally speaking, despite being an early concept, novel tools for analysis and engineering of microbial communities have been developed, including those present in synthetic biology and metabolic engineering. A common concept that is sometimes overlooked is the process of obtaining a reliable community behavior and reproducible results. Studies in the area should take advantage of the innumerous advances in the intersection of molecular biology

and biosystems engineering in order to fully develop solutions for the problems in bioprocessing, bioremediation, and even biosensing, and other many applications in which answers are urgely needed to meet the demands of a sustainable future.

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Chapter 17
Green Microalgae as Substrate for Producing Biofuels and Chlorophyll in Biorefineries

Bruna C. M. Gonçalves and Messias B. Silva

Abstract In order to develop sustainable technologies with reduced impacts in an attempt to repair previously caused damages to the environment, green alternatives have been the focus of several researches nowadays. Technological advances have enabled an effective use of natural sources to obtain clean energy, thus reducing emissions of gaseous pollutants into the environment. In this context, biofuels are promising alternatives for regulating climate change caused by an increase in the greenhouse effect, whose negative impact has been considerably perceived over the years. The use of microalgae as raw material to obtain biofuels has been proved promising. Due to the rich composition of carbohydrates, lipids and various proteins, biofuels and bioproducts can be obtained from microalgal cells, thereby contributing to bring down the final cost of products within the concept of biorefineries. Thus, this chapter aims to identify the process variables that interfere in microalgae cultivation to produce biofuels and pigments, and their impact on microalgal cell composition. Information on the most widely used culture media and the most studied species for obtaining biofuels by focusing on biohydrogen, biodiesel and bioethanol have been assessed. Furthermore, the process for obtaining these biofuels was illustrated in a simplified form in order to provide a general overview for readers.

Keywords Microalgae cultivation · Third generation biofuel · Renewable energy

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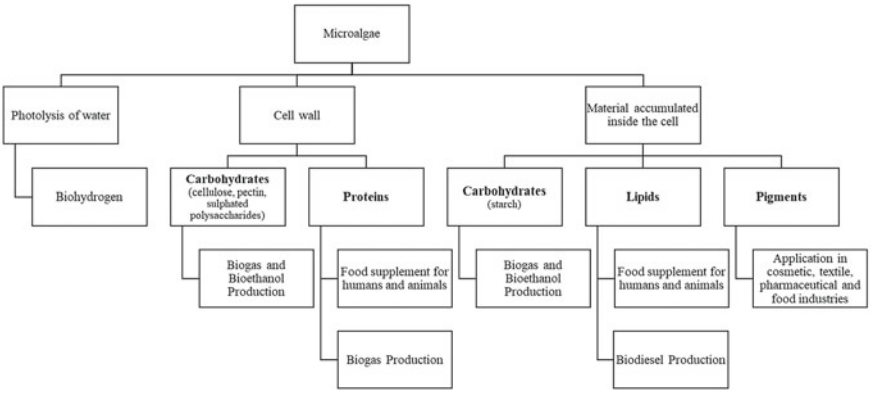

Fig. 17.1 Biotechnological possibilities of microalgal biomass utilization within the concept of biorefineries

17.1 Introduction

Microalgae are unicellular, microscopic and photosynthetic organisms found in both freshwater and seawater (Velasquez-Orta et al. 2013). They can also proliferate in industrial and urban wastewaters (Caporgno et al. 2015; Lu et al. 2015; Wu et al. 2017), and icy waters of oceans (Begum et al. 2016). These organisms are part of phytoplankton and depend essentially on sunlight, carbon dioxide (CO_2) and water for producing structural molecules, reserve molecules and pigments, consequently leading to cell proliferation (Koller et al. 2014). According to Spolaore et al. (2006) and Koller et al. (2014), microalgae and their extractives have been widely used in both human and animal nutrition, and as raw materials to be used in textile, pharmaceutical, cosmetic and food industries. In addition, they can be used as feedstock for obtaining biofuels, as shown in Fig. 17.1.

17.2 Microalgae Cultivation to Produce Biofuels

Since the beginning of the industrial revolution, technological development has been based on the combustion of fossil fuels derived from petroleum, natural gas and coal. These energy sources are not renewable and their burning promotes the emission of gases that lead to the greenhouse effect, such as CO_2. According to Alley et al. (2007) and "Global Climate Change" (2015), CO_2 atmospheric concentration has increased from 280 ppm in the year 1750 to 400.47 ppm in 2015. This 43% increase disrupted the balance between the solar radiation that is received and reflected by the Earth, thus increasing its ability to retain heat and resulting in the so-called global warming phenomenon (Höök and Tang 2013; Peters et al. 2013; Scheutz et al. 2009). This

scenario becomes extremely alarming by considering the drastic climate change that has been observed over the years, which has been clearly intensified recently.

The search for sustainable alternatives aimed at preserving the environment and controlling the increased emission of greenhouse gases (Samimi and Zarinabadi 2012), as well as a constant rise in the price of petroleum (Nazlioglu and Soytas 2012) has encouraged research in the area of bioenergy. Thus, energy resources capable of being naturally renewed at a fast pace and that are directly and indirectly obtained from energy sources such as the sun, wind, waves, plant and microalgal biomass have been explored (Jacobson and Delucchi 2011; Long et al. 2013). In this sense, three generations of biofuels, first (1G), second (2G) and third (3G) generations, can be obtained.

Both 1G and 2G biofuels are obtained from plant biomass. 1G biofuels are produced from sugar fermentation for ethanol generation, which can be obtained mainly from corn, beet, wheat and sugarcane juice, or by transesterification of lipids to be used in the food industry, especially those of vegetable origin, e.g. soybean oil, for biodiesel production. On the other hand, 2G biofuels are mainly obtained by using sugars extracted from agro-industrial waste, such as straw and bagasse, or lipids from non-food sources, e.g. jatropha oil (Naik et al. 2010). Although the production of these biofuels is classified as a sustainable process and is among the green technologies which are so encouraged as a way of preserving the environment, one of the major drawbacks of this process is the need for arable or pre-treated soil to obtain sugars, due to the recalcitrance of vegetable raw material.

3G biofuels are an alternative to 1G and 2G and, instead of being obtained from plant biomass, are the result of processing microalgae cells. The advantages of cultivation and obtainment of biofuels from microalgae in comparison with using vegetal biomass are listed in Table 17.1 (Chen et al. 2014).

Due to the rich composition of lipids and carbohydrates and the ability to fixate CO_2, (Sankar et al. 2011) several types of biofuels can be produced from microalgae (Fig. 17.1). The oil accumulated inside the cell can be extracted and used for biodiesel production (Gong and Jiang 2011). After lipid extraction, the residual biomass can be used for biogas or bioethanol production. Biogas production is carried out from an anaerobic digestion of microalgal biomass (Mussgnug et al. 2010), while bioethanol production is performed through the hydrolysis of sugars present in the cell wall and fermentation by microorganisms (Hernández et al. 2015). The stages of oil extraction and microalgal biomass processing to obtain ethanol are depicted in Fig. 17.2. The biohydrogen produced by photolysis at the photochemical stage during photosynthesis can be stored (Batyrova et al. 2015; Benemann 2000). However, studies on biofuel co-generation with the same culture have been carried out, as shown in Table 17.2.

Table 17.1 Advantages and disadvantage of using microalgae to obtain biofuels

Advantages	Disadvantage
• Microalgal cells can be cultured in small spaces • Microalgae proliferation rate of is higher than plant growth time • The absence of structures such as stem and leaves, together with the fact that they are submerged in water, make them more efficient in the conversion of solar energy into biomass when compared to superior plants • High accumulation rate of lipids, carbohydrates and proteins • Harvesting cells can be performed anywhere • They can be grown in non-potable water (wastewater) • Other products of high commercial value, in addition to biofuels, can be obtained • They are adaptable to various geographic or climate conditions	• Need for large amounts of cells to obtain a satisfactory quantity of extractives

Table 17.2 Biofuel cogeneration from the same microalgae culture

Microalgae	Biofuel	References
Chlamydomonas reinhardtii	Biohydrogen Biogas	Mussgnug et al. (2010)
Chlorella sp.	Biohydrogen Biodiesel	Dasgupta et al. (2015)
Co-culture of *Scenedesmus* sp. and anaerobic sludge in starch-rich wastewater	Biohydrogen Biodiesel	Ren et al. (2015)
Chlorella sp.	Biohydrogen Biodiesel	Sengmee et al. (2017)
Chlorococum sp.	Biohydrogen Bioethanol	Harun et al. (2010)

17.2.1 Process Variables

Microalgal cell composition may vary according to species, culture and/or environmental conditions (Brown 1991; Rhee 1978; Volkman et al. 1989). Other factors such as variation in temperature (Renaud et al. 2002), supplementation (Jiang et al. 2012; Procházková et al. 2014; White et al. 2013), luminous intensity and photoperiod (Khoeyi et al. 2012) may significantly alter microalgal cell composition. Under optimal culture conditions, microalgae multiply rapidly but do not accumulate reserve substances (carbohydrates and lipids). On the other hand, adverse conditions tend

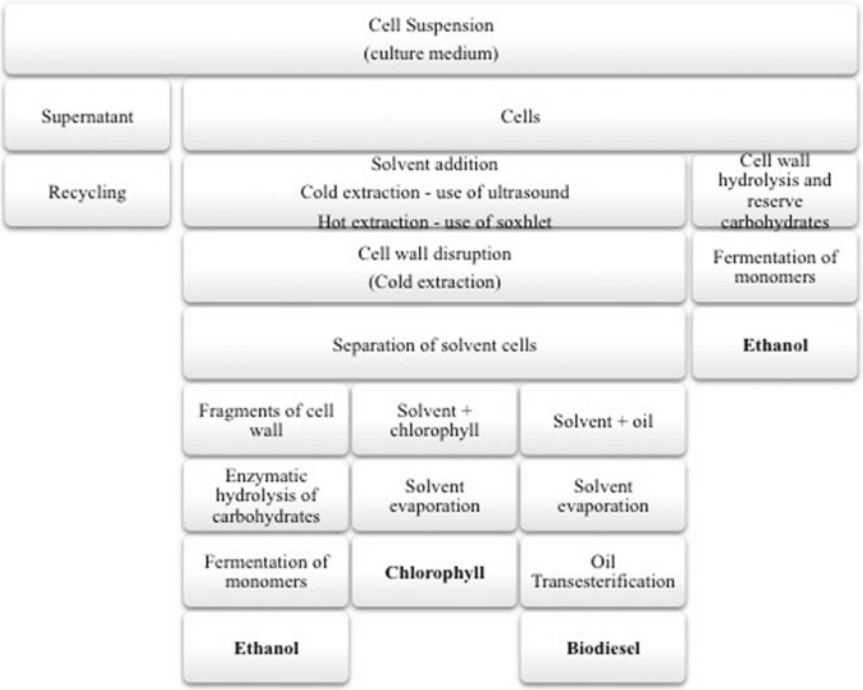

Fig. 17.2 Stages to obtain bioethanol, biodiesel and chlorophyll from green microalgae cultivation

to stimulate the accumulation of reserve substances or pigments. Thus, products or by-products of interest can be obtained by regulating experimental conditions.

Microalgae can be cultured either in the presence or absence of light, and can use both organic and inorganic carbon as an energy source. Thus, cultures can be performed in four different conditions: (i) photoautotrophic, (ii) heterotrophic, (iii) photoheterotrophic and (iv) mixotrophic (Fig. 17.3).

Photoautotrophic condition is the most used and is performed in the presence of a light source, which enables the conversion of inorganic carbon into energy through photosynthesis process. Culture illumination can be carried out by means of a natural light source (solar energy) or by light bulbs. It is emphasized that the intensity and color of light and the emitted wavelength directly interfere in the development and accumulation of biomolecules by cells (Kim et al. 2013; Zhao et al. 2013). Some microalgae are able to use organic carbon as a source of energy and carbon, thus characterizing heterotrophic culture. In such a case, light energy is not required for biochemical reactions to occur. In mixotrophic and photoheterotrophic conditions, cultures are carried out in the presence of light and an organic carbon source. The difference between these conditions is that, during mixotrophic culture, cells perform photosynthesis and use both organic and inorganic carbon to obtain energy, i.e. they are able to live in both photoautotrophic and heterotrophic conditions. On the other

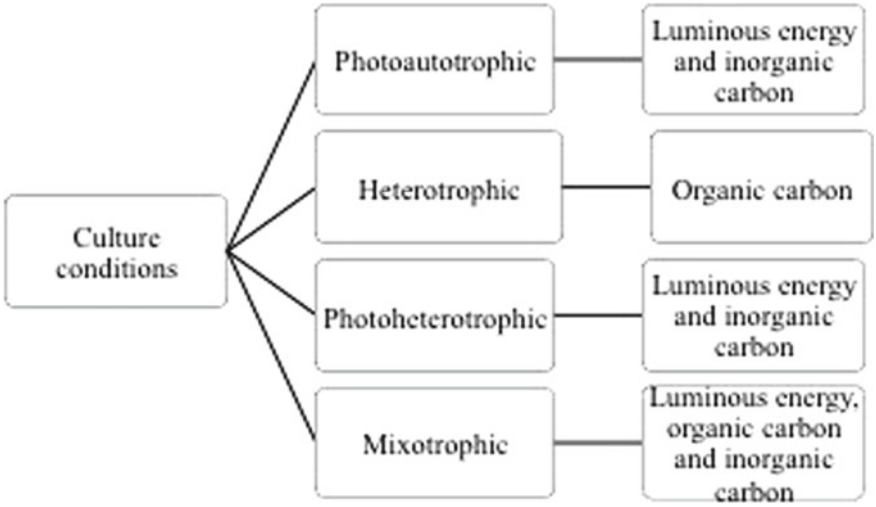

Fig. 17.3 Types of condition used for microalgae cultivation

hand, in a photoheterotrophic condition, cells need light to use organic compounds as carbon source (Chen et al. 2011). These conditions are used to study the production of hydrogen (Table 17.2), accumulation of lipids (Table 17.3) and carbohydrates (Table 17.4) by microalgal cells to evaluate biohydrogen, biodiesel and bioethanol yields, respectively.

Although the production of large quantities of microalgal biomass is required to commercially produce biofuels, most studies report cultures in laboratory scale using artificial lighting (indoor), usually in photobioreactors with 1 to 10 L of capacity. On the other hand, scale-up (Oncel et al. 2015) and pilot-scale (Chen et al. 2014; Lu et al. 2015) simulation studies using outdoor lighting have been conducted to increase productivity (Tables 17.2, 17.3 and 17.4).

Culture medium composition is of major importance for cell growth and proliferation. Different species of microalgae present different nutritional requirements, although they can adapt to different supplementation conditions. For this reason, industrial (Abdel-Raouf et al. 2012; Lu et al. 2015; Wu et al. 2017; Yu et al. 2014) or domestic (Lv et al. 2017; Reyimu and Ozçimen 2017) wastewaters which are either concentrated or diluted can be used as culture medium. However, culture media with previously established composition (synthetic) may have added or subtracted nutrients, or the concentration of one or more components may be altered in order to evaluate the response of cells to this new condition. Therefore, supplementation should be adequate to the biochemical route that is to be stimulated in order to obtain the product of interest. The use of low cost nutrient sources such as glycerol/glycerin (Li et al. 2011; Sengmee et al. 2017), human urine (Wu et al. 2017) and urea (Campos et al. 2014) has been explored. Examples of culture media used for producing H_2, lipids and carbohydrates are listed in Tables 17.2, 17.3 and 17.4,

Table 17.3 Culture media and types of photobioreactors for H_2 production from different microalga species

Microalgae	Culture media	Reactor	Agitation	Culture condition	Culture type	Hydrogen production	References
C. reinhardtii	TAP	Cylindrical reactor vessels (600 mL)	Mechanical and magnetic stirring	PA	Indoor	H_2 production begins after anaerobiosis in the culture system	Antal et al. (2003)
C. reinhardtii	High salt content media	Glass PBR with pH and O_2 sensors	Magnetic stirring	PH	Indoor	56.40 mL/L	Tsygankov et al. (2006)
C. reinhardtii CC124	TAP	Tubular PBR (110 L)	Bubbling of a mixture of air and CO_2	PA	Indoor	Productivity: 0,61 mL of H_2/L/h	Giannelli and Torzillo (2012)
C. reinhardtii Dangeard 137C mt+	TAP	Microprocessor-controlled PBR	Magnetic stirring	PA	Indoor	109.00 mL/L	Batyrova et al. (2012)
C. reinhardtii CC124 mutante	TAP	Flat plate Roux type PBR (H_2 production) (800 mL)	Mechanical stirring	MT	Indoor: simulação de condição outdoor	Productivity: 1.30 mL of H_2/L/h	Oncel et al. (2015)
Chlorella sp.	TAP glicerol	Serum bottle	Ns	MT	indoor	Maximum production: 10.31 ± 0.05 mL/L	Sengmee et al. (2017)
		Not specified (1 L)				Maximum production: 11.65 ± 0.65 mL/L	
Scenedesmus sp. NBRI012	TAP no sulphur	Rectangular glass reactor (2 L)	Magnetic stirring	PA	Indoor	17.72% $v_{H2}/v_{total\ de\ gases}$	Dasgupta et al (2015)

PBR photobiorreator; *TAP* Tris-acetate-fosfate; *PA* photoautotrophic; *PH* photoheterotrophic; *MT* mixotrophic; *Ns* not specified

Table 17.4 Culture media, types of photobioreactors and cultures to obtain oil from different species of microalgae

Microalgae	Culture media	Reactor	Culture condition	Culture type	Agitation	Composition/productivity	References
Chlorella vulgaris	Fitzgerald modified	PBR (5 L)	PA	Indoor	Aeration (air and CO_2)	Composition: 29.53% of total lipids in dry biomass Productivity: 12.77 mg lipid/L/d	Widjaja et al. (2009)
Chlorella minutissima UTEX2341	Basic N8Y	*Erlenmeyer* flasks (500 mL)	PH	Indoor	Shaking	Composition: 16.11% lipid esterifiable in dry biomass with 62.97% FAME conversion Productivity: 286.76 mg lipid/L/d and 180.68 mg FAME/L/d	Li et al. (2011)
Chlorella sp.	Bold Basal	PBR (10 L)	PA	Indoor	Aeration (air)	Composition: 12% total lipids in dry biomass, of which 42% were esterifiable	Velasquez-Orta et al. (2013)
Nannochloropsis oculata						Composition: 24% total lipids in dry biomass, of which 17% were esterifiable	
C. vulgaris ESP-31	Basal media and Bristol modified	Vertical tubular-type (50 L)	PA/PH	Outdoor	Aeration (air and CO_2)	Productivity: 48 mg lipid L/d	Chen et al. (2014)
C. vulgaris (FWM-CV)	MBL adapted to fresh water	*Erlenmeyer* flasks (5 L)	PA	Indoor	Aeration (air)	Composition: 19.27% of total lipids in dry biomass Productivity: 2.19 mg lipid/L/d	Al-Lwayzy et al. (2014)
Chlorella kessleri UTEX2229	Domestic wastewater	Flat-panel airlift (1 L)	PH	Indoor	Aeration (air and CO_2)	Composition: 7.4% of esterifiable lipids in dry biomass	Caporgno et al. (2015)
C. vulgaris CCAP211/19						Composition: 11.3% of dry biomass esterifiable lipids	
Chlorella sp.	Dairy industrial wastewater	PBR (1, 3 L)	PA	Indoor	Aeration (air)	Composition: 55.54 mg of fatty acid methyl esters/g dry weight	Lu et al. (2015)
		PBR (30 L)		Outdoor		Composition: 34.90 mg of methyl esters of fatty acid/g dry weight	

(continued)

Table 17.4 (continued)

Microalgae	Culture media	Reactor	Culture condition	Culture type	Agitation	Composition/productivity	References
Scenedesmus sp. NBRI012	TAP (no S)	*Erlenmeyer* flasks (1 L)	PH	Indoor	Magnetic stirring	410.03 mg of lipids/L	Dasgupta et al. (2015)
Chlorella sp. NBRI029						587.38 mg of lipids/L	
C. minutissima	Tamiya	tubular PBR (120 L)	MT	Indoor	Aeration (air and CO_2)	45.82% of polyunsaturated fatty acids	Aremu et al. (2015)
			PA			37.90% of polyunsaturated fatty acids	
C. minutissima UTEX2341	Artificial wastewater	*Erlenmeyer* flasks (500 mL)	PH	Indoor	Orbital shaker	Productivity: 249.36 mg of lipids/L/d in medium containing 4 mM cadmium	Yang et al. (2015)

PBR photobiorreator; *PA* photoautotrophic; *PH* photoheterotrophic*; MT* mixotrophic*; CO_2 gás carbônico; S enxofre; N nitrogênio*

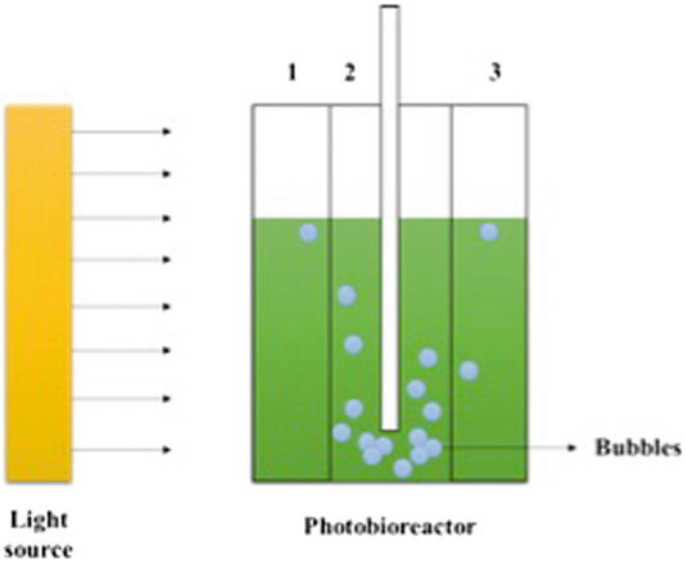

Fig. 17.4 Schematic of a bench photobioreactor with agitation by air bubbling and identification of illumination zone (1), intermediate zone (2) and dark zone (3)

respectively. It should be emphasized that the culture medium should be adequate to each species needs, so that marine microalgae can be cultivated in a high osmotic pressure environment in order to resemble seawater characteristics. In such a case, high concentrations of sodium chloride (approximately 30 g/L) are used.

Stirring in microalga cultures is essential to prevent cell sedimentation and ensure cell suspension homogenization. As regards a bench photobioreactor (PBR), stirring is essential to ensure that all cells receive the same amount of light in photoautotrophic and photoheterotrophic (indoor) cultures. In this type of culture, the light source is usually placed specifically towards the PBR (Fig. 17.4) and cells located in the illumination zone block the passage of light into the dark zone as cell proliferation progresses. One way to avoid shading between cells is by installing lighting sources on opposite sides of the PBR or wrapping it with light strips.

Flow can be produced by mechanical agitation, air and/or gas bubbling or with the aid of a peristaltic pump. Although mechanical agitation is more efficient when compared to other types of agitation, contact with the agitator can damage the cells. Another downside is the elevated cost involved in installing the agitator and operating the system, thus discouraging its use on an industrial scale. In addition to promoting lower cell damage rate, the bubbling or aeration system requires less financial investment and is easy to be installed when compared to mechanical agitators (Chisti 2008). Nevertheless, this system has the advantage of, in addition to conserving suspended cells, the culture medium being aerated. Examples of agitation types, reactor types and cultivation conditions can be seen in Tables 17.2, 17.3 and 17.4.

17.2.2 Hydrogen Production by Microalgae

According to Momirlan and Veziroglu (2002), H_2 is the most promising fuel for replacing fossil fuels in the medium and long term when compared to other known fuels, due to its high energy density per mass. In addition to being a renewable energy resource, it is considered an ideal alternative to fossil fuels since it does not increase the greenhouse effect. This is due to the fact that, upon combustion, H_2 produces only water and can be used for power generation by fuel cells, or directly in internal combustion engines. However, H_2 use as fuel is considered limited due to high production costs, and transportation and storage difficulties (Khetkorn et al. 2017).

H_2 can be produced from fossil fuels (Steinberg 1989), natural gas (Block et al. 1997) or water. H_2 production from water can be accomplished by numerous processes, including liquid water or steam electrolysis (Zeng and Zhang 2010), photolysis (Barrett and Baxendale 1960), thermochemical decomposition (Funk 2001) and photoelectrochemical process (Sivula et al. 2010). Biological H_2 production is performed by microorganisms through photosynthetic or non-photosynthetic processes (Khetkorn et al. 2017). Non-photosynthetic H_2 production can be performed under aerobic conditions using inorganic carbon, such as CO_2, or through anaerobiosis using an organic carbon source, such as starch (Sengmee et al. 2017).

H_2 can be obtained from microalgal biomass fermentation by microorganisms that produce it, or as a result of metabolic reactions of microalgal cells (Fig. 17.5). H_2 production by green microalgae is carried out during the photosynthesis process through direct or indirect water biophotolysis. Direct biophotolysis occurs in the presence of sunlight, which is captured by photosystems I and II for oxygen photosynthesis. In this process, H_2 is directly generated from breaking down the water molecule with subsequent O_2 release. In indirect biophotolysis processes, H_2 generation occurs from the carbohydrate (starch) molecule breakdown, which is previously synthesized by a biological system in the presence of water and CO_2 that is absorbed from the atmosphere. Thus, carbohydrate breakdown generates H_2 and CO_2. One of the major challenges of H_2 production by microalgae is an incompatibility between oxygen photosynthesis and anaerobic H_2 production, due to fact that hydrogenase is highly sensitive to O_2 (Benemann 2000; Khetkorn et al. 2017; Kruse and Hankamer 2010; Márquez-Reyes et al. 2015).

The microalgae *C. reinhardtii* has been used as model for studying H_2 production, although studies with other species are underway (Table 17.3). In addition, the most widely used culture medium for H_2 production by microalgae has been the TAP medium, as shown in Table 17.3. H_2 photoproduction can be favored by sulfur and phosphorus deprivation in freshwater algae cultures, and by phosphorus deprivation in seawater algae cultures (Batyrova et al. 2015; Dasgupta et al. 2015; Sengmee et al. 2017).

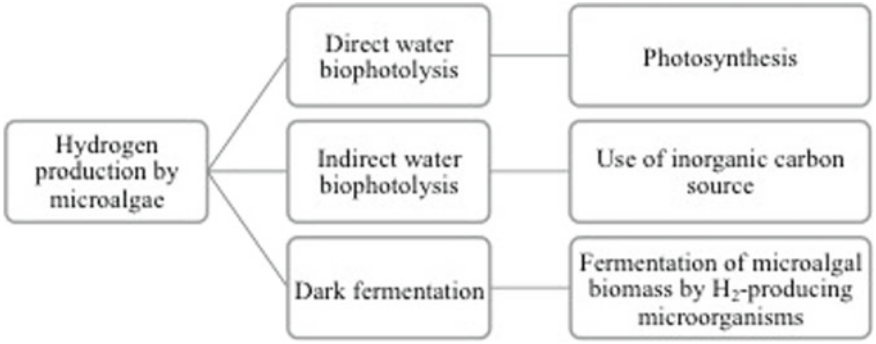

Fig. 17.5 H_2 production processes from microalgae

17.2.3 Biodiesel Production from Microalgal Biomass

The main advantage of using biodiesel as fuel instead of regular diesel lies in CO_2 emissions. With respect to biodiesel, these emissions can be considered as carbon credits, since plants and microalgae use CO_2 as an inorganic carbon source for their metabolic reactions. In addition, biodiesel can be used as fuel for generating the power necessary to produce and process microalgae (Chisti 2008).

Under unfavorable environmental conditions (stress conditions) generated by nutrient deficiency or amount of light, microalgal cells accumulate lipids in the form of triacylglycerides (Widjaja et al. 2009). Biochemical changes, such as low nitrogen supply, may reduce cell proliferation due to the scarcity of proteins that participate in cell wall formation (Aremu et al. 2015; Ördög et al. 2012). On the other hand, phosphate deprivation can negatively affect biomass production without significant losses in lipid concentration, although the concentration of unsaturated fatty acids increases significantly, which is uninteresting with respect to biodiesel production (Praveenkumar et al. 2012). Moreover, supplying organic carbon sources stimulates cell growth and lipid accumulation by the microalgal cell (Li et al. 2011).

The steps for obtaining biodiesel from microalgae culture are shown in Fig. 17.2. Triacylglycerols present in the oil extracted with the aid of solvents are cleaved in consecutive steps of reaction with methanol (methanolysis) in diglycerides and monoglycerides. Other short-chain alcohols, such as ethanol, may also be effective in this process. Acidic, basic and enzymatic catalysts or even supercritical conditions can be used, which results in increased process efficiency. In the final stages, fatty acid methyl esters (FAME) or fatty acid ethyl esters (FAEE) and glycerol (reaction byproduct) are obtained (Gong and Jiang 2011).

Solvents which are often used in the extractive process may not fully solubilize triacylglycerols, thus rendering the process inefficient and resulting in reduced oil extraction rate (Velasquez-Orta et al. 2013). In addition, biomass drying temperature can significantly interfere with oil recovery, since high temperatures promote the

oxidation of fatty acids (WIDJAJA et al. 2009). Therefore, adequate oil extraction techniques from microalgal cells are essential for good biodiesel yields.

17.2.4 Bioethanol Production from Microalgal Biomass

Bioethanol is a liquid fuel that can be produced with sugars extracted from vegetable raw material. They represent an attractive alternative to fossil fuels because they are obtained from renewable sources, thus the process of obtaining ethanol is considered a green technology. In addition, ethanol has higher octane rating, flammability limits and flame speed in comparison with gasoline, thence allowing high compression ratio and lower burning rate. The presence of oxygen in the molecular structure of ethanol improves combustion, thereby reducing particulate, hydrocarbon and carbon monoxide emissions (Balat et al. 2008).

According to Hernández et al. (2015) cell composition that is rich in lipids and proteins with low carbohydrate content, it is uninteresting to conduct research aimed at obtaining bioethanol from microalgae. In fact, obtaining ethanol from microalgal biomass has not been studied as widely as biodiesel production, although ethanol production from vegetable biomass has been increasing considerably over the years (Reyimu and Ozçimen 2017). Chen et al. (2013) classifies microalgae as a promising carbohydrate source, whose cell wall is rich in polysaccharides such as cellulose, hemicellulose, glycoproteins, pectin, agar and alginate, as well as the ability to accumulate starch. In this context, species with higher potential for carbohydrate accumulation should be used and culture supplementation should be evaluated (Dragone et al. 2011) to convert sugar into ethanol. Examples of promising microalgae species for such a purpose are listed in Table 17.4.

The steps for obtaining ethanol from microalgae are illustrated in Fig. 17.2. Structural and reserve polymeric carbohydrates must be broken down, and released monomers should be converted into ethanol by specific microorganisms. Although microalgae cell wall does not present lignin in its composition, the use of pre-treatments to disorganize its structure and expose structural and reserve sugars has been studied in order to increase ethanol yield (Chng et al. 2017). Pre-treatments have widely been used to reduce the recalcitrance of lignocellulosic materials for 2G biofuel production (Alvira et al. 2010). Chemical and physicochemical pre-treatments using acid, alkali, ozone or solvents, followed by enzymatic hydrolysis, have been reported in literature (Table 17.4). According to Keris-Sen and Gurol (2017) and Chng et al. (2017), Organosolv and Ozonolysis presented the highest sugar release rate when compared to other studied pre-treatments, thus obtaining higher ethanol production rate.

Microorganism fermentation for converting fermentable sugars into ethanol can be carried out by means of separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF). In SHF, the microorganism is added to the reaction medium that has been previously supplemented with necessary nutrients for its growth after carbohydrate hydrolysis. In this case, hydrolysis and fermenta-

tion are performed in two steps. The disadvantage of this process is the possibility of microorganism inhibition by the substrate and the long time required to obtain monomers and ethanol. In SSF, enzymes and the microorganism are incubated in the same reaction medium. As sugar monomers are released, the microorganism uses sugars as carbon source for obtaining energy. In this model, ethanol is produced while monomers are released simultaneously, and substrate inhibition is practically zero. Furthermore, total reaction time is reduced when compared to SHF (Chng et al. 2017; Ho et al. 2013). The most widely studied microorganisms that convert microalgae glucose into ethanol are *Saccharomyces cerevisiae* and *Zymomonas mobilis*, according to Table 17.5.

17.3 Pigment Production from Microalgal Biomass

Natural pigments can be produced by plants and microorganisms to be used in food, pharmaceutical and cosmetic industries. They are called natural dyes and exhibit greater sensitivity to light, temperature and oxide-reducing agents when compared to synthetic dyes. These factors contribute to a reduced use of natural pigments, thus microencapsulation techniques can be used in order to improve their stability (Özkan and Bilek 2014).

Among photosynthetic pigments produced by microalgae, chlorophylls (green), carotenoids (red, orange and yellow) and phycobilins (red and blue) are the three major classes (Begum et al. 2016). Their color is a result of a conjugate system of double bonds in the molecule structure. Electron excitation in these bonds is followed by the absorption of specific wavelengths. As a consequence, the light that is not absorbed by the molecule is reflected, thence coloring the cells (Mulders et al. 2014). For this reason, pigments are used as a criterion for microalgae classification (Serive et al. 2017).

Pigment synthesis by microalgae has been described by Mulders et al. (2014) and can be affected both by culture conditions (light, temperature, pH and salinity) (Begum et al. 2016) and by the culture medium supplementation. Examples of culture media used to obtain pigments from microalgae and supplementation are listed in Table 17.6. However, the extractive process may influence their recovery rate (Faraloni and Torzillo 2017; Ferreira and Sant'anna 2017).

17.3.1 Chlorophyll

Green microalgae have been extensively studied as raw material for biofuel production. These algae have high concentrations of chlorophyll, characterizing their coloration. Chlorophylls are essential for photosynthesis and are capable of absorbing light and converting it into chemical energy and its increased concentration in the culture medium is a sign of cellular proliferation (Benavente-Valdés et al. 2017). The

Table 17.5 Culture media, types of photobioreactors and cultures to obtain sugars and ethanol from different microalgae species

Microalgae strain	Culture media	Reactor	Culture condition	Culture type	Agitation	Process to obtain sugars	Fementation strain	Maximun ethanol yield	References
C. reinhardtii UTEX 90	TAP	PBR (2.5 L)	PA	Indoor	Ns	Liquefation and sacchaarification (α-amylase from *B. licheniforms* and amylosglucosidase from *Aspergillus niger*)	*S. cerevisiae* S288C	235 mg of ethanol was obtained from the hydrolyzed starch of 1.0 g algal biomass	Choi et al. (2010)
Chlorococum sp.	Author medium	Bag (100 mL)	PA	Outdoor	Bubbling	Cells subjected to supercritical lipid extraction	*S. cerevisiae*	3.83 g L^{-1} ethanol obtained from 10 g L^{-1} of lipid-extracted microalgae debris	Harun et al. (2010)
Chlorococum humicola	Author medium	Bag (100 mL)	PA	Outdoor	Ns	Enzymatic hydrolysis (cellulase from *Trichoderma reesei*)	*S. cerevisiae*	–	Harun and Danquah (2011a)
					Bubbling	Acid pre-treatment (H_2SO_4 1–10% (v/v))		7.20 g ethanol/L hydrolyzate	Harun and Danquah (2011b)
	Ns	Ns	Ns	Ns	Ns	Alkaline pre-treatment (NaOH0.75% (w/v))		26.1% g ethanol/g algae	Harun et al. (2011)
C. vulgaris FSP-E	Modified Basal medium	Glass vessel (1L)	PA	Indoor	Ns (300 rpm)	Enzymatic hydrolysis (hydrolytic enzymes from *Pseudomonas* sp. CL3)	*Z. mobilis*	79.9% (SHF) and 92.3% (SSF) theoretical yield	Ho et al. 2013
						Acid pretreatment (H_2SO_4 1–5% (v/v))		87.6% (SHF) theoretical yield	
Scenedesmus dimorphus UTEX 1237	Author medium	PBR (2 L)	PA	Indoor	Aeration	Organosolv pretreatmet	*S. cerevisiae* YSC2	>90% (SSF)	Chng et al. (2017)
						Acid pretreatment (H_2SO_4 4% (v/v))		80% of theoretical yield (SSF)	

(continued)

Table 17.5 (continued)

Microalgae strain	Culture media	Reactor	Culture condition	Culture type	Agitation	Process to obtain sugars	Fementation strain	Maximun ethanol yield	References
						Enzymatic hydrolysis		84% of theoretical yield (SSF)	
Scenedesmus obliquus CNW-N	Modified Detmer's Medium	PMMA-made tubular (60 L)	PA	Outdoor	Aeration (0.06 vvm)	Acid pretreatment (H_2SO_4 2% (v/v))	*Z. mobilis* ATCC 29191.	0.205 g ethanol/g biomass	Ho et al. (2017)
N. oculata	Municipal wastewater and seawater	Erlenmeyer flasks (250 mL)	PA	Indoor	Shaking incubator (150 rpm)	Alkaline pretreatment (NaOH 0.75% (w/v))	*S. cerevisiae*	Bioethanol yield of *N. oculata* and *T. suecica* ranged from 0.41% to 7.26%	Reyimu and Ozçimen (2017)
Tetraselmis suecica									
Mixed microalgal culture containing species from the *Chlorococcales* order of the *Chlorophyceae* class	BG11 medium	Glass tanks (20L)	PA	Indoor	Aeration (CO_2)	Ozonolysis (0.25–2 g O_3/g of dry weight biomass)	–	–	Keris-Sen and Gurol (2017)
						Ultrasound (ultrasonic energy intensity of 1.6 kWh/gram of biomass)			
						Alkaline (NaOH 0.75–2% (w/v))			
						Acid (H_2SO_4 concentrated)			

PBR photobiorreator; *PA* photoautotrophic; *PH* photoheterotrophic; *MT* mixotrophic; *Ns* not specified

Table 17.6 Influence of supplementation and culture conditions on pigment production from different microalgae species

Microalgae	Culture media	Reactor	Culture condition	Nutrient	Agitation	Composition/productivity	References
Chlamydomonas moewsii	Bristol modified	Pyrex glass bottles and	PA	Sodium sulphate	Aeration	Maximum production of chlorophyll a and chlorophyll b was observed at concentrations ranging between 0.1 and 3 mM of sodium sulfate. Concentrations above 5 mM exerted an inhibitory effect	Mera et al. (2016)
Trachydiscus minutus	Mineral media	Glass cylinders	PA	Ce^{3+}, Gd^{3+}, La^{3+}, Pr^{3+}, Sc^{3+}, Lu^{3+} and monazite.	Aeration CO_2 2% (v/v)	There was no significant influence of metals on pigment production	Goecke et al. (2017)
Parachlorella. kessleri						It was observed a marked decrease in the production of lutein (Lu^{3+} and PR^{3+}), chlorophyll a (Lu^{3+}, PR^{3+} and Mon), chlorophyll b (PR^{3+} and Mon) and β-carotene (Gd^{3+}, La^{3+}, Pr^{3+}, Sc^{3+}, Lu^{3+} and Mon) Ce^{3+}, Gd^{3+}, La^{3+} and Sc^{3+} have increased carotenoid and chlorophyll production; monazite increased violaxanthin production	
Porphyridium cruentum	F/2	Tubular PBR	PA	–	Bubble column and liquid circulation with the aid of a centrifugal pump	Chlorophyll a and carotenoid contents were positively affected by the drying temperature (170–190 °C) 415.88 μg/g of β-carotene and 1513.12 μg/g of chlorophyll were obtained when biomass was dried at 180 °C; higher recovery than drying at room temperature	Durmaz (2017)
C. vulgaris	Culture media for heterotrophic condition	Flat panel airlift	PA	–	Aeration	Cell growth and pigment production (carotenoids and chlorophyll) were observed in both conditions, although cell proliferation and subsequent pigment production were larger in the photoheterotrophic condition	Benavente-Valdés et al. (2017)
		Flat panel airlift and stirred tank	PH		Aeration and mechanical agitation		
		Flat panel airlift	MT		Aeration		

PBR photobiorreator; *PA* photoautotrophic; *PH* photoheterotrophic; *MT* mixotrophic

chlorophyll molecule consists of an aromatic ring, called chlorine, which contains 4 pyrrole rings surrounded by a magnesium ion. A hydrocarbon tail (phytol) can be found attached to chlorine (Mulders et al. 2014). According to Chen et al. (2010), there are 5 types of chlorophyll: *a*, *b*, *c*, *d* and *f*. Although these groups exhibit similar molecular structures, they have differences in their macrocyclic peripheral groups, thus causing their light absorption spectrum to be different.

Microalgae require favorable conditions for photoautotrophic growth, including light, water, inorganic carbon (CO_2), inorganic nitrogen (ammonia or nitrate) and phosphate. The availability of these nutrients significantly affects chlorophyll production by microalgal cells (Mulders et al. 2014). Reduced concentrations of nitrogen, sulfur, iron, magnesium and phosphorus or high concentrations of copper and zinc may reduce chlorophyll synthesis. In addition, reduced light supply limits the conversion of inorganic carbon into organic molecules, thereby limiting growth and energy uptake by cells (Ferreira and Sant'anna 2017; Mulders et al. 2014).

The process of chlorophyll extraction from microalgal cells can be observed in Fig. 17.3 and resembles lipid extraction due to the use of solvents and the need for cell disruption. The cold extraction process has been widely used, since pigments are sensitive to high temperatures. No reports of chlorophyll extraction were found in literature.

The first step for extracting pigments is cell disruption, where the dried cells are immersed in a polar solvent and the resulting suspension is incubated under ultrasound irradiation. Authors have reported the use of acetone (90–100%) (D'este et al. 2017; Mera et al. 2016; Van Heukelem and Thomas 2001), ethanol (Bertrand et al. 2002; Lv et al. 2017; Serive et al. 2017; Van Heukelem and Thomas 2001) and methanol (Goecke et al. 2017). It is recommended to use an ice bath to maintain low temperatures as a way to prevent the degradation of extractives. The extracted pigments can be quantified by spectrophotometry, using specific wavelength for each pigment (Durmaz 2017; Lv et al. 2017; Mera et al. 2016), fluorescence (Lv et al. 2017) or by high-performance liquid chromatography (HPLC) (D'este et al. 2017; Goecke et al. 2017; Serive et al. 2017; Van Heukelem and Thomas 2001).

17.4 Conclusion

Microalgal biomass represents an attractive alternative to oil for fuel obtaining once it is classified as a renewable feedstock. Different types of biofuels can be obtained from microalgal biomass in separate or co-generation process such as biodiesel, bioethanol and biohydrogen. Those biofuels are classified as green fuels due to the lower contribution to the greenhouse effect when compared to fossil fuels. Besides biofuels, microagal biomass is also a source of pigments, such as chlorophyll, which are value-added bioproducts and shows a wide range of uses in textile, pharmaceutical, cosmetics industries. In this way, microalgae are a promise feedstock to be processed is a biorefinery concept for energy generation and value-added products obtaining.

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Chapter 18
Potential Applications of Enzymes in Sericulture

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Abstract Sericulture is an important agro-industry, playing an important role in the rural and urban economy of several countries. Even though, sericulture meant for production of raw silk but entire process ends up with several by-products from chawki raring to post cocoon technology. Effective utilization of overall sericulture practice requires additional eco-friendly approaches such as application of enzymes for the better product yields. As enzymatic approaches are inexpensive and environment-friendly, there is an urgent need for more scientific studies to explore the potential applications of enzymatic technologies for improving silk production and enhanced utilization of sericulture by-products. Major sericulture by-products include mulberry straw, silkworm litter, spent pupae and degumming wastage. Some of these seri-by products are also a significant concern to the public as they cause environmental pollution. The alternative use of these by-products by enzymatic technologies needs to be developed and standardized for commercial exploitation, eventually adding-up the commercial value in sericulture. Moreover, as the applications of enzymes in sericulture is far less explored than enzymes used in agriculture and food processing technologies, more intensive studies regarding enzymatic applications for improved silk production, mulberry and silkworm waste utilization, spent pupae diversification and cocoon cooking processing needs to be undertaken. The application of enzymes in sericulture will help to strengthen and promote industry by enhancing productivity, creating additional income sources, saving resources like manpower, energy, chemicals and reducing pollution.

Keywords Enzymes · Mulberry · Sericulture · Silkworm · Sericulture by-products

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18.1 Introduction

The history of silk and its use had begun about 2000 B.C in China and afterwards it gradually spread to the other parts of the world. At present, several varieties of textile fabrics are available in the market, however silk continues to be the "queen of fabrics" due to its natural properties, availability and traditional use. In general, silks are classified as mulberry (produced from cocoons of *Bombyx mori* L. silkworm) and non-mulberry i.e. *tasar*, *eri*, and *muga*. Nearly, 90–95% of the global production of silk is of mulberry type and considered as important commercial silk produced in the world. It is well known that enzymes have various commercial applications in wide range of biobased products. However, enzymes application in sericulture have not been studied widely.

So far, mulberry plants and its silkworm are mainly exploited only for silk production. But sericulture waste or by-products being generated from major sericulture activities from silkworm raring to post cocoon technology can also be used for additional on-site income generation. These include rearing waste (mulberry shoot, silkworm larval litter), and reeling by-product (pupae and sericin protein). Appropriate technology adopted for utilization of by-products and its implementation at commercial scale is the adequate need in the sericulture industry to sustainably stand in long haul.

Utilization of biological process such as enzymes for optimum production of silk and utilization of seri-waste or by-products may give better value addition to it. With the advancement of enzyme technologies in sericulture, seri-waste such as rearing waste i.e. mulberry shoot and silkworm larval litter can be exploited for economic second generation (2G) sugars production which can be used potentially for bioethanol or commercially important biomolecules production following the concept of biorefinery. Similarly, with the help of enzymes digestibility and assimilation of mulberry leaf nutrients by silkworm larvae can be improved. In addition, these enzymes could be implemented for post cocoon technologies such as cocking and degumming process and major end products. Moreover, by products of sericulture such as spent pupae and sericin can also be exploited for the production of biopharmaceuticals such as essential fatty acids, chitosan, and novel nano gels and nano-particles. This chapter presents the details of enzymatic approaches for improving mulberry silk, silk industry by-products and mulberry feedstock (leaves and stem) for cellulosic sugars production which are the building blocks of biofuels and bio-chemicals production.

18.2 Enzymes in Mulberry Biomass Utilization

The sericulture involves the utilization of large quantity of mulberry biomass which is essentially lignocellulosic biomass comprised of plant cell wall constituents like cellulose, hemicellulose and lignin. It is estimated that one hectare of mulberry garden yields approximately about 30–35 tonnes of leaves and 12.1 tonnes of mulberry sticks

Table 18.1 Proximate chemical composition of mulberry feedstock (mulberry stem and leaves) (Lohan 1980; Eswara and Reddy 1992; Datta et al. 2002)

Cell wall component (% dry weight)	Mulberry dry stem	Mulberry leaf
Cellulose	50	20
Hemicellulose	20	10
Lignin	20	5
Protein	5	25
Mineral and other molecules	5	40

per annum (Datta 2002). Currently it is estimated that worldwide approximately 10 lakh hectares of mulberry are being cultivated in a year. It generates around 36 million tonnes of mulberry sticks as a by-product after shoot raring every year. Presently, these materials are being used for compost, house-hold application or biogas production. Application of mulberry biomass can be turned into the production of second generation sugars which are considered as building blocks for biofuels and biochemicals. As mulberry straw is mainly composed of cellulose (50%), hemicelluloses (20%), and lignin (20%) (Jorgensen et al. 2007) and the availability so it can be a good feedstock for economic cellulosic sugars production. Table 18.1 presents the cell wall composition of mulberry leaves and shoot. The cell wall chemical composition of mulberry feedstock may vary depending upon the species, cultivation conditions, climate change and several other factors (Majumdar et al. 1967a, b; Subba Rao et al. 1971; Lohan 1980; Eswara and Reddy 1992; Datta et al. 2002).

Enzymatic approaches and microbial contribution to the conversion of waste mulberry lignocellulosic biomass into bioethanol and biochemicals would be one of the most promising eco-friendly alternatives to fossil fuels or petroleum-based products (Balan et al. 2014; Chandel and Silveira 2017). Although almost all the current bioethanol is generated from edible sources (sugarcane juice, molasses and maize grains), use of mulberry lignocellulosic biomass may draw much attention in future as these materials not compete for edible sources.

In order to obtain the cellulosic and hemicellulosic sugars so called second generation sugars (2G sugars), chemical pretreatment plays key role in enhancing the subsequent enzymatic conversion of lignocellulosic biomass into monomeric sugars (Chandel and Silva 2013). Alkaline pretreatment being the most effective chemical method may results into delignification of mulberry residues which leads to breakage of ester bonds cross-linking lignin and xylan, and thus increases the porosity of biomass for subsequent enzyme activity (Sun and Cheng 2002). In case of acid pretreatment, acid concentration, particle size, temperature, reaction time and liquid-to-solid ratio are the major influencing factors affecting the overall process yield and productivity (Zhu and Pan 2010). Using H_2O_2 in delignification of biomass via oxidative reactions to fractionate and solubilize the lignin polymer causing the weakening of lignocellulosic matrix, eventually improving enzyme digestibility of cellulosic fraction of lignocellulosic biomass (Silverstein et al. 2007). As hemicelluloses are severely cross linked with lignin hence chemical pretreatment is necessary to

modify or remove lignin to some extent finally hemicelluloses fractionation, accessing the cellulases towards carbohydrates portion of cell wall (Zhu and Pan 2010). The key plant cell wall degrading enzymes include primarily cellulases, hemicellulases, laccases and pectinases efficiently depolymerizing carbohydrate fraction of lignocellulosic materials into second-generation sugars production (Chandel and Singh 2011). All these enzymes are also known as carbohydrate-active enzymes (CAZymes) and are classified into various families and sub-families of glycoside hydrolases (GHs), glycosyl transferases (GTs), polysaccharide lyases (PLs), carbohydrate esterases (CEs) and other enzymes having auxiliary activities (AAs) such as cellobiose dehydrogenase (CDH) and lytic polysaccharide monooxygenase (LPMO, formerly GH61), concomitantly acting on polysaccharide (Levasseur et al. 2013; Lombard et al. 2014). Additionally, there are other non-hydrolytic cellulose active proteins (NHCAPs) aiding hydrolysis of plant cell wall with low protein loadings (Ekwe et al. 2013).

Laccases are poly phenyl oxidases which are generally produced by fungi, more commonly by Ascomycetes, Deuteromycetes, and Basidiomycetes. Particularly, white rot fungi from basidiomycetes are more commonly involved in lignin metabolism eventually causing lignin degradation (Kunamneni et al. 2007). Laccases are group of enzymes which include manganese peroxidases (MnP), lignin peroxidases (LiPs) and hybrid enzymes known as versatile peroxidases (VPs) (Ohm et al. 2014). Release of optimum levels of cellulose and hemicelluloses from lignocellulose polymer requires laccase treatment as it causes lignin degradation (Madhavi and Lele 2009). Laccases obtained from white rot fungi are usually recovered by by solid state fermentation and could be used for treating mulberry shoot material under desired environmental conditions in order to remove lignin sustainably (Lopez et al 2010).

Degradation of hemicellulose fraction requires a greater diversity of multiple enzymes such as xylanases, xyloglucanases and beta-xylosidases, which are involved in degradation of the main hemicelluloses backbone into monomeric constituents (Chandel and Singh 2011). Additionally, other accessory enzymes such as 4-O-glucuronoyl methylesterases, arabinofuranosidases, alpha-galactosidases and acetylxylan esterases also have been recognized as key ancillary enzymes breaking hemicellulosic fraction in turn increasing amenability of cellulases to the cellulose fibers and thus yielding efficient amount of sugars in the reaction mixture (Chandel and Singh 2011).

Cellulase is an important enzyme in degrading cellulose fraction of mulberry lingocellulosic substrate into glucose. Some of the microorganisms that have been reported to hydrolyze this insoluble polymer into soluble monomeric glucose units by the cellulases action (Alam et al. 2009; Sehnem et al. 2006; Sohail et al. 2009). Mainly three enzymes are involved for the cellulolytic enzymes action. Endo-β-D-glucanase catalyzes the random hydrolysis of both soluble and insoluble cellulose polymer. Exo-β-D-glucanase releases cellobiose from reducing and non-reducing ends of cellulose. β-glucosidase then hydrolyse the cellobiose into glucose (Bhat 2000; Sohail et al. 2009; Chandel and Singh 2011) (Fig. 18.1; Table 18.2).

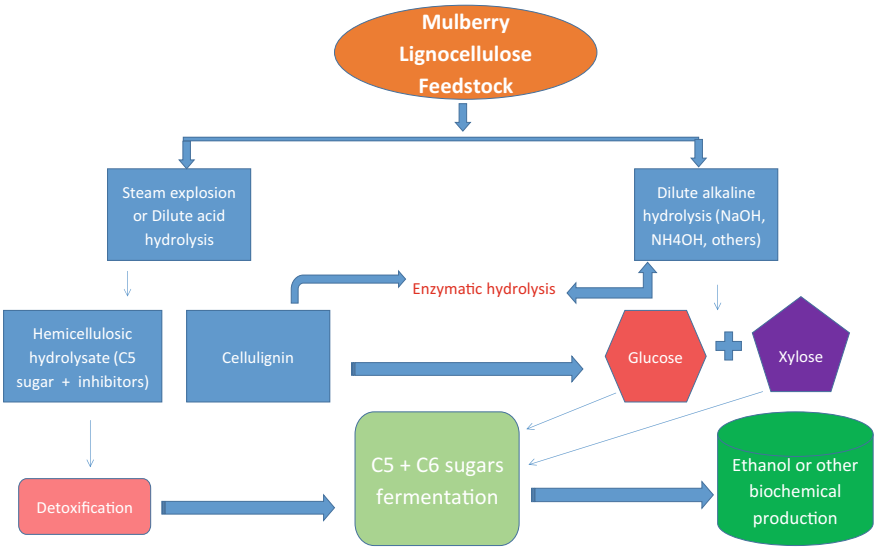

Fig. 18.1 General outline of the bioconversion of mulberry lignocellulosic feedstock (shoot or leaves) into bioethanol and bio-chemicals production

18.3 Enzymes in Silkworm Rearing

There are many factors (environmental and biological) that directly influence the successful silkworm rearing and production of quality silk. Enzymes are essential for the digestibility of mulberry leaves in silkworm larva, enhancing its activity influencing the growth, development and resistance to disease of silkworm. These factors subsequently affect the silkworm capacity to produce good quality cocoons and silk (Esaivani et al. 2014). The key digestive enzymes like cellulases, amylases, proteases help in silkworm breeding programme for improvement of cocoon characters and disease resistance. In recent years, supplements having various enzymes are successfully used to enhance prawn, dairy, poultry production etc. However, these supplements have not been tried in sericulture which can bring the significant changes in economic parameters, disease resistance potential in silkworm.

18.4 Application of Enzymes in Post Cocoon Technology

The natural silk filament secreted by the silkworm is composed of two fibroin filaments held together by an adhesive cementing layer called as silk gum and sericin. This is necessary to remove sericin (degumming) which cover on the silk fiber surface yielding final reelable silk threads. Presently, the conventional methods are used for the degumming of silk under alkaline conditions at a pH of 10–11 near

Table 18.2 Potential applications of enzymes in sericulture industry (Borah and Baruah 2009; Nakpathom et al. 2009)

Enzyme	Microbial producers	Function/applications
Laccases	Fungal species (*Trametes versicolor, Trametes villosa, Rhizoctonia praticola Pycnoporus cinnabarinus, Botrytis cinerea, and Myceliophthora thermophila* etc.)	i. Breakdown of Lignin by oxidation of polyphenols, methoxy-substituted monophenols, and aromatic amines
Cellulases	Bacterial and Fungal species (*Bacillus pumilus*, *Pseudomonas* sp, *Trichoderma reesei, Trichoderma harzianum*, Penicillium *echinulatum, Aspergillus niger* etc.)	i. Breakdown of cellulose ii. Suppliment in silkworm rearing for improving mulberry digestion iii. Mulberry biomass utilization
Xylanases, xyloglucanases, β-xylosidases	White and brown-rot fungal species, *Myceliophthora thermophila* etc.	i. Degradation of the hemicelluloses fraction into simple sugars ii. Utilization of mulberry biomass
Exo-β-D-glucanase, and β-glucosidase	Bacterial and fungal species	i. Release of cellobiose from reducing and non-reducing ends of cellulose, and hydrolysis of cellobiose to glucose ii. Mulberry biomass utilization
Amylases	Several Species of genus *Bacillus* and Aspergillus	i. Conversion of starch into glucose ii. Supplement in silkworm rearing for digestion of starch
Proteases	Bacterial and fungal species	i. Useful in cooking of silkworm cocoons and degumming of silk fibers/yarn ii. Supplement in silkworm feeding for mulberry assimilation
Lipases	*Aspergillus* and *Candida* species	i. Extraction of essential fatty acids from silkworm pupae oil ii. Removes waxes and fats in silk gum
Pectinases	Fungi	i. Removal of pectin in mulberry leaf and fruit juices for medicinal value
Lysozyme and Chitinases	Egg white, viruses, fungal and bacterial species	i. Act against silkworm bacterial and fungal pathogens ii. Chitin removal from spent pupae

boiling point. However, alkaline conditions have shown to be adverse effect on the silk fiber because silk has poor resistance to alkalinity. These adverse effects can be overcome by applying proteolytic enzymes for cooking and degumming process instead of chemical treatment. However, enzymes pose some practical problems in

this process due to its high costs and requires specific environmental conditions for optimum activity, as enzymes are large molecules do not penetrate properly into silk threads. These challenges require to be solved out for using proteases in post cocoon technological processing.

18.4.1 Enzymes in Cocoon Cooking

In nature, some insects produce enzymes for attacking silkworm coccon by breaking the sericin cross bridge of the silk strand for making a hole to eat pupae (Kafatos and Williams 1964). Proteases isolated from *Antheraea polyphemus*, *Antheraea pernyi* and *Antheraea mori* have been well studied and are found to be acts like trypsin (Hruska and Law 1970). Interestingly, Pandey et al. (2011) demonstrated the possible use of protease from *Antheraea mylitta* in cocoon-cooking. It was concluded that temperature around 35–40 °C and pH range in 8.5–9.0 is quite suitable for cooking of cocoons. Generally, Papain protease of papaya is commonly used for cooking the cocoons and maximum silk recovery was obtained. Use of proteases could be more beneficial in cocoon cooking than conventional boiling alone (Sinha et al. 1989; Borah and Baruah 2009). Plant protein- Bromelain extracted from pineapple is also being used for softening of the cocoons (Devi et al. 2011). Commercial preparations of several proteolytic enzymes like Anilozyme-P, Biopril-50, Trypsin and Pepsin have also been used for softening of the cocoons (Goel and Rao 2004). However, application of enzymes in cooking process is less frequent than enzymes used in degumming process (treatment of fiber/yarn) due to high cost of enzymes.

18.4.2 Enzymes in Degumming Process

Degumming is the silk refining process which includes removal of sericin (major portion), natural wax, some colouring components and minerals along with any other particles from silk fibre/yarn/fabric (Gulrajani 1992). Enzymes such as trypsin, papain, bacterial proteases and lipases are used for degumming process (Johnny et al. 2012). Devi et al. (2012) reported the use of plant protease- Bromelain in degumming showing positive impact on silk degumming process. Moreover, bacterial alkaline protease has also been found to be more effective than trypsin and papain for removal of sericin (Lee et al. 1986; Nalankilli 1992). More recently, fungal proteases have been standardized for degumming process and economically more viable (Thirupathaiah et al. unpublished work). Application of enzymes in cooking and degumming have several advantages over chemical methods, because of specificity and at the same time gives minimum damage to fibroin during cooking or degumming process. These proteases do not readily attack on silk fibroin because the protein chains in silk are densely packed without bulky side chains. It has a minimum risk by degumming than using alkaline soaps. Another advantage of enzymatic method is

the less consumption of energy, as silk fiber is treated at low temperature if enzymes are used and restoring fiber strength (Sonthisombat and Speakman 2004). Nevertheless, enzymes-based applications are considered to be eco-friendly process due to its biodegradability in nature.

18.4.3 Application of Enzymes in Utilization of Silkworm Reeling By-products

Silkworm spent pupa is a major byproduct of sericulture industry, obtained after removal of the silk from the cocoon. Presently, worldwide approximately 6,00,000 tons of the spent pupae is generated from the cocoons of the domesticated mulberry silkworm alone (Savithri et al. 2016). This spent pupa is being used for several commercial applications includes cooking oils, snacks, paints, animal feeds, varnishes, soaps, candles, plastic, biofuels, fertilizers, and chitosan production (Suresh et al. 2012; Trivedy and Murthy 2008). However, the utilization of silkworm spent pupal oil can be exploited for the commercial production of essential fatty acids, biodiesel and several other high value-based products eventually adding the value in sericulture industry by applying enzyme technologies.

18.5 Conclusions

Applications of enzymes offer several specific advantages over the conventional mechanical and chemical process in all stages of sericulture industry. Sericulture industry primarily have following steps: mulberry cultivation, silkworm rearing and post cocoon technology. As mulberry feedstock is basic raw material in sericulture, so use of mulberry lignocelluse into biofuels and biochemicals production bring the value-addition in sericulture. Plant cell wall degrading enzymes (cellulases, xylanases, laccases and other auxiliary enzymes) have been studied in detail exploring sugarcane lignocellulose residues, corn stover and others feedstock. However, mulberry waste biomass has not been explored rationally for ethanol or biochemicals production. Looking at the sizeable generation of mulberry feedstock, harnessing of this feedstock could play a pivotal role in sustainable bio-economy. The enzymatic hydrolysis can be applied to convert entire mulberry biomass into renewable sugars production which could be economic base material for bioethanol and biochemical production. There is scope for application of enzymes like amylase, cellulose, proteases, and lipases in silkworm rearing directly or as microbial formulations to produce enzymes for silkworm growth, development and economic characters of silk. Novel and ELISA based enzyme assay can be used for disease monitoring in mulberry and silkworm. Enzymes can be applied as environmentally friendly alternatives to chemical process used in post cocoon technology of cocking

and degumming process. Several alkaline, acidic, and neutral proteases have been used in degumming process for removing sericin for improving silk surface quality. In future, several potential applications of enzyme technologies may arise for better sericulture practice and its by-product utilization for commercial exploitation.

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Nature offers abundant *renewable* resources that can be used to replace fossil fuels but issues of cost, technology readiness levels, and compatibility with existing distribution networks remain. Cellulosic ethanol and biodiesel are the most immediately obvious target fuels, with hydrogen, methane and butanol as other potentially viable products. This book continue to bridge the technology gap and focus on critical aspects of lignocellulosic biomolecules and the respective mechanisms regulating their bioconversion to liquid fuels into energy and value-added products of industrial significance. This book is a collection of research reports and reviews elucidating several broad-ranging areas of progress and challenges in the utilization of sustainable resources of renewable energy, especially in biofuels. This book comes just at a time when government and industries are accelerating their efforts in the exploration of alternative energy resources, with expectations of the establishment of long-term sustainable alternatives to petroleum-based liquid fuels. Apart from liquid fuel this book also emphasizes the use of sustainable resources for value-added products, which may help in revitalizing the biotechnology industry at a broader scale. This book intends to design for scientists involved in the basic and advance biofuel research, biotechnology and pharmaceutical industries. This book also provides a comprehensive review of basic literature and advance research methodologies to graduate students studying environmental microbiology and microbial biotechnology.

O. V. Singh and A. K. Chandel (eds.), *Sustainable Biotechnology- Enzymatic Resources of Renewable Energy*, https://doi.org/10.1007/978-3-319-95480-6

Author Index

A
A. Arun, 399
A. K. Lavanya, 83
Agapi Dima, 307
Akhilesh K. Singh, 57
Akhilesh Kumar Singh, 355
Alessandra Verardi, 117
Anamika Sharma, 83
Anuj K. Chandel, 1, 57, 463
Anurup Adak, 83
Aravindan Rajendran, 423
Argyro Bekatorou, 307
Athanasios A. Koutinas, 307
Avinash P. Ingle, 289

B
Bijender Kumar Bajaj, 5
Bilqeesa Bhat, 5
Bo Hu, 423
Boopalan Thulasinathan, 399
Bruna C. M. Gonçalves, 439

C
Catia Giovanna Lopresto, 117
Cecilia Nicoletti, 117
Cristiano E. Rodrigues Reis, 423
Cristiano Reis, 247

D
D. Vasanth, 205
Daniela V. Cortez, 247
Debolina Mukherjee, 117

G
Gregorio Rafael-Galindo, 325

H
Heizir F. De Castro, 247
Heizir F. de Castro, 423

I
Inty Omar Hernández-De Lira, 325

J
Júlio César dos Santos, 155
Janmejai Kumar Srivastava, 355

K
Konstantina Boura, 307

L
Lata Nain, 83
Latika Bhatia, 57
Laxuman Sharma, 355
Lilia E. Montañez-Hernández, 325

M
Mahak Gupta, 5
Mahendra Rai, 289
María de Lourdes Froto Madariaga, 325
Marcos Moacir de Souza Junior, 155
Maria Kanellaki, 307
Messias B. Silva, 423, 439
Mohammad Israil Ansari, 355
Muhammad Ajaz Ahmed, 155

N
Nagamani Balagurusamy, 325
Nirupama Mallick, 355
Nivedita Sharma, 219

O. V. Singh and A. K. Chandel (eds.), *Sustainable Biotechnology- Enzymatic Resources of Renewable Energy*, https://doi.org/10.1007/978-3-319-95480-6

O
Om V. Singh, 1, 57
Özgür Seydibeyolu, 399

P
Panagiotis Kandylis, 307
Parushi Nargotra, 5
Paulo R. F. Marcelino, 155
Poonam Sharma, 219
Pornkamol Unrean, 177
Priti Paralikar, 289

R
Ruly Terán Hilares, 155

S
Satbir Singh, 5
Shikha Sharma, 5
Silvio S. da Silva, 155
Silvio Silverio da Silva, 289
Stefano Curcio, 117
Sudhakar Muniyasamy, 399
Sudip Chakraborty, 117
Surbhi Vaid, 5
Surender Singh, 83

V
V. Sivaprasad, 463
Victor H. Perez, 247
Vikas Sharma, 205
Vincenza Calabro, 117
Vishal Sharma, 5

Y
Yeruva Thirupathaiah, 463