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Keikhosro Karimi *Editor*

Lignocellulose-Based Bioproducts

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Preface

Lignocelluloses are widely available and nonfood-based materials considered for the replacement of a major part of fossil-derived fuels and chemicals in the future. They have a high potential for the production of variety of products. However, lignocelluloses are naturally designed to protect plants against physical and biological attacks. Thus, their component separation as well as chemical and biological conversions is challenging and not easily possible. During the last decades, worldwide research resulted in the understanding of their characteristics, composition, components distribution, and linkage between different parts. Recently, processes, environmental issues, economy, policies, and challenges are the subject of a large number of studies.

Nowadays, uncountable studies are conducted on the production of valuable chemicals and biochemicals from lignocelluloses. Dominant constituents of lignocelluloses are cellulose, hemicellulose, and lignin. Once cellulose is separated it can be used for the production of a large number of products including pulp and paper, textiles, cellulose derivatives, and nanocellulose. Hemicelluloses can be used for the production of films, coatings, and hydrogels. Xylitol can also be produced from hemicellulosic xylose by both chemical and biological methods. Lignin has the potential for production of different valuable products including fuels, adhesives, dispersants, aromatics, carbon fiber, fillers, resins, and activated carbon.

Fermentable sugar mixtures can be produced by chemical or enzymatic hydrolysis from cellulose and hemicellulose. Biofuel production from lignocellulosic hydrolysates is among the most attractive routes. Production of bioethanol, biobutanol, bioacetone, and biodiesel not only reduces the need for fossil fuels but also controls environmental pollutions. Moreover, organic acids, polysaccharides, microbial biomass, and single-cell proteins and oils are other valuable products. Furthermore, without hydrolysis, it is possible to produce biohydrogen and biomethane from lignocellulose. Moreover, a large number of valuable materials, e.g., furfural, hydroxymethyl furfural, formic acid, levulinic acid, and syngas, can be produced from lignocelluloses by chemical conversions.

This book is aimed at researchers and engineers who need theoretical basis for technical knowledge of using lignocelluloses in different biofuels and chemicals. It is particularly prepared for teaching biofuel courses at graduate level and for those interested in conducting research on this topic.

I hope you enjoy reading this book.

October 2014 Keikhosro Karimi

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Contents

Chapter 1
Introduction to Lignocellulose-based Products

Akram Zamani

Abstract Lignocellulosic materials are naturally designed composites that play crucial roles in the survival of plants. Considering their broad availability, there is a general agreement on the replacement of a major part of fossil-derived fuels and chemicals with lignocellulosic products. Additionally, some products are solely prepared from lignocelluloses and have no fossil-based equivalent. Several chemical, enzymatic, and thermal treatments are available to fractionate the lignocelluloses to their constituents, i.e., cellulose, hemicellulose, and lignin. Once separated, they are employed for the production of a wide variety of products. Pulp and paper, textiles, and nanocellulose are produced directly from cellulose. Cellulose may undergo chemical modifications to prepare cellulose derivatives. Hemicelluloses are used as a source of films, coatings, and hydrogels. Cellulose and hemicelluloses are hydrolyzed to sugars. Biofuel production from lignocellulosic sugars is among the most attractive routes. The consumption of ethanol, biobutanol, biohydrogen, biomethane, and biodiesel not only reduces the need to fossil fuels but also controls the environmental pollutions resulted by fossil fuels. Organic acids, polysaccharides, microbial biomass, and single cell proteins are other valuable products of fermentation of lignocellulosic sugars. Moreover, these sugars can be chemically converted to some other important chemicals, i.e., furfural, hydroxymethyl furfural, and levulinic acid. Xylitol is produced from hemicellulosic xylose by both chemical and biological methods. Lignin has a high potential for the production of several products including fuels, aromatics, carbon fiber, activated carbon, fillers, adhesives, resins, and dispersants. This chapter presents a brief overview of importance, applications, and production processes of different lignocellulosic products.

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1.1 Introduction

Amazing micro- and macro-structure of the plants cell wall, i.e., lignocellulosic materials, is the key factor for plants survival. During the growth of plant cells, multilayer cell wall is formed around their plasma membrane which protects the plant cells against mechanical, chemical, and microbial stresses (Taherzadeh and Jeihanipour 2012). Lignocellulosic materials are mainly composed of cellulose (35–50 %), hemicellulose (15–35 %), and lignin (10–35 %). The concentration of the mentioned components varies with plant types. For example, higher concentrations of lignin are found in higher plants, whereas lower lignin is present in annual plants (Azadi et al. 2013). Cellulose, hemicellulose, and lignin have very close interactions with each other in the cell wall. This results in a unique three-dimensional structure of cell wall. The presence of a high number of OH groups on the cellulose chain, polymer of glucose, makes this biopolymer susceptible for formation of strong hydrogen bonds. This ends with formation of high crystalline cellulose micro-fibrils in plant cell wall. Cellulose micro-fibrils are surrounded by hemicellulose (a heteropolymer of five and six carbon sugars) and lignin (a complex polymer of phenolic compounds). The high crystallinity of cellulose is crucial for exhibition of a high mechanical strength by plant cell wall. Lignin and hemicellulose protect the cellulose against microbial and chemical attacks. Moreover, the hydrophobic nature of lignin shields plant cells against moisture loss and death due to drying. The lignocellulosic biomass, the direct product of photosynthetic reactions by plants, is indeed the most abundant renewable material in the world (Karimi et al. 2013; Taherzadeh and Jeihanipour 2012; Xu et al. 2013).

Besides the critical function of lignocellulosic materials in plants life, they have a potential to play a significant role in development of modern sustainable societies as they have been introduced as a widely available feedstock for the production of energy and chemicals. Annually, several million tons of lignocellulosic materials are produced in the form of forest trees, grasses, and agricultural residues (Skog and Stanturf 2011). Dependence of human life to lignocellulosic materials goes back to several thousand years ago where human used wood as a source of energy (by direct burning) as well as construction materials. By discovery of fossil fuels, especially petroleum, the consumption of lignocellulosic materials as a source of energy was declined namely in the developed countries. However, limited sources of fossil fuels as well as serious environmental concerns arise from the unlimited consumption of these sources have returned the attentions to lignocellulosic materials in recent years. Indeed, lignocellulosic materials will be one of the most important sources of energy and materials in the future (Ma et al. 2012). Thanks to clever design and build up of lignocellulosic materials in the nature, we have access to huge amounts of these materials with low costs. Nowadays, the strategic goal in several developed and underdeveloped countries is to have a sustainable society by employing lignocellulosic materials as a source of material and energy.

There are two perspectives for application of lignocellulosic materials in the human life:

- Direct consumption.
- Conversion to secondary value-added products.

High-quality wood is still a major component used as a construction material for houses and furniture in many countries (Cambria and Pierangeli 2012). Getting the benefit of high nutritional values, many of agricultural residues are nowadays directly used as animal feed (Walker and Kohler 1981). Additionally, lignocellulosic biomass is still used for heat generation through the direct combustion in several rural areas. However, what can have a significant impact on the reduction of environmental concerns is indirect application of these materials as feedstock for the production of energy careers and chemicals. In this indirect route, chemical, thermal, and biological approaches are employed to convert lignocellulosic materials to several value-added products (Menon and Rao 2012). In this road, physicochemical properties of individual constituents of lignocellulosic materials are considered for creation of new materials. For example, high-strength fibers and textiles are produced from cellulosic fraction of lignocellulosic materials. For such applications, the component with the desired properties (cellulose in this example) should be purified from its complex in lignocellulosic materials and used for different applications. On the other hand, the decomposition of different components of lignocellulosic materials has opened up several opportunities for the production of new chemicals. Biofuels and several other chemicals are some examples which are categorized in this group. In an ideal biorefinery, the goal is complete fractionation of lignocellulosic biomass to several value-added products (Garver and Liu 2014).

In this chapter, an overview of different products which can be obtained from cellulose, hemicellulose, and lignin through biological and chemical routes is presented (Fig. 1.1).

1.2 Products Derived from Cellulose and Hemicellulose

Polysaccharides, i.e., cellulose and hemicellulose, make up 45–80 % of lignocellulosic biomass (Azadi et al. 2013). Taking into account the enormous amounts of existing low-cost lignocellulosic materials, e.g., forest residues, agricultural wastes, and a major part of municipal solid wastes, considerable amounts of these polysaccharides are annually produced which can be employed for preparation of several products. Cellulose- and hemicellulose-based products can be categorized into two groups. The first group refers to the products which are directly obtained from these biopolymers. Paper, textiles, cellulose derivatives, packaging films, and superabsorbent materials are a few examples of such products. In contrast, the process of production of second groups of products goes through a hydrolysis step in which polysaccharides are converted to their building blocks, i.e., simple six and

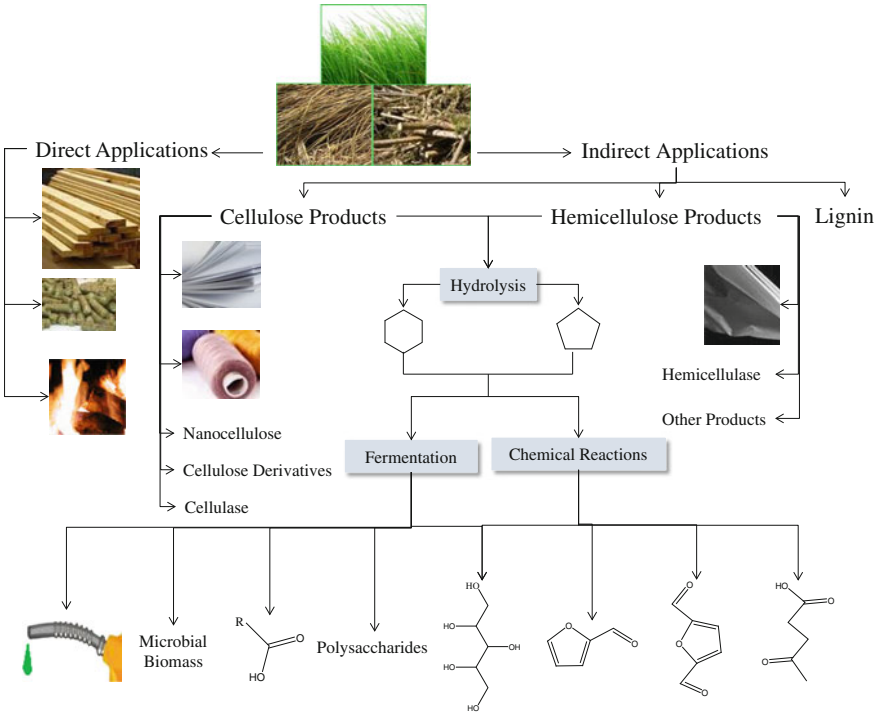

Fig. 1.1 Different products obtained from lignocellulosic materials

five carbon sugars. Sugars and all of their derivatives belong to this group. Different thermal, chemical, and biological approaches are employed for conversion of lignocellulosic sugars to final products (Rødsrud et al. 2012; French et al. 2000).

1.2.1 Products Directly Obtained from Cellulose

1.2.1.1 Pulp and Paper

Indeed, pulp and paper are the most well-known products obtained from lignocelluloses. Although before 1800s cotton and linen rags were the only resources for paper production, woody biomass is nowadays the dominant source (90 % of total production). The process of paper production from wood chips has seven major steps of pulping, washing, beating, blending with additives, fiber mat formation, water drainage, and drying (Shmulsky and Jones 2011).

The pulping is referred to mechanical, thermal, or chemical processes in which cellulose fibers are separated from their complex mixture in lignocellulosic materials. The progress of lignin and hemicellulose removal from the cellulosic fibers

(pulp) is the key factor determining the quality of the final product. The obtained pulp is then washed to remove the impurities. Due to presence of low amounts of lignin in the produced pulp, the pulp color is brown. The unbleached pulp is a suitable raw material for applications where color is not important, e.g., for paper bag production. However, for writing and book papers, the pulp should be bleached to remove or lighten the remaining lignin. After the polishing step, to enhance the strength of the paper, the cellulose micro-fibrils are mechanically flattened and disentangled. This step is called beating or refining. Afterward, the pulp is mixed with different additives such as starch and clays to increase the bond strength and brightness, respectively. Finally, a thin mat is formed using the pulp. The pulp slurry is poured onto a horizontal screen, the water drains away and the mat is remaining on the screen. The mat is finally dried and used for different applications (Hocking 2005; Shmulsky and Jones 2011; Young et al. 2003). The whole process of paper production from woody materials is illustrated in Fig. 1.2.

It should be mentioned that during the pulping process, lignin and hemicelluloses are mainly separated leaving a product with high cellulose purity. The separated lignin and hemicelluloses can subsequently be used for different purposes as discussed in the coming sections.

1.2.1.2 Fibers and Textiles

Cellulosic fibers typically contribute to over 40 % of different textiles used for clothing, home furnishing, and different industrial applications. Cotton, , and are the most important cellulosic textiles in terms of production volume. Although the

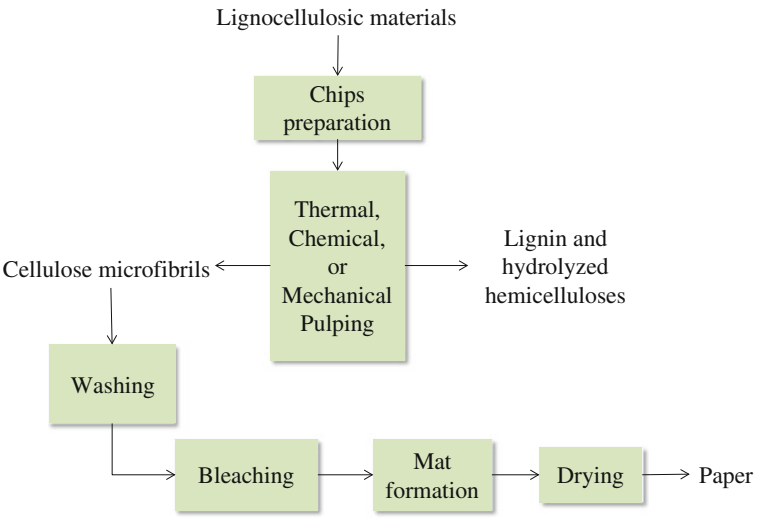

Fig. 1.2 The overview of paper production process from lignocellulosic materials

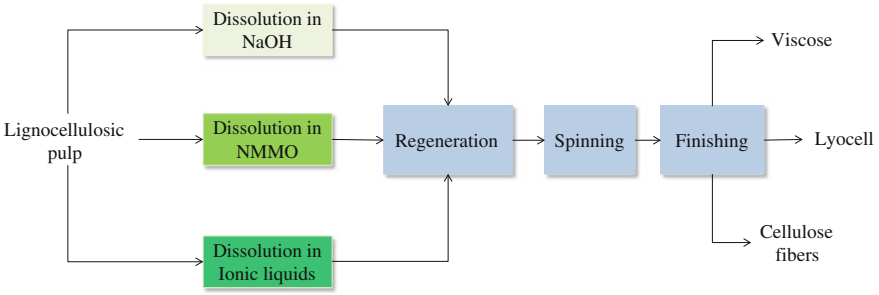

Fig. 1.3 Production of cellulosic fibers from lignocellulosic materials

seeds of gossypium plant are the only recourses for cotton production, viscose and lyocell textiles can be produced from lignocellulosic materials. The latter textiles are produced via a process including dissolution of cellulosic fraction of lignocellulosic pulp in a solvent, regeneration of cellulose, spinning of fibers, and finishing. The solvents used for preparation of viscose and lyocell are sodium hydroxide solution and N-Methyl morpholine-N-Oxide (NMMO), respectively (Mather and Wardman 2010; Perepelkin 2007; Wilkes 2001). Recently, application of ionic liquids as more efficient solvents compared to NaOH and NMMO has been proposed for production of cellulose fibers (Moniruzzaman and Ono 2012). A schematic of cellulosic fiber production process from lignocellulosic pulp is presented in Fig. 1.3.

1.2.1.3 Nanocellulose

Besides extensive application of cellulose for the production of paper and textiles for a very long time, some new aspects of application of cellulosic fraction of lignocellulosic materials, in nano-scale, have been recently proposed. Getting the benefit of inherent properties of cellulose, e.g., high hydrophilic nature and potential for chemical modifications together with unique properties of materials at nano-scale, namely extremely large surface areas, nanocellulose exhibits outstanding sole characteristics. Nanocellulose has been produced from pure cellulose as well as the cellulose available in wood and agricultural residues (Dufresne 2008).

The process of production of nanocellulose from lignocellulosic materials usually includes two major steps of cellulose purification and nanocellulose formation. In the purification step, a process similar to pulping is performed to remove lignin and hemicellulose from cellulose. Accordingly, lignin and hemicellulose removal is accompanied using chemical or enzymatic reagents together with mechanical treatment. The product of this step is subjected to a bleaching process to remove the remaining lignin and produce a purified cellulose pulp. This is then subjected to a process called fibrillation where cellulosic fibers are transferred to nanocellulose. Originally, fibrillation was performed through extensive mechanical share to

disintegrate the large cellulose fibers and liberate cellulose nanofibers. However, this is nowadays performed in commercial scale by passing of cellulose–water suspensions through mechanical homogenizers under very high pressure drops. Besides homogenization, cryocrushing can also be employed for fibrillation. In this method, the cellulose slurry is quickly frozen in liquid nitrogen and the frozen sample is subjected to very high share forces. The presence of ice crystals exert extremely high pressures and result in cellulose rupture and nanocellulose liberation. Additionally, some modified types of grinders have been used for cellulose fibrillation in which the cellulose suspension is placed between two grind stones (one static and one rotating) and nanocellulose is formed (Dufresne 2008; Eichhorn et al. 2010; Klemm et al. 2011; Rebouillat and Pla 2013; Siró and Plackett 2010).

All types of mechanical forces for nanocellulose generation suffer from the bottleneck of very high energy consumption (higher than 25,000 kWh/ton product). This negatively affects the economy of nanocellulose production. Therefore, recently pretreatment steps have been suggested to reduce the energy consumption during the fibrillation. The pretreatment of cellulose with acid (HCl or H_2SO_4) or cellulosic enzymes considerably reduce the energy consumption in subsequent mechanical fibrillation process (Klemm et al. 2011). Figure 1.4 represents the major steps performed during the nanocellulose formation process.

Although there is not a long time since nanocellulose has been introduced, different applications are known for this material today. Several features such as very high tensile strength and stiffness, high viscosity, film and foam forming properties, very high barrier characteristics, possible surface modifications, hydrophobicity, and high biocompatibility of nanocellulose have made this biomaterial an ideal choice for different purposes. Most commonly, nanocellulose is used as reinforcement in nanocomposites. In biomedical industry, nanocellulose is used in wound dressing, artificial skins, implants, bandages, face masks, artificial blood vessels, supports for drug delivery, cell and enzyme carriers, and cosmetic tissues. In food industry, it is widely used as thickeners, flavor carriers, and suspensions stabilizer. In other industries, nanocellulose can be used in different forms such as foams, membranes, filters, coatings, adhesives, packaging and sealing materials, superabsorbent, and nonwoven materials (Dufresne 2008; Eichhorn et al. 2010; Klemm et al. 2011; Rebouillat and Pla 2013; Siró and Plackett 2010).

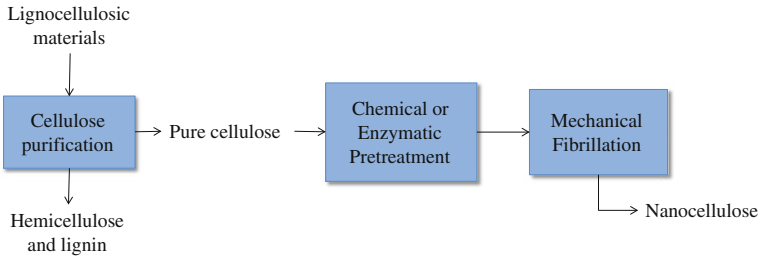

Fig. 1.4 The production of nanocellulose from lignocellulosic materials

1.2.1.4 Cellulose Derivatives

Today, besides direct application of cellulose, different derivatives of this biopolymer are also used in various sectors. Presence of three hydroxyl groups on each building block of cellulose chain creates a high potential for chemical modification of cellulose. Cellulose derivatives are commonly produced through esterification or etherification reactions (Majewicz and Podlas 2000). These reactions are traditionally performed in heterogeneous phase. However, due to difficulties in control of the reactions at heterogeneous conditions, many studies have been dedicated to perform the cellulose modification reactions in a homogenous phase where cellulose is dissolved in an appropriate solvent and the reaction takes place in the solution (Blanchi et al. 1997).

Among the wide variety of cellulose derivatives, cellulose acetate, carboxymethyl cellulose, cellulose nitrate, methyl cellulose, and hydroxyethyl cellulose are the most important ones in terms of production volume. Cellulose derivatives have versatile applications in preparation of coatings, films, membranes, and absorbents. Moreover, they are employed as additives to enhance the characteristics of food, pharmaceuticals, and cosmetics (Majewicz and Podlas 2000; Bidgoli et al. 2014; Barba et al. 2002; Toğrul and Arslan 2003).

1.2.2 Products Obtained Directly from Hemicelluloses

Although cellulosic-based products are commercially used for a very long time, application of hemicellulosic polymers for different purposes has recently got a high attention. One reason for the latter discovery of hemicellulosic characteristics and its wide application may be extensive degradation of hemicelluloses during the course of most cellulose purification processes. For example, when lignocellulosic materials are subjected to pulping process, the strong chemical environment leads to considerable hydrolysis of hemicelluloses to sugars and their degradation products (Spiridon and Popa 2008). Therefore, hemicellulosic polymers are not recovered in nearly all industrial pulping and fiber formation processes.

However, in order to get the benefit of the whole polysaccharide fraction of lignocellulosic materials, i.e., hallocellulose, hemicelluloses should be recovered prior to the harsh treatments. Strong interaction of hemicelluloses with cellulose and lignin renders the easy liberation of hemicelluloses during the extraction processes. Several approaches have been proposed for effective recovery of hemicelluloses. Most commonly, alkali treatments are used for effective extraction of hemicelluloses (Peng et al. 2012; Spiridon and Popa 2008). However, the extracted hemicelluloses are usually contaminated with significant amounts of lignin. On the other hand, hemicelluloses are considerably deacetylated during the course of alkali extraction. Employment of alkali peroxide pretreatment significantly enhances the purity of extracted hemicellulose with respect to lignin contamination (Peng et al. 2012; Spiridon and Popa 2008). Organic solvent treatment, especially treatment

with dimethyl sulfoxide, not only effectively extracts the hemicelluloses but also does not alter the structure of these polymers (Hansen and Plackett 2008; Peng et al. 2012). Mechanical treatments, including ultrasonication and extrusion, and thermal treatments including microwave irradiation enhance the performance of hemicellulose chemical extraction process (Sedlmeyer 2011; Peng et al. 2012). Although hemicelluloses are nowadays commercially extracted and used for different purposes, there is still active research aiming at improvement of the hemicelluloses extraction process to achieve a material with desired properties.

Nowadays, different applications of hemicelluloses are known in food, medical, and cosmetic industries. Emulsifying, stabilizing, and binding properties are among the important characteristics of hemicelluloses in different applications (Spiridon and Popa 2008). Additionally, hemicelluloses have shown promising potential in the enhancement of immune system in human body (Peng et al. 2012). Moreover, hemicelluloses exhibit excellent film-forming properties. Currently, hemicelluloses are commercially applied as packaging films and coatings for food products. However, researches are still conduced to improve the oxygen and water vapor barrier properties of the films and coatings (Hansen and Plackett 2008). The production of hydrogels from hemicelluloses in combination with other biopolymers such as chitosan is another recently proposed use for hemicelluloses. The obtained hydrogels have a high potential to be used in biomedical applications, e.g., drug delivery systems (Hansen and Plackett 2008; Sedlmeyer 2011).

Because of relatively short-term research on hemicellulose-based products compared to that of cellulose, new routes may be investigated in the future suggesting new aspects for application of hemicellulosic polymers.

1.2.3 Lignocellulosic Enzymes

In the nature, degradation of lignocellulosic materials is performed through the simultaneous action of several different enzymes specifically cellulases, hemicellulases, and ligninases. Cellulases are usually referred to a group of enzymes that act synergically on crystalline cellulose and hydrolyze it to glucose. Moreover, hemicelluloses catalyze the hydrolysis of hemicelluloses to their building blocks, i.e., five and six carbon sugars. In contrast, ligninase promotes depolymerization of the complex structure of lignin (Martins et al. 2011). Interestingly, several applications for these enzymes have been recognized in different industries. Among different lignocellulosic enzymes, cellulases are the most important group such that the need for these enzymes shares 8 % of the total demand for different enzymes in the world (Acharya and Chaudhary 2012).

Microbial cellulases are nowadays broadly used in food, textile, and paper processing (Acharya and Chaudhary 2012; Martins et al. 2011). A new application for cellulases has recently got increasing attentions in parallel to the growth of second generation of biofuels from lignocellulosic materials. The enzymatic hydrolysis of cellulose by cellulases is one of the most important and challenging

steps in production of biofuels, e.g., ethanol, from lignocellulosic materials. Nowadays, one of the major bottlenecks of lignocellulosic ethanol production is high cost of cellulases. Therefore, there is an increasing demand for production of cellulases with lower costs and higher activities (Hahn-Hägerdal et al. 2006; Percival Zhang et al. 2006; Quinlan et al. 2010).

Pure cellulose as well as lignocellulosic materials can be employed as a substrate for the production of cellulases. Although several fungal and bacterial strains are able to produce different levels of cellulases, nowadays, cellulases are industrially produced using fungal strains specifically *Trichoderma* spp (Fig. 1.5). This is because of the high cellulases production ability of fungi and less complexity and easier recovery of the fungal cellulases compared to the bacterial ones (Acharya and Chaudhary 2012; Martins et al. 2011; Quinlan et al. 2010; Quiroz-Castañeda et al. 2011).

The Danish company, Novozymes, as the largest enzyme producer, has succeeded to significantly reduce the cellulases cost and enhance its activity in recent years (Acharya and Chaudhary 2012; Quinlan et al. 2010). However, the enzyme production industry still needs to pave the way to achieve lower prices and higher activities to meet the requirements for lignocellulosic ethanol production.

The production of cellulases by fungi can be performed both in solid state and submerged fermentations. Although the submerged fermentation is industrially used for production of cellulases, solid-state fermentation is expected to be a competitive method getting benefit of a number of advantages including lower working volumes, higher productivities, higher concentrations, and easier recovery and purification of the enzymes (Acharya and Chaudhary 2012; Martins et al. 2011).

Although the fungal cellulases are nowadays industrially dominant, bacterial cellulases have been the subject of several researches in recent years. Compared to

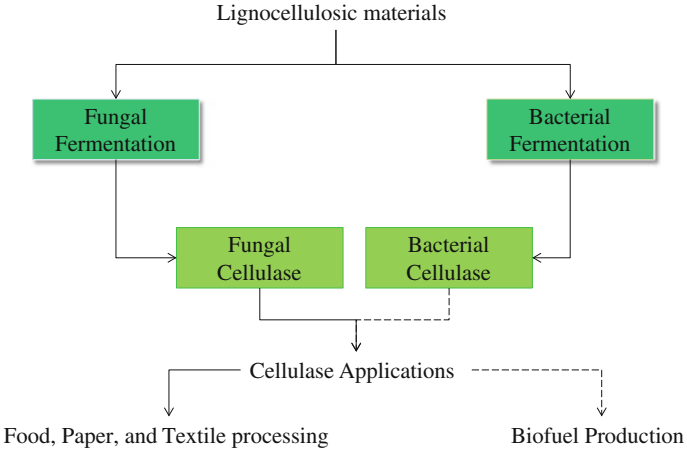

Fig. 1.5 Production of microbial cellulases and their current and future applications

fungi, bacteria generally have a higher growth rate. Moreover, especial strains of cellulase-producing bacteria are enormously resistant to environmental stresses and can withstand extreme conditions of temperature and pH. More complex structure of bacterial cellulose may result in increased synergy and effectiveness (Sadhu and Maiti 2013). Even though promising results have been achieved in this area, there is still a long way until bacterial cellulases get enough commercial impacts (Fig. 1.5).

1.2.4 Products Obtained Through the Hydrolysis of Cellulose and Hemicellulose

Although cellulose and hemicellulose are directly employed for the preparation of different polymer-based products, a wider range of products are obtained through hydrolysis of these polysaccharides. Once cellulose and hemicellulose are cleaved to their building blocks, i.e., simple sugars, they can either be used directly or converted to a broad spectrum of secondary products. Conversion of sugars to the end products can be performed through chemical, thermal, and biochemical methods.

1.2.4.1 Lignocellulosic Sugars

Glucose is the sole building block of cellulose, while hemicelluloses are composed of different five and six carbon sugars (e.g., xylose, mannose, and glucose). These sugars are released in hydrolysis reactions. Generally, hemicelluloses are much more susceptible to different hydrolytic agents compared to cellulose. For example, because of hydrolysis of hemicelluloses during the pulping process, usually 15–22 % of total sugars are available in the liquor of pulping processes where cellulose remains almost unchanged (Taherzadeh et al. 2003). This sugar mixture can be employed for the cultivation of different microorganisms and biosynthesis of different fermentation products (Helle et al. 2008). However, unlike hemicelluloses, the cellulosic fraction of lignocelluloses cannot be easily hydrolyzed (Karimi et al. 2013).

Very high crystallinity along with high protection by lignin and hemicelluloses hinder the chemical and enzymatic hydrolysis of cellulose. Therefore, for the efficient production of glucose from cellulose, a pretreatment step is needed prior to enzymatic or chemical hydrolysis. Up to now, several physical, chemical, and biological treatments have been proposed for enhancement of cellulose saccharification. Although there is a general agreement that pretreatment is a key step toward profitable production of some strategic fermentation products from lignocelluloses, e.g., ethanol, however, up to now, no unique pretreatment is available, which is optimized for all types of lignocellulosic materials (Karimi et al. 2013; Menon and Rao 2012; Taherzadeh and Jeihanipour 2012; Taherzadeh and Karimi 2008b; Xu et al. 2013). Different leading pretreatment techniques and their performance are discussed in detail in Chap. 3 of this book.

At ideal conditions, pretreatment removes lignin and hemicellulose from cellulose and reduces its crystallinity. The ready to digest pretreated cellulose can be either subjected to chemical (e.g., by acid) or enzymatic hydrolysis to liberate glucose. The enzymatic hydrolysis of cellulose is usually ended with a much higher conversion yield compared to chemical hydrolysis, e.g., by acid (Taherzadeh and Karimi 2007a, b). However, high price of enzyme is a major limiting factor. Details of different aspects of enzymatic hydrolysis are thoroughly discussed in Chap. 4.

The sugars obtained from lignocellulosic materials can be potentially recovered, purified, and used as pure sugars; however, since the purification is difficult, they are usually converted to other products through chemical and biological reactions. This is probably due to the fact that the production of edible sugars from other sources such as sugar beet, sugar cane, and starch is much easier and safer than production from lignocellulosic materials (Sjöman et al. 2008; Aguedo et al. 2013). Therefore, lignocellulosic sugars are usually converted to other value-added products.

It is worth mentioning that due to the recalcitrance of lignocellulosic materials and high costs of pretreatment and enzymatic hydrolysis steps; there is still a long way until these processes become widely commercial (Gnansounou and Dauriat 2010; Klein-Marcuschamer et al. 2010).

1.2.4.2 Products of Lignocellulosic Sugars Obtained by Biological Reactions

Numerous biological products can be obtained in fermentation processes where microorganisms consume sugars as carbon and energy sources. Therefore, lignocellulosic materials can be potentially employed as low cost and widely available substrates for production of a very broad range of bio-products. Some of the most important examples of these products are biofuels, organic acids, microbial biomass, proteins, and polysaccharides. A brief overview of production process as well as applications of these materials is discussed in this section.

Biofuels

Currently biofuels are the most strategic products which can be obtained from lignocellulosic materials. The limited resources for fossil fuels as well as irreparable negative side effects of these fuels on the environment dictate the necessity of biofuels. Production of biofuels from lignocellulosic materials is referred to as the second generation of biofuels which was proposed as a solution for food–energy conflict resulted by the first generation, i.e., the production of biofuels from edible sugars and starches (Zhu and Zhuang 2012; Menon and Rao 2012).

Annually, considerable amounts of lignocellulosic materials are produced which are regarded as waste materials and have no especial applications. Agricultural residues such as rice and wheat straws are examples of these waste materials. These materials can be employed as a low-cost feedstock for the production of biofuels

(Hahn-Hägerdal et al. 2006; Taherzadeh and Karimi 2008b). Different aspects of biofuel production from waste materials are presented in Chap. 2.

The overall process for the production of biofuels from lignocellulosic materials consists of three major steps, i.e., the pretreatment of feedstock, saccharification of polysaccharides through enzymatic, chemical, or microbial routes, and fermentation of sugars to biofuels by microorganisms (Fig. 1.6) (Karimi et al. 2013). The most well-known biofuels are bioethanol, biomethane, biobutanol, and biohydrogen.

Saccharomyces cerevisiae and *Mucor indicus* are among the best microorganisms for ethanol production. The former is nowadays the industrial ethanol producer (only from hexoses), while the later has recently been introduced as an alternative which assimilate both hexoses and pentoses and produce ethanol with high yields (Karimi and Zamani 2013). Indeed, ethanol is the most important biological product in terms of the volume of production. Although there are a high number of large scale ethanol production companies in the world and this product is extensively used in the transportation section, research about production of this valuable biofuel from low-cost feedstock, i.e., lignocellulosic waste materials, is still a very hot topic (Taherzadeh and Karimi 2008a). Different aspects of ethanol production from lignocellulosic materials are thoroughly discussed in Chap. 5 of this book.

Colestridium acetobutilicum is the famous microorganism that ferments sugars to a mixture containing acetone, butanol, and ethanol with approximate ratio (w/w) of 3, 6, and 1 (Ni and Sun 2009). Butanol can be used as a biofuel which has a higher heating value and miscibility with gasoline in car engines than ethanol. Similar to ethanol, industrial butanol production processes were originally developed based on sugar and starch feedstocks. However, the employment of lignocellulosic materials is expected to improve the economy of butanol production process (Tashiro et al. 2013). The production of butanol from lignocellulosic materials is the subject of Chap. 8.

For both ethanol and butanol, lignocellulosic materials can be either hydrolyzed by chemical or enzymatic catalysts to obtain fermentable sugars. Then, the appropriate microorganism converts the sugars to the biofuels. The process of biomethane production from lignocellulosic materials is different from that of ethanol and butanol production. A consortium of several hundreds of microorganisms works together to convert lignocelluloses to this biofuel. The first group of these microorganisms hydrolyzes the polysaccharides. After the hydrolysis reactions, three groups of microorganisms subsequently perform acidogenesis, acetogenesis, and

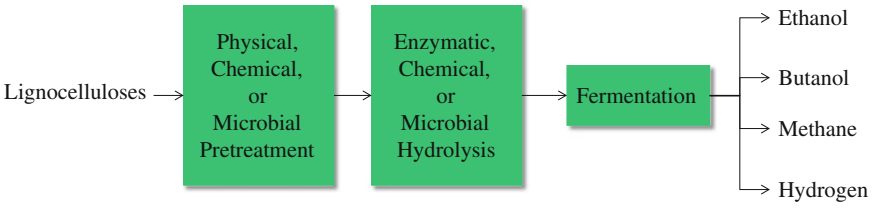

Fig. 1.6 The production of biofuels from lignocellulosic materials

methanogenesis reactions. The final product is a mixture of methane and carbon dioxide which is called biogas (Taherzadeh and Jeihanipour 2012). Biogas production from lignocellulosic materials is described in detail in Chap. 6.

Hydrogen can also be produced from lignocellulosic materials via a process comparable to that of biogas. Hydrogen is produced in a process called dark fermentation. In this process, anaerobic microbial consortiums hydrolyze the feedstock and convert it to hydrogen (Sigurbjornsdottir and Orlygsson 2012; Brynjarsdottir et al. 2013; Lo et al. 2011). Details of this process are presented in Chap. 7.

Although ethanol, butanol, methane, and hydrogen can be obtained as the final products of fermentation processes, biodiesel, the other commercially important biofuel, can also be produced from lignocellulosic materials but not through the pathway shown in Fig. 1.6. Because of limited amounts and high price of oil resources, especially edible oils, the conventional biodiesel production process from plant oils currently has faced crucial problems. Therefore, the production of oils by microorganisms, single cell oils, has got growing attentions. Among different possible feedstocks for single cell oil production, lignocellulosic sugars have been highly recommended because of low cost and wide availability (Zeng et al. 2013). Different oleaginous microorganisms can be employed for single cell oil production. Several yeast and fungi have been reported to store considerable amounts of lipids in their body during their growth phase. For example, *Trichosporon fermentans*, *Rhodotorula glutinis*, and *M. indicus* have been reported to contain 40, 29, and 24 % lipids in their biomass (Karimi and Zamani 2013; Yousuf 2012). Oil production is performed through the aerobic cultivation of oleaginous fungi. Microbial oil can be extracted by mechanical forces or appropriate solvents. The obtained microbial oil should be subjected to a process called transesterification where the oil molecules (triglycerides) are reacted with methanol in the presence of a catalyst which is usually a base (e.g., NaOH and KOH) or acid, and consequently methyl esters (biodiesel) and glycerol are formed (Fig. 1.7). The obtained biodiesel can be used as an alternative to fossil diesel in the car engines (Galafassi et al. 2012; Yousuf 2012).

Process of biodiesel production from lignocellulosic materials still paves the preliminary steps toward industrialization. Costly pretreatment and hydrolysis processes are among the bottlenecks of this process.

Microbial Biomass and Proteins

Lignocellulosic hydrolysates have also been employed for cultivation of different microorganisms, and the obtained microbial biomass has been used for different purposes, e.g., as a source of animal feed. Different fungal strains such as *Rhizopus oryzae*, *Rhizomucor pusillus*, and *M. indicus* have been successfully cultivated on lignocellulosic hydrolysates and spent sulfite liquor. The obtained biomass has shown superior characteristics for being used as a fish feed (Taherzadeh et al. 2003;

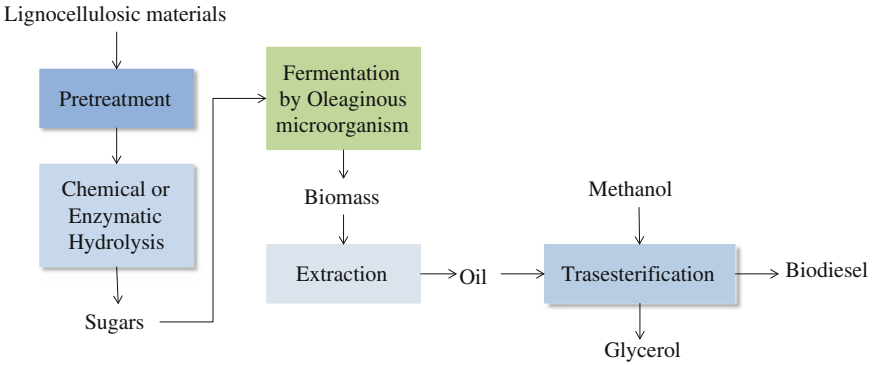

Fig. 1.7 Production of biodiesel from lignocellulosic materials

Lennartsson et al. 2009, 2011; Ferreira et al. 2013). Moreover, the obtained fungal biomass is shown to have a high potential for heavy metal removal from wastewaters (Karimi and Zamani 2013; Rouhollahi et al. 2014).

Integration of the first and second generation of bioethanol, where zygomycetes and ascomycetes fungi are cultivated on a mixture of lignocellulosic hydrolysates and the residue of ethanol production process (from starch) has been recently proposed. The employment of the fungi not only enhances the ethanol production yield but also improves the quality of distiller's dried grains and soluble (DDGS), the byproduct of ethanol process, for being used as an animal feed (Lennartsson et al. 2014).

Proteins comprise around 50 % of the microbial biomass. Lignocellulosic sugars can be fermented to prepare single cell proteins, which have very high nutritional values (Ferreira et al. 2013; Chandel et al. 2012).

Organic Acids

Nowadays organic acids allocate a big part of the market of biological products. Different microorganisms produce various organic acids such as citric, lactic, gluconic, itaconic, kojic, succinic, and acetic acids during their growth phase. Like any other fermentation product, the employment of vastly abundant and low-cost feedstock such as lignocellulosic materials may significantly enhance the large scale production of organic acids.

Citric acid

Citric acid is one of the most important organic acids in terms of production volume. This versatile acid has numerous applications especially in food, pharmaceutical, and cosmetic industries. Biological processes for the production of citric acid are generally more favorable than chemical routes, and today 99 % of

food grade citric acid is obtained via fermentation processes. *Aspergilus niger* is the most applied industrial strain for citric acid production (Max et al. 2010).

Nowadays, the commercial substrates for citric acid production are sugars and starchy materials which usually have high costs. Moreover, because of the growing demand for this acid in different industries, its annual production is continuously rising. Therefore, lignocellulosic materials have been considered as an alternative feedstock for citric acid production. Different strains of *A. niger* usually do not display a high potential for hydrolysis of polysaccharides present in lignocellulosic materials. As a result, similar to biofuels, the production of citric acid should be carried out through pretreatment, enzymatic hydrolysis, and fermentation steps (Singh Dhillon et al. 2011).

Co-cultivation of a cellulase-producing microorganism such as *Trichoderma reesei* and *A. terreus* together with a citric acid-producing microorganism such as *A. niger* and *Yarrowia lipolytica* has been suggested for direct conversion of lignocellulosic materials to citric acid. In this process, the cellulosic fraction of the substrate stimulated the cellulase production. Then, the obtained cellulase hydrolyzes the cellulose to glucose which is finally converted to citric through the fermentation (Liu et al. 2014).

The employment of some especial strains of *A. niger* with cellulase synthesis ability, in the presence of a co-substrate such as sucrose and an alcoholic stimulator such as methanol, is another proposed approach for direct production of citric acid from lignocellulosic materials (Bari et al. 2009). Genetic modification of a more conventional *A. niger* strain to achieve a strain with high ability for cellulose hydrolysis has also been performed for production of citric acid from pure cellulose (Watanapokasin et al. 2007).

Moreover, the employment of lignocellulosic materials for citric acid production in solid-state fermentation systems has recently received high attentions. In such systems which are more favorable for citric acid production in operational and economical points of view, lignocellulosic materials are not only used as a substrate but also as a support to avoid wash out the acid-producing microorganism (Bari et al. 2009; Grewal and Kalra 1995; Khosravi-Darani and Zoghi 2008).

Despite considerable research and promising results, the production of citric acid from lignocellulosic materials has not still come to the commercial market.

Lactic acid

Lactic acid is another organic acid which is broadly used in food, cosmetics, pharmaceutical, leather, textile, and other chemical industries. The production of polylactic acid, as a promising substitute for synthetic plastics, is a new application which has increased the need for this acid.

Nowadays, around 90 and 10 % of lactic acid production is performed through biological and chemical routes, respectively. The chemical method has a limited potential for expansion, and therefore increased demand for lactic acid should be supplied through fermentation processes. Higher production volumes dictate the need for a low cost and widely available feedstock for the replacement of current sugar and starchy substrates. Similar to citric acid, lignocellulosic materials have

been considered as suitable alternative substrates for lactic acid production (Hofvendahl and Hahn–Hägerdal 2000).

Different strains of lactic acid bacteria as well as *Rhizopus* fungi are among the well-known microorganisms for lactic acid production (Hofvendahl and Hahn–Hägerdal 2000; Zhang et al. 2007). The production of lactic acid from lignocellulosic materials can be considerably improved if pentose-fermenting microorganisms are employed. Unfortunately, many strains of lactic acid bacteria are not able to ferment pentose sugars, a major building block of hemicellulose. To overcome this limitation, screening of lactic acid bacteria as well as genetic modification of the available strains has been performed (Ou et al. 2011; Hofvendahl and Hahn–Hägerdal 2000). *Rhizopus* fungi have the ability for fermentation of both pentoses and hexoses to lactic acid. Additionally, they have some other advantages such as lower nutrient requirement and more valuable biomass compared to the bacteria (John et al. 2009; Zhang et al. 2007).

The process of lactic acid production from lignocelluloses comprises three main steps of pretreatment, hydrolysis, and fermentation. Although this process still has not came to the industrial scale, several researches have been performed aiming at improvement of the economy of this process. Performing the hydrolysis and fermentation processes in one single stage (simultaneous saccharification and fermentation) (John et al. 2009), employment of membrane bioreactor to carry out continuous fermentation and product purification by electrodialysis (Neureiter et al. 2004), and employment of thermo-tolerant and genetically modified bacterial strains with high lactic acid productivity (Ou et al. 2011) are among the attempts for enhancement of the yield of lactic acid production.

Succinic acid

Succinic acid, another important organic acid, is the precursor of several chemicals used in food, chemical, and pharmaceutical industries. Several specialty chemicals (e.g., detergents, surfactants, chelators, and corrosion inhibitors), additives (e.g., pharmaceutical intermediates, food ingredients, and flavor additives), and commodity chemicals (e.g., adipic acid, fumaric acid, and esters) are nowadays produced from succinic acid (Akhtar et al. 2014; Borges and Pereira 2011; Li et al. 2011; Liu et al. 2013).

Although succinic acid is produced through a petrochemical process, nowadays there is increasing attentions for biological production of this acid where sugar and carbon dioxide are employed as raw materials. Using lignocellulosic substrates as a feedstock has been proposed for improvement of profitability of the biological production of succinic acid. Separate as well as simultaneous saccharification and fermentation of lignocellulosic materials has been successfully evaluated for the production of this acid while the latter is more preferred because of lower enzyme consumption and higher succinic acid productivity (Akhtar et al. 2014).

There are several succinic acid-producing microorganisms which are able to ferment glucose. However, there are limited strains that can consume several carbon sources including hexoses and pentoses. *Actinobacillus succinogenes* is among those microorganisms (Borges and Pereira 2011). Generally, xylose uptake by

succinic acid producer is delayed due to the presence of glucose. Therefore, a recombinant strain of *Eschercia coli* has been developed which can simultaneously ferment both glucose and xylose available in the hydrolysate of hemicelluloses (Akhtar et al. 2014; Borges and Pereira 2011; Liu et al. 2013).

Similar to citric, lactic, and succinic acids, lignocellulosic materials have also been recommended as alternative raw materials for biological production of other acids such as acetic, gluconic, itaconic, and kojic acids. A general overview of organic acid production from lignocellulosic materials is presented in Fig. 1.8.

Polysaccharides

Polysaccharides are the other group of products can be obtained from lignocellulosic sugars through in vivo biological reactions. A general outline of the process for production of these biopolymers is shown in Fig. 1.9. Chitosan, xanthan, bacterial cellulose, and microbial polyesters are among the most important polysaccharides which can potentially be produced from lignocellulosic materials.

Chitosan

Chitosan is one of the cationic biopolymers with superior characteristics such as antimicrobial activity, metal-binding capacity, and gel- and film-forming characteristics. This biopolymer has a number of current and potential applications namely

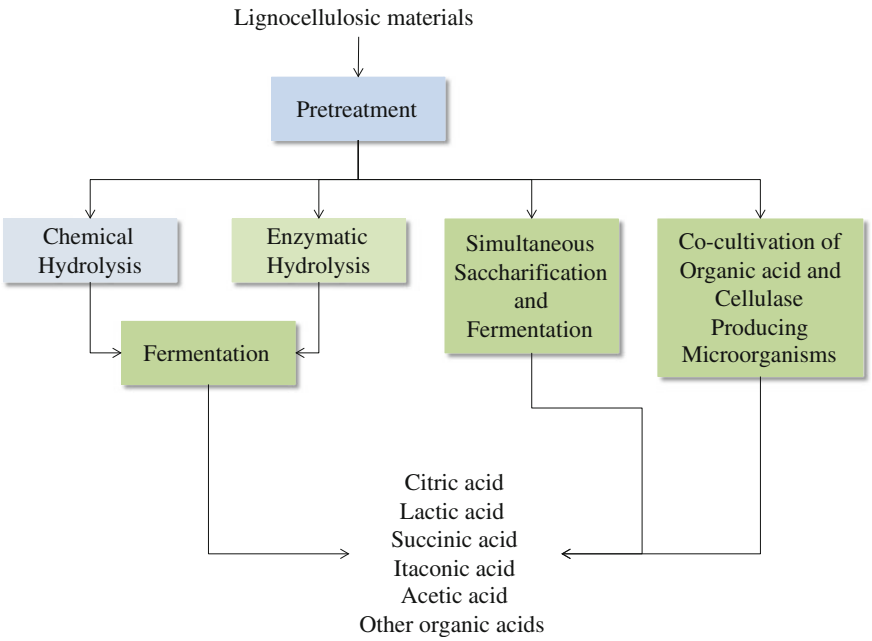

Fig. 1.8 Outline of organic acid production from lignocellulosic materials

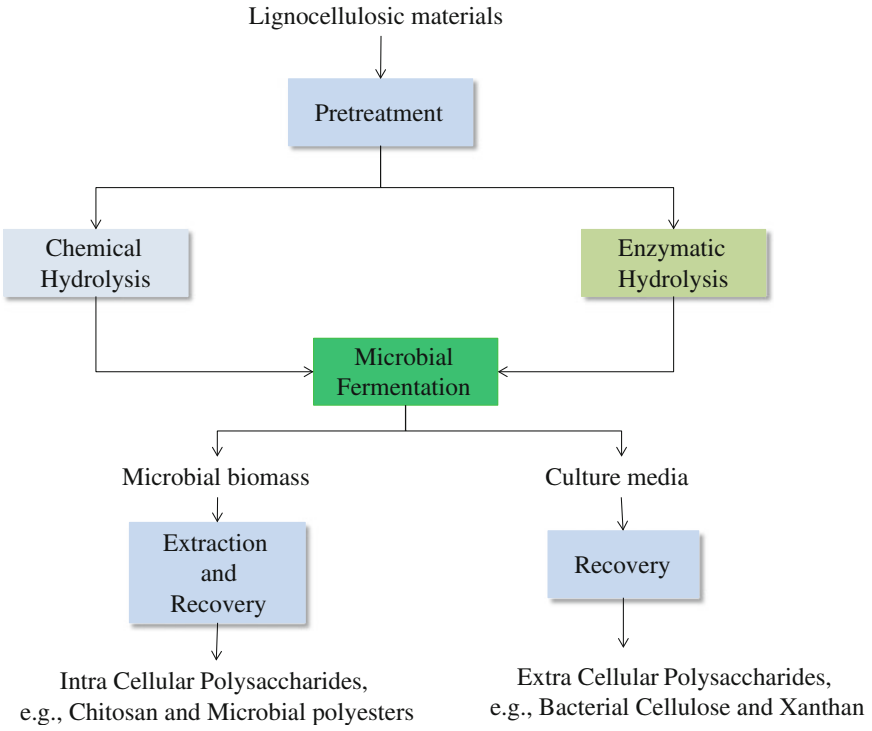

Fig. 1.9 Production of polysaccharides from lignocellulosic materials

in food, medical, cosmetic, agricultural, paper, and water treatment industries (Zamani 2010).

Nowadays, chitosan is commercially produced from shellfish wastes through chemical deacetylation. However, due to environmental drawbacks of the existing process, attentions have been paid to the production of this polysaccharide by fermentation processes. Chitosan biosynthesis takes place in a family of fungi called zygomycetes. Chitosan is produced during the fungal growth and stored in cell wall. Zygomycetes fungi are usually able to consume different five and six carbon sugars. Therefore, hydrolysates of lignocellulosic materials seem to be a suitable low-cost feedstock for chitosan production by fermentation (Karimi and Zamani 2013; Zamani 2010; Zamani et al. 2010; Millati et al. 2005; Asachi et al. 2011).

Interestingly, some strains of these fungi which store considerable amounts of chitosan in their cell wall have been recognized as efficient ethanol producers with comparable yield and productivity to that of industrial *S. cerevisiae*. *M. indicus*, *Mucor hiemalis*, and *R. oryzae* are examples of these strains (Heidary Vinche et al. 2012; Karimi et al. 2005, 2006; Lennartsson et al. 2009; Millati et al. 2005, 2008). Therefore, current researches about the production of ethanol from lignocellulosic materials by zygomycetes fungi are directly connected to chitosan production.

In other words, using zygomycetes fungi, all lignocellulosic sugars are efficiently consumed and ethanol with high yield is obtained as the main product, while chitosan is produced as a valuable byproduct. This method has been introduced as one of the promising routes for industrialization of the process of lignocellulosic ethanol (Karimi and Zamani 2013). So far, bench- and pilot-scale studies have resulted in hopeful results (Lennartsson et al. 2011); though much more is needed to be done before industrialization. Once chitosan is synthesized in the fungal cells, appropriate extraction process should be employed for its extraction and recovery (Zamani et al. 2010; Naghdi et al. 2014).

Xanthan

Xanthan is another broadly used biopolymer which can be produced from lignocellulosic sugars. Unlike chitosan, xanthan is an extracellular product which is commercially produced by *xanthomonas* bacteria. Indeed, the process of production of this exopolysaccharide was one of the earliest industrial biological processes. Because of unique rheological characteristics, xanthan gum has several applications in food, cosmetic, petroleum, and paper industries (Palaniraj and Jayaraman 2011).

Some attempts for improvement of the economy of xanthan production process are performed through the searching for alternative substrates with lower cost and higher availability. Accordingly, lignocellulosic hydrolysates have been considered as a suitable feedstock for xanthan production. However, the rate of xylose uptake by the famous xanthan producer, i.e., *Xanthomonas campestris*, is not as high as that of glucose. It has been reported that xanthan produced from acid hydrolysates of lignocelluloses has higher stability toward changes of temperature, pH, and ionic strength compared to xanthan produced from glucose. Xanthan is directly dissolved in the culture medium after biosynthesis and therefore can be recovered by precipitation (Gunasekar et al. 2014; López et al. 2004; Woiciechowski et al. 2004; Zhang and Chen 2010).

Bacterial cellulose

The third biopolymer which can be produced from lignocellulosic materials is bacterial cellulose. The employment of plant cellulose and hemicelluloses to produce bacterial cellulose has recently got significant attentions. Compared to plant cellulose, bacterial cellulose exhibits several unique features such as nano-structure and higher purity, crystallinity, degree of polymerization, water binding capacity, tensile strength, and biocompatibility. Getting benefit of these features, new applications are continuously proposed for bacterial cellulose in food, pharmaceutical, cosmetic, paper, biomedical, electronic, paint, coating, and membrane industries. However, novel applications can be commercialized only if bacterial cellulose is produced with high volume and low costs (Huang et al. 2014).

The product of sugar fermentation by *Acetobacter xylinum* is bacterial cellulose which is formed as an insoluble layer above the liquid cultivation medium of the bacterium. Costs of sugars have a considerable effect on the total cost of bacterial cellulose production. Therefore, using lignocellulosic sugars, obtained by chemical or enzymatic hydrolysis, considerably improve the economy of the process. Recovery of bacterial cellulose is easier than that of chitosan and xanthan since this

biopolymer is already formed as an insoluble layer which is easily collected and washed to remove the bacterial cells, e.g., by alkali solutions (Cavka et al. 2013; Chen et al. 2013; Guo et al. 2013; Hong and Qiu 2008; Hong et al. 2011).

Microbial polyesters

Microbial polyesters, classified as polyhydroxyalkanoates, are other big group of biopolymers that are produced by fermentation processes. Low concentrations of some nutrients together with excess amounts of carbon source lead to the formation of intracellular granules of microbial polyesters by several bacterial strains. There is a very high potential for the replacement of synthetic plastics by polyhydroxyalkanoates if these biopolymers can be broadly produced with reasonable prices (Pan et al. 2012).

Fortunately, many of polyester-producing microorganisms consume xylose even better than glucose, and therefore lignocellulosic hydrolysates seem to be ideal substrates for production of these biopolymers. The production of microbial polyesters from lignocellulosic materials is usually performed in two steps of hydrolysis (chemical or enzymatic) and fermentation. Recombinant strains of *E. coli* have been also developed with the ability to hydrolyze hemicelluloses (namely xylan) and convert the obtained xylose to microbial polyesters. After fermentation, polyesters are separated and purified by extraction from microbial biomass using suitable solvents (Lee and Na 2013; Zhang et al. 2013; Salamanca-Cardona et al. 2014).

Xylitol

Xylitol is a five-carbon sugar alcohol which has got growing attentions in food and pharmaceutical industries. The most important characteristics of xylitol are strong sweetness with lower calorie and higher tolerance by diabetic people compared to sucrose (Parajó et al. 1998).

Although, the present industrial production of xylitol is performed through the chemical hydrogenation of the dominant hemicellulosic sugar, i.e., xylose, high production costs limit the growth of applications of this sugar. In contrast, biological conversion of xylose to xylitol has the potential to be performed at high volumes. In this method, microorganisms directly uptake the xylose from lignocellulosic hydrolysates, and therefore the costly xylose purification step, that is needed in the chemical process, is omitted. Several bacteria, filamentous fungi, and yeasts can perform the biotransformation of xylose to xylitol. Different strategies such as entrapment of xylitol-producing microorganisms, cell recycling, fed batch cultivation, and using metabolically engineered cells have been suggested for improvement of the yield and hence the economy of xylose production by fermentation (Carvalho et al. 2005; Chandel et al. 2012; Cheng et al. 2010; Granström et al. 2007; Sjöman et al. 2008; Su et al. 2013; Wang et al. 2013).

Up to now, the chemical conversion of xylose is still used for production of xylitol; however, recently, some research-based companies try to commercialize the biotechnological production method. Once xylitol is produced by fermentation, it

can be recovered by crystallization from lignocellulosic hydrolysates (Martínez et al. 2007).

1.2.4.3 Products of Lignocellulosic Sugars Obtained by Chemical Reactions

Although the biological conversion of sugars to value-added products is generally preferred especially because of lower environmental impacts, some important chemicals are nowadays obtained by chemical conversion of lignocellulosic sugars. A brief overview of the process for the production of these materials is described in this section. Some of lignocellulosic-based products are prepared in the course of dehydration of sugars under acidic conditions, usually at high temperatures. Furfural, hydroxymethyl furfural, formic acid, and levulinic acid are among the most important examples of these products (Fig. 1.10) (Peng et al. 2011).

Furfural

Furfural is a colorless oily organic liquid with almond odor which turns to yellow color upon air exposure. A wide range of applications are industrially known for furfural including solvents (e.g., for petroleum refining) and precursors for production of different chemicals (e.g., furfuryl alcohol and tetrahydrofuran) (Peng et al. 2011).

Nowadays, furfural is mainly produced from pentoses available in hemicellulosic hydrolysates through the cyclic dehydration reactions. These reactions are usually catalyzed by sulfuric acid. The possibility of extensive dehydration of furfural after formation in the reaction mixture, which leads to considerable reduction of furfural yield, is among the major drawbacks of this process. Therefore, in industrial furfural production processes, a high pressure steam is injected to the reactor to heat up the reaction mixture and at the same time strip the formed furfural and remove it from the reaction mixture (Fig. 1.11). This noticeably avoids unwanted side reactions. Annually, around 300,000 ton of furfural is produced by this method in batch and continuous processes (Peng et al. 2011; Agirrezabal-Telleria et al. 2014).

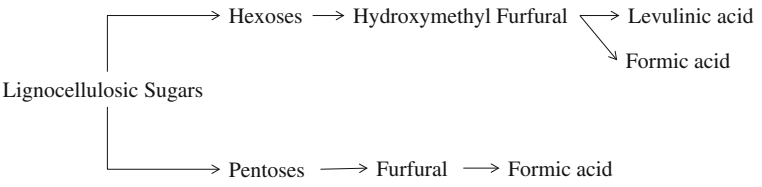

Fig. 1.10 Acid-catalyzed dehydration of sugars to new products

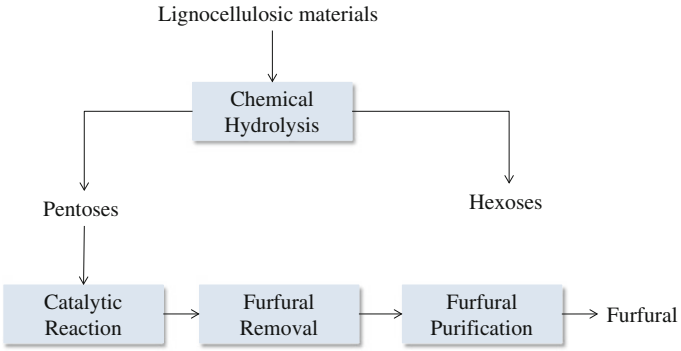

Fig. 1.11 Production of furfural from lignocellulosic materials

Despite presence of a fully developed industrial process for furfural production, extensive research is still conducted primarily to improve the process conditions toward higher yields and to find new catalysts with better performance than that of sulfuric acid. Accordingly, performing the acid-catalyzed dehydration of pentoses in hemicellulosic solvents (instead of aqueous solutions) (Campos Molina et al. 2012), using two-phase (aqueous-organic) solvent system for simultaneous removal of furfural after formation (Peng et al. 2011), removal of furfural from the reaction mixture by supercritical carbon dioxide (Gairola and Smirnova 2012), and hydrothermal conversion of pentoses at high pressures and temperatures (Gairola and Smirnova 2012) are among the new suggested processes for improvement of the process yield. Besides, supplementation of sulfuric acid with salts, e.g., NaCl, using other mineral acids as catalyst, and conducting the furfural formation reaction in heterogeneous phase employing solid catalysts such as $CrCl_3$ and zeolites have been proposed to enhance the catalyst performance and reduce the downstream purification costs (Zhang et al. 2014; Yoo et al. 2012; Agirrezabal-Telleria et al. 2014).

The production of furfural from lignocellulosic sugars is the only accepted method for industrial production of this versatile material. The economy of this process is expected to be greatly enhanced when the results of the above-mentioned researches are applied in industrial scale. Furfural is usually purified from the reaction mixture by azeotropic distillation.

Hydroxymethyl Furfural

Hydroxymethyl furfural (HMF) is one of the most important derivatives of lignocellulosic hexoses obtained by chemical dehydration. This is a very high potential intermediate which can be converted to high-quality fuels, resins, adhesives, composites, binders, solvents, monomers, and pharmaceuticals (Peng et al. 2011).

A number of different catalysts including organic acids, inorganic acids, salts, ion exchange resins, zeolites, and Lewis acids accelerate the dehydration of hexoses to HMF (Peng et al. 2011). Generally, dehydration of fructose to HMF is performed very easily. In contrast, direct conversion of glucose to HMF requires especial types of catalysts, and this conversion is performed in two steps of isomerization of glucose to fructose and conversion of fructose to HMF. It should be mentioned that from economical point of view, glucose is a more preferred substrate for HMF production than fructose (Ståhlberg et al. 2010; Mascal and Nikitin 2010). Therefore, considerable attentions have been paid to enhance the yield and rate of reactions of HMF production from glucose. Accordingly, attempts for finding new effective catalytic systems, e.g., lanthanide catalysts, incorporation of other solvents, e.g., ionic liquids, and using bi-phasic solvents have shown promising results in production of HMF from glucose (Ståhlberg et al. 2010, 2011; Choudhary et al. 2013; Bali et al. 2012; Agirrezabal-Telleria et al. 2014; Mascal and Nikitin 2010).

However, more research is still needed to be conducted before commercialization of HMF production from lignocellulosic sugars. It should be mentioned that prolonged reaction times lead to conversion of HMF to another value-added chemical, i.e., levulinic acid.

Levulinic Acid

Levulinic acid is a five-carbon chain fatty acid which has a ketone carbonyl and an acidic carboxyl group. Levulinic acid has a very high potential for synthesis of alternative fuels, fuel additives, solvents, dyes, flavoring agents, and different resins (Peng et al. 2011).

The most well-known method for production of levulinic acid is degradation of hexoses in the presence of mineral acids. As mentioned in the previous section, HMF is formed as an intermediate in this process. Biofine process which was developed by Biofine Corporation is an improved process for production of levulinic acid with considerably higher yield compared to traditional methods. In this process, a continuous system with two reactors is used for production of levulinic acid (Fig. 1.12) (Bozell et al. 2000).

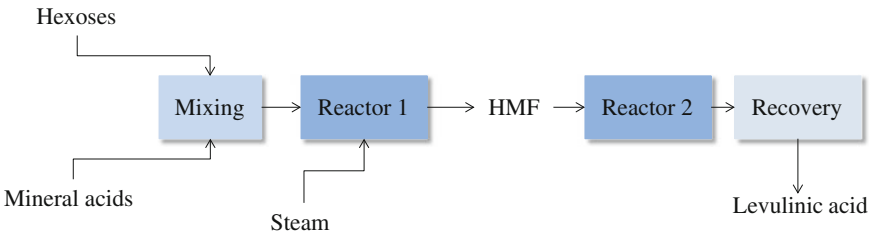

Fig. 1.12 Production of levulinic acid from hexoses

In this system, hexoses are fed to the first reactor where they are converted mainly to HMF in the presence of 1.5 % mineral acid. HMF is continuously removed using steam and fed to the second reactor. The residence time in the first reactor is rather short (15–25 s), while the reaction occurs at 210–230 °C. Longer residence times (15–30 min) as well as lower temperatures (145–230 °C) are applied in the second step to convert HMF to levulinic acid (Bozell et al. 2000).

Although using mineral acids as catalyst efficiently convert sugars to levulinic acid in this economically feasible process, alternative catalysts have also been evaluated for the production of levulinic acid. Accordingly, transition metal chlorides have shown a high catalytic activity in this reaction. Chromium chloride is among the best catalysts recommended for production of levulinic acid. Application of chromium chloride has the advantage of easier separation and recovery of catalyst, since it is partially dissolved in the reaction medium (Chang et al. 2007; Galletti et al. 2012; Bozell et al. 2000; Mascal and Nikitin 2010).

As levulinic acid can be easily produced from lignocellulosic sugars with high yields and low costs, new researches are more concentrated on the production of new value-added chemicals from this material.

Xylitol

As mentioned in Sect. Xylitol, the production of xylitol by fermentation has not yet come to the industrial market. Therefore, nowadays the only industrial method for xylitol production is chemical reduction of xylose obtained from lignocellulosic materials (Peng et al. 2011). An overview of the industrial process is shown in Fig. 1.13.

It should be emphasized that the purification of xylose from hemicellulosic hydrolysates is a crucial and costly step in this process. A combination of ion exchange chromatography and activated carbon treatment is used to remove dissolved salts, charged degradation products, and color from the hydrolysates. However, the presence of other sugars in the hydrolysates which are not removed

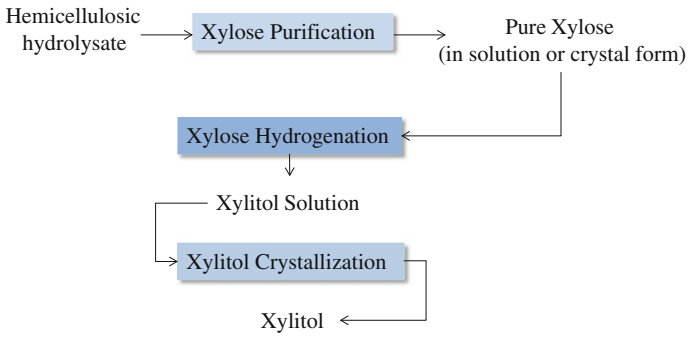

Fig. 1.13 Chemical production of xylitol from xylose

by chromatography is a problem for this process. Different metal-based catalysts (Pt, Ru, Pd, and Ni) have been proposed to assist the hydrogenation of xylose to xylitol. Most commonly nickel-based catalysts are used for the production of xylitol. This process is performed at relatively high temperatures and pressures (80–130 °C and more than 40 bar, respectively) (Peng et al. 2011).

1.3 Products Derived from Lignin

Besides cellulose and hemicellulose, lignin is another main part of lignocellulosic materials. Despite abundant resources of lignin in the nature, its application has not been developed as broadly as cellulose and hemicelluloses. The main reason for the limited applications is very complex chemical structure of lignin. Lignin has a cross-linked macromolecular structure of phenylpropanoid monomers. These monomers have the same phenylpropanoid skeleton but different degrees of oxygen substitution on the phenyl ring (one, two, or three hydroxyl or methoxyl groups which are referred to as hydroxyphenyl (H), guaicyl (G), and syringyl (G) monomers, respectively). The share of each monomer varies depending on the plant type and growing conditions. Therefore, generally lignins not only exhibit a very complex structure but also show a very wide diversity. This makes the development process of lignin-based products very difficult (Doherty et al. 2011; Silva et al. 2009).

However, appreciated amounts of lignin are available in lignocellulosic materials, and therefore it is the main byproduct of the industries which are based on cellulose, hemicelluloses, and their derivatives. Additionally, if the biofuel production from lignocellulosic materials comes to the commercial market, huge amounts of lignin will also be produced as an unavoidable byproduct of such industries.

Therefore, the employment of lignin as a source of energy and materials is among the important goals for improvement of the economy of cellulose- and hemicelluloses-based industries. Because of very high heating value of lignin, compared to polysaccharides, traditionally, this material is directly burned in the paper making facilities to produce electricity and steam. However, lignin has a much higher potential for the production of value-added products as well as energy which may not be obtained from other biological materials. Therefore, although direct combustion of lignin improves the economy of pulp and paper industry, it is not the most reasonable way for lignin application in the future (Azadi et al. 2013; Silva et al. 2009).

Recently, besides direct burning, lignin has got other small- and big-scale applications. Different applications are designed based on the lignin characteristics. It should be mentioned that lignin purification process significantly influences the lignin properties. Many of purification processes lead to cleavage of several bonds on lignin macromolecular structure. Therefore, the purified lignin does not exhibit exactly the same characteristics as it does in original form in plant cell wall (Doherty et al. 2011; Lora 2008).

Generally, lignin purification method is categorized into two groups: (a) the methods in which lignin is dissolved and removed from cellulose and (b) the methods in which cellulose is dissolved and lignin is recovered as insoluble fraction. It is worth mentioning that due to rather instable nature of hemicelluloses, these polysaccharides are usually dissolved and sometimes hydrolyzed during the lignin purification processes (Azadi et al. 2013; Doherty et al. 2011).

All pulping processes (Fig. 1.14) are belong to the first group while some pretreatments of lignocelluloses, such as pretreatment with cellulose solvents, as well as dilute acid hydrolysis of lignocellulosic materials are examples of the second group. Sulfite, kraft, and soda lignins which are, respectively, obtained in sulfite, kraft, and soda pulping processes are nowadays commercially available sources of purified lignin (Lora 2008; Azadi et al. 2013; Doherty et al. 2011; Silva et al. 2009; Baurhoo et al. 2008).

The sulfite process is used for preparation of pulp from woody biomass. In this process, wood chips are treated in aqueous solutions of bisulfite or sulfite salts (Na, Mg, Ca, or NH_4 salts) at 140–170 °C. In this process, several complex reactions take place resulting in dissolution of lignin, hydrolysis of hemicelluloses, and recovery of cellulose. The spent sulfite liquor obtained in this process contains hemicellulosic sugars and the dissolved lignin. The sulfite lignin, lignosulfonate, contains high amounts of sulfur (4–8 %) and is soluble in almost full range of pH (Lora 2008; Doherty et al. 2011).

Before recovery of sulfite lignin, it is worth to employ hemicellulosic sugars, e.g., for cultivation of microorganisms. These sugars have been used for cultivation of different zygomycetes fungal strains to achieve fungal biomass which can be

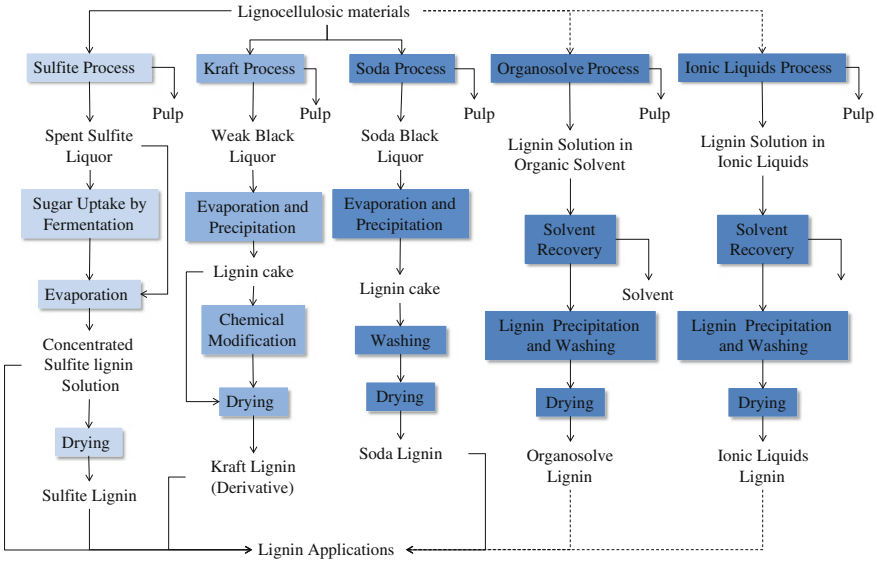

Fig. 1.14 Lignin recovery and purification during different pulping processes

used as a source of fish feed, fungal chitosan, and fungal oil (Helle et al. 2008; Taherzadeh et al. 2003; Zamani et al. 2010; Lora 2008). After consumption of sugars, spent sulfite liquor is evaporated to obtain concentrate sulfite lignin. This can either be used directly or further be dried to get a powder form (Fig. 1.14) (Lora 2008).

Sulfite lignin is used as a dispersant in concrete, gypsum wallboards, agrochemicals, dyes, bitumen, pigments, and drilling fluids for oil recovery. Moreover, it is used for several binding applications, e.g., in animal feed, fertilizers, refractory materials, and phenol-formaldehyde resins. Crude spent sulfite liquor can also be utilized for soil stabilization and control of dust. Furthermore, sulfite lignin is used for production of vanillin, a flavor agent and an intermediate of pharmaceutical products, through a catalytic oxidation reaction (Lora 2008; Silva et al. 2009).

Although the sulfite process was the main method of pulping in the past, discovery of more efficient pulping processes, such as kraft process, led to the replacement of this method. Currently, only 10 % of the pulp is obtained by sulfite method. However, it is important to know that recovery of lignin in new pulping processes has not as broadly applied as the sulfite process. Therefore, today, still a significant part of the commercially available lignin is sulfite lignin (Azadi et al. 2013; Doherty et al. 2011; Lora 2008).

Kraft pulping process is nowadays the most commonly used method of pulping. In this method, wood is treated with sodium hydroxide and sodium sulfide to remove lignin and hemicelluloses. The obtained pulp has enhanced characteristics compared to the pulp obtained in sulfite process (Fig. 1.14) (Hocking 2005; Lora 2008).

Despite wide application of kraft process, only a small portion of the kraft lignin is recovered, and the rest is directly burned to balance the costs of the pulping process. On the other hand, several researches are conducted to find alternative ways for kraft lignin utilization which can compete the burning process in economical point of view. Kraft lignin is recovered from the weak black liquor of pulping process by precipitation as low pH. The lignin can either be dried directly or chemically modified (e.g., by sulfonation or amination) prior to drying (Fig. 1.14) (Lora 2008).

Although the kraft lignin is commercially available at low volumes, it has already got several applications in different industries. For example, kraft lignin can be used as a dispersant for dyes and agrochemical products. It can also be employed to enhance air entrainment in mortar and concrete giving a microstructure to these products. Application of kraft lignin as an expander in lead–acid batteries has also been reported. Another interesting proposed application of this lignin is asphalt emulsifier where lignin stabilizes the asphalt at a very broad range of temperature. Kraft lignin has also shown antioxidant and UV protection activities. In addition, the production of dimethyl sulfoxide has been reported (Azadi et al. 2013; Doherty et al. 2011; Lora 2008).

The sulfite and kraft processes have been mainly developed for woody feedstock. In contrast, soda (or alkali) process has been proposed for pulp production from annual plants. Annual plants provide only 5 % of total world pulp production.

These plants usually have lower lignin contents compared to wood and are delignified using only sodium hydroxide solutions. The dissolved alkali (soda) lignin is recovered by acid precipitation (Azadi et al. 2013; Doherty et al. 2011; Lora 2008).

Absence of any sulfur reagent during the course of pulping process leads to the recovery of a sulfur-free lignin. Therefore, soda lignin is much more similar to native lignin than sulfite and kraft lignins. This similarity provides the opportunity for some new applications of soda lignin. For example, soda lignin has shown promising performance for the replacement of fossil-based phenol, e.g., in phenol-formaldehyde resins. Additionally, soda lignin is a better choice for animal health and nutrition products compared to sulfite and kraft lignin. Moreover, soda lignin has shown interesting activity for replacement of antibiotics (Doherty et al. 2011; Lora 2008).

In addition to the sulfite, kraft, and soda processes, currently some new pulping methods have been proposed which are called organosolv processes (Fig. 1.14). Although none of these methods have been broadly commercialized yet, they have a high potential for being used for pulping in the future. The organosolv treatments are performed using a low boiling point organic solvent such as ethanol and methanol in the presence or absence of acidic catalysts to dissolve lignin. In this processes, hemicelluloses can be hydrolyzed to their sugars while cellulose is remained intact. Prior to lignin recovery, the solvent is recovered by evaporation. Then, the organosolv lignin can be precipitated by pH alteration and recovered. Since relatively low structural changes occur on lignin during these processes, organosolv lignin has been recognized as one of the most suitable lignins for production of lignin-based materials (Doherty et al. 2011; Sarkanen et al. 1981).

Organosolv methods are very effective not only for pulp production but also for biofuel production from lignocellulosic materials. As mentioned in Sect. Biofuels, a pretreatment step is necessary prior to hydrolysis of lignocellulosic materials for biofuel production.

Organosolv pretreatments are among the most promising methods for pretreatment of lignocellulosic materials, e.g., for ethanol production (Lai et al. 2014; Azadi et al. 2013). Therefore, in both pulp and biofuel industries, organosolv processes are expected to improve the economy of the whole process.

Organosolv methods are not the only new proposed solutions for improving the efficiency of pulping process and obtaining of a high-quality lignin. Currently, considerable attentions have been paid to separation of lignin using ionic liquids. Early investigations indicate that ionic liquids may play an important role in lignin purification in the future because of low boiling point, high efficiency in lignin dissolution, and very high recovery (Prado et al. 2013b; Pu et al. 2007).

As mentioned earlier, pulping processes are not the only methods of lignin purification. Dilute acid hydrolysis of lignocellulosic materials at elevated temperatures results in the hydrolysis of cellulose and hemicellulose. The intermediate products of carbohydrates hydrolysis are sugars while the end products are furfural, HMF, levulinic acid, and formic acid. Lignin and ash contents of the substrate are recovered as a solid residue called char. Char has a very high heating value (26 MJ/kg) and is mostly used for heat generation (Azadi et al. 2013).

Applications of lignin are not limited to the fully developed ones and continuously new aspects and opportunities are reported getting benefit of the sole structure of lignin in the nature. Indeed, lignin is the only material with biological origin which is composed of aromatic residues. Therefore, this can be considered as the sole feedstock for replacement of fossil-based aromatics (benzene, toluene, and xylene). Ideally, depolymerization of lignin would end up with formation of lignin aromatic constitutes which can be employed as substitutes for fossil-based aromatics. However, up to now, there is no optimized depolymerization process which result in high selectivity and yield for a certain component. However, it has been reported that even partial depolymerization of lignin and subsequent product separation, based on, e.g., boiling point, may be economically feasible. This is due to the fact that the obtained bulk grade aromatics also have a high potential for different applications, e.g., high-quality liquid fuel production. Although extensive studies have been conducted aiming at development of appropriate methods of lignin depolymerization for aromatic formation, there is much to be done to achieve this goal. Therefore, the production of aromatics from lignin is considered to be a very long-term objective (Azadi et al. 2013; Nanayakkara et al. 2014; Prado et al. 2013a).

Meanwhile, lignin can be employed in some other new applications. The production of carbon fiber, activated carbon, polymer fillers, resins, and adhesives from lignin have got considerable attentions in recent years. On the other hand, lignin can be completely converted to syngas (a mixture of mainly hydrogen and carbon mono-oxide) through well-designed gasification processes. The obtained syngas can be either directly combusted in gas turbines to create heat and electricity or be converted to methanol and Fischer–Tropsch fuels. Furthermore, syngas can be upgraded to pure hydrogen (Azadi et al. 2013).

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Chapter 2
Perspective of Biofuels from Wastes

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Abstract In the world today, with an everyday increase in global population, transitioning the society to a more sustainable atmosphere would be the only solution for guaranteeing a long-lasting life in this planet. Despite the fact that the earth is armed with various natural resources, it should be accepted that they would not last forever. By converting useless wastes and residues to a new source for supplying energy rather than wasting the existed energy for their disposal, not only the concerns for the depletion of fossil fuels would be reduced but also the environment dares to breathe. Concerning this issue, the present chapter has tried to depict a clearer perspective for waste-based biofuels which are known as second-generation ones. The discussed products in this chapter are biodiesel, bioethanol, biobutanol, biogas, and biohydrogen. The focus is mostly on new researches which have introduced new waste as feedstock and their usage feasibility, though production processes and challenges ahead are included as well.

2.1 Introduction

Today, the population growth and the need for energy together with the fossil fuel depletion and environmental pollutions have urged countries to seek for more newer and cleaner sources of energy (Balat and Balat 2009). Around 60 % of world's oil consumption and one-fifth of global CO_2 emissions are related to transportation sector (Kırtay 2011). Hence, replacing fossil fuels by renewable

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energy sources, specifically in developing countries where the rate of energy consumption is faster than industrial ones, is a must (Balat and Balat 2009).

In 2012, the global petroleum consumption was estimated as 89 million barrels per day from which about a half was used for gasoline production. At this rate of consumption, the oil resources are predicted to run out within the next 50 years (Arifin et al. 2014). Furthermore, fossil fuel usage can cause environmental problems such as air pollution, greenhouse gas emissions, and global warming, consequently, which makes the society worried. Thus, many countries are trying to find renewable resources as alternatives which are capable of balancing the GHG emissions. Biofuels seem a key solution for the present challenge since it is produced from renewable resources and have a great influence on GHG mitigation as 23 % of CO_2 emissions are related to transportation sector (Fiorese et al. 2013). Biofuels can be categorized into three main groups, first-, second-, and third-generation biofuels which can be obtained from food, non-food feedstocks, and microalgae, respectively. The biomass potential for supplying energy has been estimated as 10^{20} J/year of which 40 % is being utilized nowadays (Ragauskas et al. 2006).

First-generation biofuels, the most common type, are mainly produced from agricultural crops such as corn, sugarcane, sugar beet, wheat, rapeseed, soybean oil, sunflower, and palm oil, having a good access to a highly mature technology in converting crops to biofuels. In Europe, it was first emerged as a result of agricultural stagnation in 1990–1994, encouraged farmers to grow crops for non-food purposes, thanks to tax exemptions supported by governments. In the Netherlands for instance, two public transport companies invested on bioethanol and biodiesel production for bus consumptions. In 1995, environmental issues and biomass energy gained an importance leading to the investments of two boating companies on biodiesel followed by the 10-year tax exemptions' demand for the Nedalco with the aim of producing 30 L bioethanol from agriculture crops. In 1998, Kyoto treaty was signed, and the climate issues became more and more important. On the one hand, different investigations revealed that it is worthy to develop the projects in which CO_2 emissions are reduced up to more than 80 %. On the other hand, as a result of the growing world population, by 2050, the agriculture will need to provide food for 9 billion people, an enormous challenge from an agronomic perspective (Jeihanipour 2011). The consideration of using crops for food or for biofuel may be referred to as the "food versus energy" conflict (Suurs and Hekkert 2009). Furthermore, recent studies disclosed that an increase in the production of biofuels from food resources might cause a substantial "carbon debt," since the reduction of GHG emission by replacing fossil fuels is less than the CO_2 released from direct or indirect changes in land use (Williams et al. 2009). Therefore, decision makers are trying to devise more research on second- and third-generation biofuels (advanced biofuels) which can be obtained from non-food crops, wastes, and algae. Unlike the former one and without considering dedicated crops, these new feedstocks do not require extra land and water, as they are mainly consisted of residues of agriculture (straw, stover, husks and cobs, marcs and lees, bagasse, empty fruit bunches, and nutshells) and forestry (treetops, branches, stumps, leaves,

sawdust, cutter shaving, scrap wood, and wood pulp). Even the aquaculture fishery (algae, fish scales, viscera, and scrap) residues, sewage sludge, and industrial and municipal wastes are classified in this category. Furthermore, it is believed that GHG emissions caused by second-generation biofuels are much lesser than the former ones. As an example, in comparison with fossil fuels, the cellulosic ethanol can reduce CO_2 emission up to 75 %, whereas the sugarcane/cassava-based ethanol decreases it by 60 %. Similarly, thermochemical-based biodiesel obtained from wastes can reduce carbon release by 90 % compared to the 75 % reduction offered by usual biodiesel (Patumsawad 2011). However, the conversion processes of such biomass are much more complicated and costlier in comparison to first-generation ones (Fiorese et al. 2013). As a matter of fact, the worldwide production of advanced biofuels is less than 1 billion gallon per year at present (Yue et al. 2013).

There are two major methods of bio/thermopath for converting biomass to second-generation biofuels (Fig. 2.1). In thermopath, the biomass gets heated, and depending on the temperature range, finally three different products are obtained. Torrefaction (250–350 °C/anaerobic), pyrolysis (550–750 °C/anaerobic), and gasification (750–1,200 °C/limited oxygen) are different processes, applied in this path leading to the production of solid (biochar), liquid (bio-oil), and gaseous (syngas)

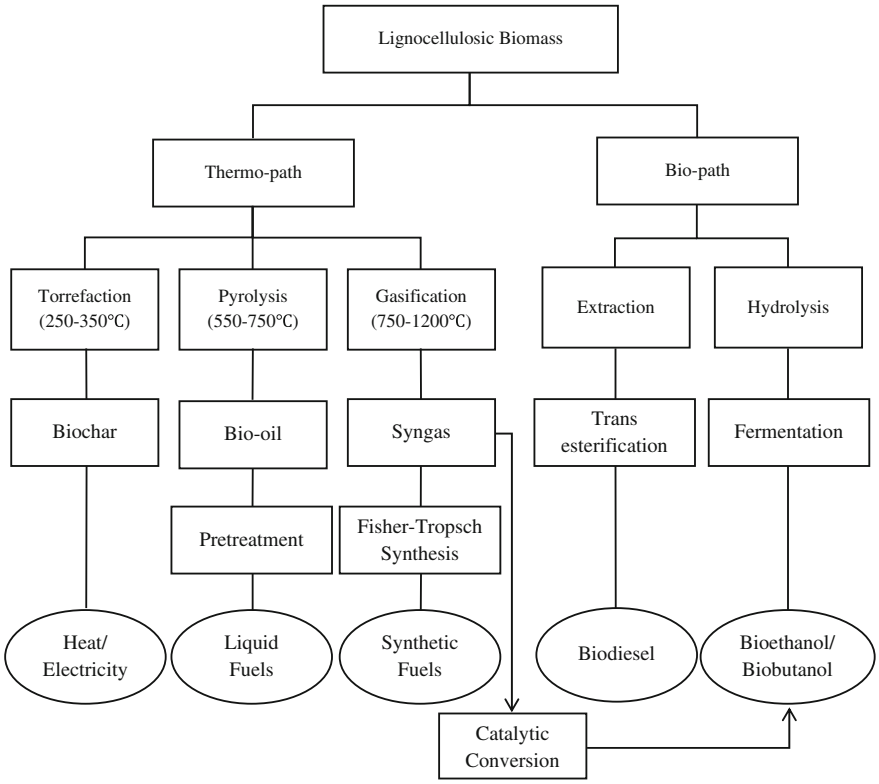

Fig. 2.1 Conversion processes of second-generation biofuels

products. However, within syngas production, a few amounts of biochar and bio-oil are formed as well. Furthermore, thermal processes are exothermic; thus, they can supply their own energy requirement for such high temperatures. Moreover, bio-oil and syngas are more appropriate to be used as a fuel than biochar since they have fluid's characteristics; however, they need some pretreatments before being able to be used in vehicles. In all thermopath processes, the price of biomass is a key factor. Therefore, when agricultural residues or municipal wastes are used instead of dedicated crops, the process would become more cost-effective (Lee and Lavoie 2013). In contrast, biopaths entirely lead to the formation of liquid and gaseous biofuels through fermentation and anaerobic digestion processes.

It is predicted that lignocellulose-based biomass can produce ethanol up to 442 billion L/year, though there is still no large-scale plant for this purpose (Festel et al. 2014). These biomasses are usually comprised of cellulose (crystalline polymers made of glucose), hemicellulose (amorphous polymers of several pentoses and hexoses), and lignin (a complex and large polyaromatic compound). With the aid of pretreatments such as steam explosion and ammonia treatment, hemicelluloses and lignin can be separated from the cellulose and further enzymatic or acidic hydrolysis would lead to glucose separation as well (Taherzadeh and Karimi 2008). Different types and details of leading pretreatment processes are presented in Chap. 3. The glucose is then converted to ethanol via fermentation, and finally, ethanol is separated by distillation. Lignin has the potential to be used as a solid fuel or an H_2 source in biorefineries. However, its aromatic monomer consisted of valuable chemical compounds that can be separated for plastic or adhesive production industries. This issue would open a new market for second-generation bioplastics and bioadhesive manufacturing based on biomass (Lee and Lavoie 2013).

From another aspect, second-generation biofuels will not only overcome the first-generation problems but also could moderate the waste management hardships (Zah 2010). In 2012, the global urban population was estimated as 3 billion, which is predicted to increase to 4.3 billion by 2025. The municipal solid waste production rate of this population was 1.3 billion ton/year, which is estimated to increase to 2.2 billion ton/year by 2025 (Hoornweg and Bhada-Tata 2012). According to Kelessidis and Stasinakis (2012) the annual production of sewage sludge in EU-12 in 2005 was estimated as 1.1 million ton dry solid which is predicted to exceed 13 million ton dry solid by 2020. Currently, the most commonplace methods for disposing solid wastes in many countries are incineration and landfilling which are faced with barriers such as air pollution, ash disposal, land availability, and financial issues. However, by separating the organic fractions of the municipal solid wastes or using other sources of wastes, e.g., agriculture, forestry, and sewage sludge, these barriers would be eliminated, and in addition, it would be considered as a new source for energy and value-added products.

The aim of this chapter is to introduce the biofuels having the potential of being produced from different types of wastes as well as discussing the available processes and probable challenges ahead.

2.2 Which Waste? Which Biofuels?

Wastes, residues, and co-products are new and widely available alternatives for biofuel production, while municipal solid wastes, used cooking oil, industrial wastes, and sewage sludge are examples of wastes that can be used for second-generation biofuel production. These new sources can be used for the production of biofuels such as biodiesel, bioethanol, biobutanol, biogas, biohydrogen, and a number of biochemicals such as citric acid, xanthan, lactic acid, and acetic acid. Here is a brief introduction to some of the important ones.

2.2.1 Wastes to Biodiesel

Biodiesel, shortly introduced in Chap. 1, is a renewable alternative for the fossil fuel diesel having the potential to reduce the emission of toxic gases such as carbon monoxide (Mandolesi de Araújo et al. 2013). It can be obtained via transesterification of oils in which glycerides are converted into esters resulting in viscosity drop, better combustion, and less emission in engines (Canakci 2007). The main challenges of biodiesel production are the high cost of vegetable oils constituting 70 % of the total costs (Haas and Foglia 2005). In August 2012, the price of soybean oil, palm oil, and canola oil, which are the main sources of biodiesel in the present market, was US$ 1,230/ton, 931/ton, and 1,180/ton, respectively. Based on the price of canola oil, each ton of oil is capable of producing 1,000–1,200 L biodiesel with the market price of US$ 0.85/L; nevertheless, the methanol price should also be considered (US$ 0.35/L) since about 125–150 L of methanol is required per ton of oil (Lee and Lavoie 2013). Therefore, it is essential to find cheaper oil sources to make the biodiesel production more profitable. These oils can be supplied from different wastes and residues, e.g., used cooking oil, oils and fats extracted from animal tallow, sewage sludge, crude or waste fish oil, fish canning industry, leather, winery and agro-industrial wastes, restaurant waste lipids, olive pomace oil, sorghum bagasse, and meat industry residues, directly or indirectly. Indirect use of wastes for biodiesel production is referred to those in which the oleaginous microorganisms are cultivated on municipal, industrial, or agricultural wastes to store lipids. Then, the lipids can be extracted and applied for biodiesel production as well.

There are usually three main steps for biodiesel production from waste oils including pretreatment, transesterification, and separation processes. However, depending on the type of waste, lipid extraction should be considered as well. About 13 % of Brazil annual biodiesel requirements can be supplied from used cooking oil. This type of oil usually needs some pretreatments to reduce the viscosity and the content of water and free fatty acids (FFAs) which can influence transesterification process negatively (Mandolesi de Araújo et al. 2013). Even by carrying out a pretreatment on used oil, it is still more economical and can reduce the direct production costs up to 45 % in comparison with pure oils (Zhang et al. 2003). In fish canning

and processing industry, there is also a possibility of biodiesel production from oily fishes such as codfish, salmon, mackerel, and tuna. The annual production rate for fish oil is about 150 ton of which more than 50 ton would be disposed as sewage (Costa et al. 2013). In 2005, the estimated world fish production was 142 Mt which increased to 144 Mt in 2008. About 25 % of this amount can be considered as a waste, and its oil content has the potential of biodiesel production (Yahyaee et al. 2013). Waste chicken fat has also been used as a feedstock for biodiesel production. In 2006, 10.5 million chickens were produced in China from which 115,500 ton fat was recoverable. However, it should meet the pretreatment requirements as well (Shi et al. 2013). Chakraborty and Sahu (2014) used waste goat tallow as an oil supplement for biodiesel production using a novel method for transesterification. Using infrared radiation method, they could not only enhance the product yield due to an increase in heat and mass transfer but also reduced the reaction time significantly which is considered as a challenging task if the catalytic methods are used. Furthermore, in different countries, some industries are more developed, of which the residual and wastes can be utilized for biodiesel production. For instance, in countries where the leather industry is developed, there is a possibility to use fleshing oil wastes as a raw material for biodiesel production (Alptekin et al. 2012). Spain, France, and Italy are large producers of wine, and the oils exist in grape wastes can be converted to biodiesel. The global estimation of annual grape's production in 2005 was 67 million ton of which more than 20 % is regarded as waste. By extracting the oil that exists in waste grape's seed (10–20 %), biodiesel production would be feasible. However, it should be pretreated as most of the wastes (Fernández et al. 2010). Besides, in Mediterranean countries, such as Greece where the annual production of olive pomace oil is about 40,000 ton, there is a possibility to convert this oil to biodiesel after performing a pretreatment on it (Che et al. 2012). Meat industry has also been evaluated for biodiesel production. Since 49 % of cattle, 44 % of pig, and 37 % of fowl weight are not consumable by human, the yellow fat obtained from these sorts of wastes, estimated as 1.38 kt/year, would lead to a biodiesel yield of 95 %. This means that it is possible to produce 1.3 kt/year biodiesel from such fats, providing 51.2 GJ/year energy. It is estimated that the obtained biodiesel blending with fossil fuel diesel (2 % biodiesel and 98 % fossil fuel diesel) would cover 25 % of the fuel consumption of the passenger vehicles and trucks registered in 2007 in Baja California, Mexico (Toscano et al. 2011). Besides, some of the industrial and agro-industrial wastes and residuals have the potential to be used as a carbon source or an organic supplement for oleaginous microorganisms which are capable of lipid production. It is estimated that for producing 10,000 ton microbial oil from glucose, the unitary cost is about US$ 3.4/kg. Crude glycerol is a by-product of biodiesel production and is used in producing alcoholic beverages as well as saponification of oils and fats. However, it is contaminated to chemical catalysts and cannot be used in pharmaceutical, toothpastes, or cosmetic purposes since the purification costs are significantly high and the process is considered as energy-consuming one. Therefore, it is more economical to use it as a carbon source for lipid production. Lignocellulose materials obtained from agriculture residues are expected to be consumed as carbon supplement by lipid-producing microorganisms after pretreatment and conversion to

simple sugars as well. However, the strains resistance to inhibitors, formed after pretreatments, should be considered (Leiva-Candia et al. 2014).

Apart from all waste types, municipal and industrial wastewater sludge has also the potential to be used for biodiesel production. In 2010, each gallon of biodiesel produced from primary and secondary sludge was estimated to cost 3.11 and 3.23 US$, respectively (Siddiquee and Rohani 2011). Lipids present in sewage sludge contain triglycerides, diglycerides, monoglycerides, phospholipids, and FFAs which are usually absorbed directly to sludge or are consisted of microorganism's cell membranes made of phospholipids (Kargbo 2010). These lipids can be extracted from sludge through boiling solvent, supercritical CO_2, and solo or a mixture of solvent extraction methods. However, lipid extraction from raw sludge is not economically feasible since it needs a large amount of organic solvent and bulky tanks equipped with mixers and heating systems. Since the dewatered sludge is very sticky and can hinder the extraction process, dry sludge has been widely used for lipid extraction (Siddiquee and Rohani 2011). It has been reported that all types of sludge, i.e., primary, secondary, blended, and digested sludge, are capable of being used as a biodiesel production source; however, primary sludge has shown the maximum biodiesel yield (Olkiewicz et al. 2012). By integrating lipid extraction and transesterification process in half of the US wastewater treatment plants, 0.5 % of its annual petroleum diesel would be supplied (about 1.8 billion gallon). Besides extracting lipids from sewage sludge, there is the possibility of microalgae cultivation in wastewater treatment plants to store the lipid and produce biodiesel after the harvesting and lipid extraction step. Microalgae, like other types of microorganisms, are phototrophic or heterotrophic and can supply their energy and carbon requirements from sunlight and CO_2 or by metabolizing organic carbons, respectively. Since they can grow fast and yield high amounts of lipid at controlled conditions, they can be used in large-scale biodiesel production processes. However, the energy balance and greenhouse gas emissions (GHG) would seem rather challenging. The cultivation can be performed on industrial wastewaters, e.g., starch industry wastewater. In producing biodiesel using starch industry and cellulose industry wastewater, by applying heterotrophic microalgae, the energy gain is 27.2 and 11.8 GJ for production of 1 ton biodiesel , respectively (Zhang et al. 2013).

Pretreatment is the first step in biodiesel production from waste oils in order to remove the impurities and solid particles or decrease the water content and acidity value (Costa et al. 2013; Mandolesi de Araújo et al. 2013). The required pretreatments in most cases are filtration and heating; however, pretreatments such as steam injection, neutralization, vacuum evaporation, and vacuum filtration have been applied as well (Kulkarni and Dalai 2006).

In the second step, transesterification reaction should be performed in which glycerides are converted into esters in the presence of a catalyst and an alcohol. However, in this step, using non-catalytic conversion techniques are also possible. In fact, each mole of lipid reacts with 3 mol alcohol and produces 3 mol alkyl esters (biodiesel) and 1 mol glycerol (Zhang et al. 2013).

Methanol is the most common type of alcohol used for performing the esterification reaction, as it is the least expensive industrial alcohol. In comparison with ethanol, it has a lower price, higher reactivity, simpler recoverability, preventing soap property, and azeotrope formation tendency (Siddiquee and Rohani 2011). Catalysts can deprotonate the alcohol in order to make it more nucleophile while reacting with lipid (Kargbo 2010). They are categorized as alkaline, acidic, and enzymatic types. Alkaline catalysis is the most common catalysts which is fast (less than 1 h) but extremely sensitive to the presence of water and FFAs. Water can change the saponification process of ester into alkaline conditions, and FFAs may react with the catalyst leading to the formation of water and soap or emulsions that influence the removal of biodiesel negatively (Mandolesi de Araújo et al. 2013; Canakci 2007). To overcome this problem, acidic esterification and heating can be applied on waste oils prior to transesterification process. El-Mashad et al. (2008) used H_2SO_4 1 wt% to reduce the acidity of the salmon oil to the standard range of alkaline catalysts (the maximum concentration of 2 mg KOH g^{-1}) followed by heating at 52 °C for 1 h within stirring condition of 600 rpm. The molar ratio used for methanol:oil was selected as 6:1. Alptekin et al. (2012) used animal fat obtained from leather industry fleshing wastes for biodiesel production and achieved ester yield of 92.6 % using KOH 1 wt% and a methanol molar ratio of 7.5:1 at optimum conditions. However, they had to pretreat the oil by heating it up to 110 °C to remove the water followed by a filtration to remove the suspended solids. NaOH has also been used for production of biodiesel from used coconut oil as an alkaline catalyst, leading to the transesterification performance of 94.5 % at the optimum catalyst concentration of 0.08 % and heating temperature of 60 °C for 20 min (Chhetri et al. 2008). Costa et al. (2013) produced biodiesel using the oil extracted from Portuguese fish canning industry. In order to dehydrate and esterify the oil, they heated the oil up to 100 °C and added it into a round-bottomed vessel and immersed it in a temperature-adjustable water bath equipped with a water-cooled condenser (4 °C) and and was mixed at 900 rpm. Then, the catalyst (1, 2, and 3 wt% H_2SO_4) and methanol (with molar ratio of 6:1) were added to the oil. The reaction temperature kept constant at 65 °C for 1 h. The maximum and minimum transesterification yield was obtained as 73.9 and 66.4 wt% with methanolic solution of 60 and 90 vol.% and catalyst concentration of 1 and 3 wt%, respectively. The results showed that catalyst concentration can negatively influence the biodiesel yield as a result of purification of lipid phase during the washing process leading to emulsion formation and loss of the product. Yahyaee et al. (2013) produced 0.91 L biodiesel out of 1 L oil extracted from fish processing waste using 1 wt% KOH and a methanol:oil ratio of 1:4 at 60 °C and 300 rpm for 2 h. Shi et al. (2013) used the integrated catalytic composite membrane (CCMs) process and sodium methoxide as a novel approach to produce biodiesel from waste chicken fat. Heterogeneous solid catalysts, i.e., sodium methoxide, SrO, MgO, and CaO, have been used for transesterification of animal oils since they are recyclable and more efficient in the presence of FFAs and water. They esterified the oil with CCM and methanol first, to reduce the acidity value of chicken fat, and the initial value was 39.52 KOH/g fat. However, the more CCM layers resulted in more reduction in acidity of esterified

product since more active sites were involved during the reaction between methanol and chicken oil. The membrane porosity and the methanol:oil ratio used were 68 % and 3:1, respectively, with a reaction temperature of 338 K. Sodium methoxide was used as the alkaline catalyst at 1 wt%, and the transesterification yield of 98.1 % was achieved (Shi et al. 2013). The biodiesel produced from waste winery grape's seed had a yield of 97.8 wt% after pretreating the extracted oil through acid conditioning and deacidification. The first step was performed by heating the oil at 60 °C and adding phosphoric acid and water with molar ratio of 0.05 and 1:6, respectively, mixing for 30 min. This was done in order to convert non-hydrated phospholipids to the hydrated form since H_3PO_4 is able to break down the metal/phospholipid complexes. Then, after separating the phases with centrifugation at 6,000 rpm and 20 °C for 20 min, the oil was mixed with NaOH and water (molar ratio of 0.2 and 6, respectively), and the temperature was held at 60 °C for 15 min until the formation of emulsion. Then, the formed soap and residual water were separated by centrifugation and vacuum evaporation. The maximum oil extraction yield from grape's seed (17.5 %) was achieved via soxhelt extraction and hexane: acetone ratio of 1:1 as the extracting solvents at 60 °C with the oxidation stability of 16.3 h (Fernández et al. 2010).

Che et al. (2012) could reduce the acid content of olive pomace oil to 1.38 % from the initial value of 22.11 % by acid esterification for 60 min, choosing catalyst:oil and methanol:oil ratios of 1 % and 0.45 v/v, respectively. However, the acidity obtained after pretreatment was still out of the standard range (1 %). They concluded that the most feasible and economical way to reduce the acidity would be achieved after using extra amount of alkaline catalyst during the esterification; however, an increase in methanol:oil ratio or reaction time and temperature, even performing an additional pretreatment step, can improve the acid value reduction up to some extent.

Cheirsilp and Louhasakul (2013) studied the cultivation possibility of four types of *Yarrowia lipolytica* on four different industrial wastes such as palm oil mill, serum latex obtained from rubber latex industry, crude glycerol of biodiesel-producing plant, and molasses that comes from sugarcane processes. They concluded that the first two wastes can be used as a cultivation media and the two others are more feasible as an additional carbon source for lipid production. In fact, in 4 % concentration of glycerol added to palm oil, the maximum lipid was produced. They also found out that direct transesterification of wet microbial cells is the most economical and easiest way to achieve biodiesel with a yield of 72 % in 1 h and methanol to oil ratio of 209:1 in comparison with direct transesterification of dried cells or transesterification of extracted lipids from dried cells, since the latter are more expensive and energy consuming.

Sorghum bagasse hydrolysates have been utilized for producing microbial lipids by Liang et al. (2012). They could achieve the lipid yield of 8.74 g per 100 g bagasse after pretreating with lime and enzyme. The lime pretreatment step was suitable for removing the lignin and hemicellulose, whereas the enzymatic procedure could degrade xylane and glucan.

Olkiewicz et al. (2012) evaluated different sludge types (primary, secondary, blended, and digested) for biodiesel production and obtained the highest yield (13.9 %) from primary sludge based on dry weight, since it mainly consisted of floated grease, while secondary sludge is composed of microbial cells obtained during biological treatment of wastewater (Kargbo 2010). In addition, digested sludge produced in an anaerobic digester showed the least biodiesel yield as a result of organic matter mineralization during the anaerobic digestion process. Furthermore, Angerbauer et al. (2008) could successfully store lipid by *Lipomyces starkeiy* up to 0.4 and 1.2 g/L on raw and ultrasonicated sludge. However, they did not report any biodiesel production yield in their study. In order to extract lipid from sludge, Boocock et al. (1993) used soxhlet and boiling solvent extraction method (chloroform and toluene) and extracted 12 and 17–18 % lipid from 50 to 100 g dry sludge, respectively. They concluded that both solvents are efficient, but toluene is more environmental compatible and economical. Dufreche et al. (2007) also reported a lipid yield of 27.0, 3.5, and 13.7 % for extraction methods of solo or three-solvent mixture (60 % hexane, 20 % methanol, and 20 % acetone), supercritical CO_2, and the combination of methanol and supercritical CO_2, respectively. Li et al. (2011) investigated nutrient removal and biodiesel production from microalgae *Chlorella* sp. cultivated on highly concentrated municipal wastewater and obtained the removal efficiency of 93.9, 89.1, 80.9, and 90.8 % for ammonia, total nitrogen, total phosphorous, and COD, respectively, as well as biodiesel yield of 11.04 % of dry biomass.

Unlike the alkaline catalysts, acidic ones are not sensitive to the presence of water nor FFAs but can esterify them about 4,000 times slower and require higher oil:alcohol ratio (Canakci 2007; Siddiquee and Rohani 2011). Thus, they can be used for the oils with acidity more than 1 % (Freedman et al. 1984).

Enzymatic catalysts in which lipase is the most common type are recovered easily, and unlike the former ones, they do not lead to the by-product formation and are not sensitive to water and FFA content as well (Mandolesi de Araújo et al. 2013). However, low reaction rate, high price, and product contamination by enzymatic activity make its usage challenging (Siddiquee and Rohani 2011). Recently, the application of lipase to catalyze palm oil sludge is investigated as a green technology for biodiesel production. By using enzymes, not only the need for multistep catalyst's removing processes would be eliminated but also a wider range of lipids such as FFAs, monoacylglycerides, diacylglycerides, and triacylglycerides are converted to biodiesel. However, the selection of proper solvent is of crucial importance since polar solvents like methanol can reduce the enzyme activity by removing the water molecule from the surface of enzymes. Thus, ethanol that does not have such effect and has the potential for being produced from other wastes can be used instead. The FFA conversion and biodiesel yield obtained in optimal condition in this method are 21.7 and 54.4 % w/w, respectively (Nasaruddin et al. 2014).

In non-catalytic approaches, it is possible to use a solvent which is miscible in both methanol and oil instead of using the catalyst. The reaction occurs fast without any catalytic residue remaining, or by applying the BIOX, a patented production process, FFAs and triglycerides are converted into esters through a two-step process

in less than 90 min, resulting in more than 90 % conversion in a case that the feedstock contains more than 10 % acidity (Mandolesi de Araújo et al. 2013). So far, microwave radiation, static mixers, microchannel, cavitational method, and infrared radiation have been investigated as well. For instance, Chakraborty and Sahu (2014) using an infrared radiation assisted reactor, obtained 96.7 wt% FFA conversion and 98.5 wt% biodiesel yield from waste goat tallow in 2.5 h, at optimal conditions.

As the third and last step, the produced biodiesel should be separated and purified which can be regarded hard if the feedstock is supplied from wastes. In this case, the alcohol (methanol) would be separated via distillation and the catalyst and the glycerol are aimed to be washed away by hot water (50 °C). After washing the mixture by hot water, two phases form. The biodiesel form the top phase, with a water content of 1 g per liter of biodiesel, which will be separated by distillation later on. The bottom phase would be glycerol, catalyst, and water. The catalyst would be separated in a neutralizing reactor, and the glycerol then would be dried via distillation (El-Mashad et al. 2008; Lin and Li 2009; Fan et al. 2010; Zhang et al. 2013).

2.2.2 Wastes to Bioethanol

Bioethanol is currently the most common biofuel in terms of produced volume which is industrially produced mainly from corn and sugarcane. For decades, it was used as a source of energy in a lamp oil, for cooking, or known as spirit oil before being applied in an internal motor combustion by Samuel Morey early in the nineteenth century. Later on, it was introduced to the automobile market and in agricultural machines. However, emergence of oil and its derivatives in twentieth century drove it aside until the Arab oil embargo of 1970s (Lee and Lavoie 2013). Today, the depletion of fossil fuels and its environmental drawbacks have enforced the policy makers to focus on renewable sources of energy. Hence, many countries enacted several directions for enhancing the share of biofuels in their energy basket (Zah 2010).

The annual production of ethanol increased to more than 85.6 billion liter in 2010 worldwide (Carriquiry et al. 2011) since it has some advantages over other fuel additives. Its high octane number and flexibility in blending with petrol have made it a well-suited additive in automobile's engines without almost any modification requirement. By blending ethanol with petrol, not only the emissions of GHGs, unburned hydrocarbons, and carcinogens would be reduced but also the sulfur oxides which are the main cause of acid rains will be decreased significantly (Nigam and Singh 2011). These features can make the ethanol as the best environmental-friendly candidate in transportation sector. There are three main sources of biomass that can be used as a feedstock for bioethanol production: sucrose-based sources (sugarcane, sugar beet, and sweet sorghum), starchy biomass (wheat, corn, and barley), and lignocellulose materials (wood, straw, and grass) (Soccol et al. 2010). Although the first two (first generations) benefit a highly simple and mature

technology in converting the sugars to ethanol, they suffer the controversy of food versus energy as well. For instance, the price of raw sugar in August 2012 was US$ 0.2/pound, while the ethanol price was US$ 0.68/L. This meant that production of 1 L ethanol from raw sugar would account for US$ 0.3–0.35 and it is more beneficial for market to produce sugar rather than ethanol. The story goes the same for the corn and other food-based ethanol sources (Lee and Lavoie 2013). Thus, lignocellulose-based material can be a better replacement for them. However, more complex processes are required to convert them to ethanol.

In most countries, there is a good potential for bioethanol production from various wastes and residues including forestry and agricultural residues (forest woody feedstocks, corn stovers, cornstalks, rapeseed residue, sugarcane bagasse, citrus peels, empty bunches of fruits, straws, etc.), water hyacinth and seaweeds as well as industrial or municipal solid wastes (waste papers, newspapers, money bills, household food and kitchen wastes, coffee residue, waste textiles, etc.). In Iran for instance, the bioenergy potential from agricultural, animal, and municipal wastes has been estimated as 8.78×10^6, 7.7×10^6, and 3×10^6 t, respectively, while a potential of annual ethanol production of 4.91 GL from agricultural residues was reported before (Avami 2013). About 600–900 million ton rice straw is produced annually in the world of which 205 billion liter ethanol might be possible to be produced (Sarkar et al. 2012). This means that most of the Asian countries can benefit this sort of biomass. Around 20 % of sweet potato grown worldwide remains as waste on fields. It is stated that sweet potato can yield higher concentration of ethanol, nearly 114 %, than corn (Dewan et al. 2013). In India, growing bamboos is well established, and annually, 32 million ton of this herb is cultivated. Since bamboo contains a very small amount of lignin and a large share of cellulose, its residues, which are estimated as 5.4 million ton, can be utilized for bioethanol production. It has been reported that the surplus amount of bamboo residues, i.e., 3.3 million ton, have the potential to produce 473 million liter ethanol (Kuttiraja et al. 2013). An herbal plant, called *Coleus forskohlii Briq*, is cultivated for its root in large scale in India due to its medical advantages. In 2011, 100 tons of this herb was produced in an area as wide as 100 ha. The remained biomass after the extraction of *forskolin* still consisted of 90 % carbohydrate and can be used for ethanol production (Harde et al. 2014). In the USA, each year 10 million dollars is spent on disposal of apple pomace. However, it is more economical to reutilize it as a feedstock for ethanol production. In fact, most of the food wastes have the same potential to be utilized in ethanol industry rather than being composted or consumed for animal feed in which the market does not seem interested (Van Dyk et al. 2013). Similarly, in Mediterranean countries, olive cake, usually called olive mill solid waste (OMSW), formed after oil extraction can be applied as a promising source of ethanol production. The global production of this waste is reported as 4×10^8 kg dry weight. Besides, it can yield 3 g ethanol/100 g dry OMSW (Abu Tayeh et al. 2014). Gaining a giant industry in instant noodle production, Korea produced more than 2,106 ton instant noodle residue in 2011. By treating this residue through boiling, washing by hexane and then filtration, a solid and a liquid phase was produced which can be used for ethanol and biodiesel production, respectively

(Yang et al. 2014). In addition to this, Korea has the potential to profit a 40,000 ton log waste remained after being used for mushroom cultivation annually (Kim et al. 2010). Palm oil provides a wide industry in different continents such as Asia, Africa, and Latin America. The empty fruit bunches left after oil extraction are capable of being used for ethanol production (Chiesa and Gnansounou 2014).

The main steps of producing bioethanol from lignocelluloses include pretreatment, saccharification or enzymatic hydrolysis, fermentation, and distillation. However, for developing these steps, it is of crucial importance to learn about the nature and structure of the feedstocks first (Taherzadeh and Jeihanipour 2012). Lignocellulosic biomass consisted of three main components including cellulose (30–50 %), hemicellulose (15–35 %), and lignin (10–30 %). Cellulose is a crystalline polymer, made of long-chained glucose units that should be broken down to its monomers to become consumable by microorganisms for ethanol production. In contrast, hemicellulose is comprised of five-carbon sugars. Despite the fact that hemicelluloses can easily be degraded to their building blocks, i.e., xylose and pentose, their fermentation is complicated since most strains are naturally able to ferment only six-carbon sugars to ethanol. The lignin part of lignocellulosic biomass is made of non-fermentable phenolic compounds which can be recovered and applied as a solid fuel for electricity and heat supplement in ethanol or butanol production plants (Nigam and Singh 2011). Therefore, in order to convert lignocelluloses to fuel ethanol more efficiently, applying an effective pretreatment is in priority since it can break the recalcitrant structure of lignocellulose (Limayem and Ricke 2012). However, it should be noticed that while the pretreatments enhance the efficiency of enzymatic hydrolysis and enable the better digestion of released sugars by increasing the biomass surface area or changing its chemical structure to a more accessible material, it would affect the economical feasibility of a process as well. In fact, it is stated that pretreatment step constitutes 20 % of the overall process operational costs. Thus, finding a proper pretreatment can significantly economize the lignocellulosic ethanol production which is still in pilot scale and needs further investigation to become economically commercial, the same as first-generation bioethanol (Paulová et al. 2014).

Physical, solvent fractionation, chemical, and biological pretreatments are different options that can be applied to lignocelluloses. However, thermochemical conversion processes are also available to convert them to other fuels as well (Taherzadeh and Karimi 2008). Figure 2.2 shows a schematic diagram of various pretreatments that deal with lignocellulosic material leading to ethanol production.

Through physical pretreatments, i.e., ball milling, grinding, chipping, and freezing, the biomass size is reduced by mechanical stress, making it more susceptible for further enzymatic hydrolysis by improving the surface area to the volume ratio. In fact, this type of treatment prepares the biomass for better digestion, though it is not able to hydrolyze recalcitrant structure solely (da Costa Sousa et al. 2009). Solvent fractionations act as a hydrogen-binding disrupter to solubilize the components. Organosolv process, phosphoric acid fractionation, and ionic liquids (ILs) are some of the examples (da Costa Sousa et al. 2009). For instance, organosolv method is usually applied to high lignin biomass providing the opportunity of removing the

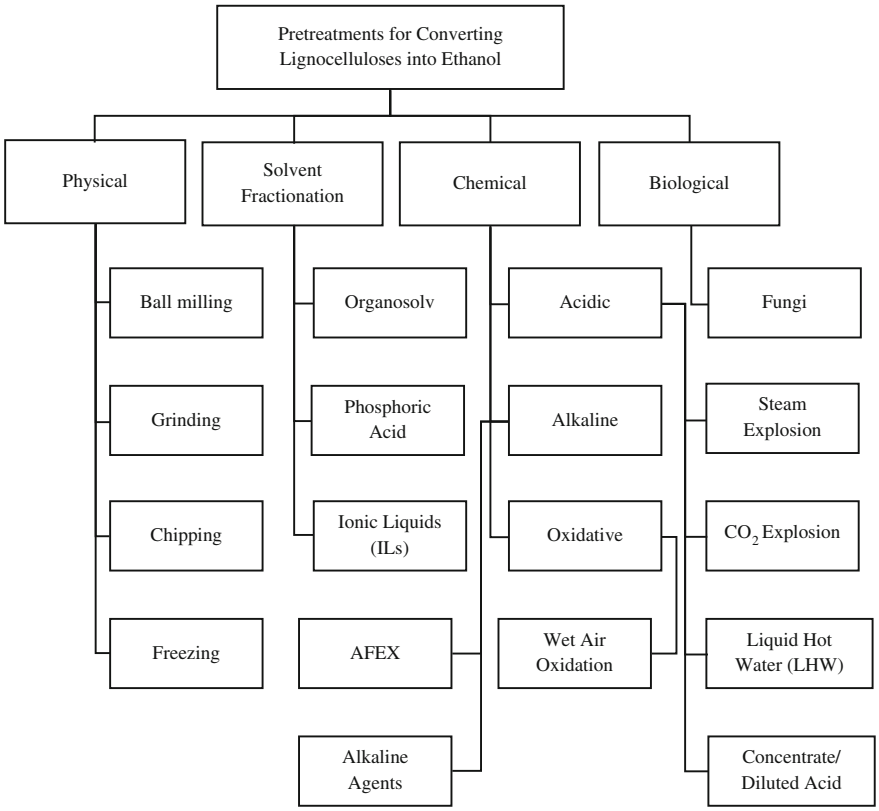

Fig. 2.2 Various pretreatment methodologies for lignocellulosic materials

pure lignin as a by-product. This happens by aiding an organic solvent, usually alcohols, in the presence or absence of a catalyst. Catalyst concentration, temperature, and time range are selected depending on the structure of lignocellulose, and the percentage of lignin and hemicellulose exist in that (da Costa Sousa et al. 2009; Haghighi Mood et al. 2013). This type of pretreatment is capable of producing considerable amount of inhibitors, furfural and hydroxymethylfurfural (HMF), and is less efficient in hydrolyzing hemicellulose sugars (Limayem and Ricke 2012). In phosphoric acid fractionation, different components of lignocellulosic biomass dissolve in various solvents (phosphoric acid, acetone, and water) at 50 °C resulting in its fractionation into amorphous cellulose, hemicellulose, lignin, and acetic acid. ILs are organic salts consisted of cations and anions. The anions present in ILs are capable of forming hydrogen bond with the hydroxyl protons of the sugars present in lignocellulosic biomass. Thus, the linkages between cellulose, hemicellulose, and lignin degrade, and the antisolvents would recover ILs via flash distillation (da Costa Sousa et al. 2009; Haghighi Mood et al. 2013). Chemical pretreatments are more common than the former and can be categorized into three main methods, i.e., acidic,

alkaline, and oxidative. Acidic methodologies are carried out based on applying concentrated or dilute acid, steam or CO_2 explosion, and liquid hot water (LHW) through which mostly lignin and hemicellulose get involved in a chemical hydrolysis process. However, during the pretreatment, some inhibitor compounds such as furfural, 5-hydroxymethylfurfural (5-HMF), phenolic acids, aldehydes, levulinic acid, and aliphatic acid form which limit the enzymatic hydrolysis (da Costa Sousa et al. 2009). Despite the fact that concentrated acids result in less toxic and inhibitor compounds with the sugar yield of 90 %, they are mainly corrosive, toxic, and hazardous, requiring corrosion-resistant reactors as well as acid recovery considerations which increase the operational costs. Thus, it is more preferable to use dilute acids; however, they are more likely to form toxic compounds and phenolic substances which are problematic to saccharification (Limayem and Ricke 2012). Steam explosion is the most common pretreatment for plant biomass since less dangerous chemicals are used and it has the ability to improve enzymatic hydrolysis, reduce the inhibitor formation, and remove hemicelluloses completely (Nigam and Singh 2011). Through this method, the grounded biomass is being heated by a high pressure steam for a few minutes, and in a sudden process, the pressure will get back to the atmospheric condition via an adiabatic expansion, leading to the hydrolysis of hemicelluloses and partly depolymerization of cellulose and lignin as well (Soccol et al. 2010; Kahr et al. 2013).

Among all explosion methods available as pretreatment, CO_2 explosion has gained more popularity since it needs a lower operational temperature and costs less. It is categorized in acidic pretreatments as a consequence of its ability to form carbonic acid (behaving like an acid-catalyzed process) while dissolving in water (Nigam and Singh 2011; Haghighi Mood et al. 2013). The same feature is accessible by using LHW. In fact, water exhibits acidic characteristics at high temperatures, solubilizing most of the hemicellulose and improving the digestibility of cellulose. Moreover, during LHW treatment, inhibitor formation and sugar degradation would be avoided if the pH is controlled at 4–7 (Roy et al. 2012a; da Costa Sousa et al. 2009). By applying alkaline approaches, intermolecular ester bonds cross-linking xylane hemicellulose and other components would go through a saponification process (Nigam and Singh 2011). This pretreatment can mostly expose cellulose and hemicellulose to enzymatic hydrolysis and extract the lignin existed in agricultural residues or herbaceous crops (Roy et al. 2012a; Soccol et al. 2010). Alkaline catalysts, i.e., sodium hydroxide, potassium hydroxide, calcium oxide (lime), and ammonia, are usually applied as well as ammonia fiber explosion (AFEX) as an alkaline methodology. The AFEX is an alkaline process at high pressure (1.72–2.06 MPa) and moderate temperature (60–120) acting like other explosion methods (Haghighi Mood et al. 2013). However, the ammonia supplement and recovery's cost, its handling, and high energy requirement during recompression are of major drawbacks (Roy et al. 2012b). Oxidizing agents such as air/oxygen or hydrogen peroxide as an oxidant can remove hemicellulose and lignin as well as solubilizing the cellulose at temperatures higher than 120 °C and pressure range of 0.5–2 MPa which are considered as wet oxidation methods. The presence of alkaline during this process increases the yield of monosaccharide sugars and

decreases the formation of inhibitor compounds (da Costa Sousa et al. 2009; Haghighi Mood et al. 2013). Biological methods are also available to deal with the recalcitrant structure of lignocellulosic materials without requiring much energy. During this pretreatment, fungi (usually white rot) are used to secrete the extracellular enzymes such as lignin peroxidase or laccase to remove lignin (da Costa Sousa et al. 2009).

Pretreated lignocelluloses have to be hydrolyzed with enzymes, so that its sugars get saccharified, being available as monosaccharides such as glucose or xylose depending on the structure of the feedstock. Enzymatic hydrolysis usually occurs by using cellulase, endo/exoglucanase, β-glucosidase, and xylanase (Kahr et al. 2013). If the pretreatments are not done well and the amount of lignin is still high, the accessibility of cellulase to cellulose would be reduced and more enzymes will be required since lignin has a non-productive adsorption effect on the enzyme. Moreover, phenolic groups formed after degradation of lignin make cellulase enzymes inactive (Limayem and Ricke 2012). Thus, usually surfactants are added to avoid the side effect of lignin on enzymes (López-Linares et al. 2014). However, high amount of glucose and cellobiose can cause inhibition to endo/exoglucanase and β-glucosidase (Limayem and Ricke 2012). In order to overcome this drawback, the fermentation step can be combined with the enzymatic hydrolysis.

Separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and consolidated bioprocessing are the main fermentation processing procedures for bioethanol production. However, some modifications such as the combination of SHF and vacuum evaporation (SHEF) (Choi et al. 2013), delayed SSF (dSSF) (Paulová et al. 2014) and prehydrolysis and simultaneous saccharification and fermentation (PSSF) (López-Linares et al. 2014) have also been applied to improve saccharification. In SHF, the hydrolysis and fermentation take place at separate vessels leading to product inhibition of enzymes (cellulase) as a result of glucose accumulation. However, since it is done at 50 °C in which the cellulase has higher activity, hydrolysis rate would increase and microbial contamination decreases. Moreover, it is more likely to remove lignin from the sugars as a solid fuel in SHF (Soccol et al. 2010). In comparison with SHF, SSF is more rapid and economical since the saccharified glucose is fermented into ethanol in the same tank simultaneously. Additionally, the rapid consumption of glucose prevents its accumulation and consequently enzyme inhibition. In fact, during SSF, more cellulose would be hydrolyzed to its building block sugars. Anaerobic condition, the presence of ethanol, and continuous process of glucose formation and consumption lessen the contamination risks as well. However, the optimum temperature for the enzymes (45–50 °C) and fermenting microorganisms (28–35 °C) is different, and also limitation in accessibility of carbon source for microorganisms might happen at the beginning of the process (Paulová et al. 2014). There are two options for overcoming the mismatch temperature problem in SSF by using PSSF or dSSF. Through PSSF, presaccharification is carried out at enzymes' optimum temperature (usually 50 °C) for 24 h followed by SSF at a lower temperature (40 °C) to facilitate the fermentation and decrease the viscosity of solid–liquid mixture before the addition of microorganisms (López-Linares et al. 2014).

The dSSF or delayed SSF is designed not only to eliminate the mismatch temperature limitations but also to omit the low glucose availability in the early stages of SSF and improve the ethanol productivity as well. Paulová et al. (2014) found out that by delaying the inoculation for 12 h, the cellulose and ethanol concentration can increase to 33.3 and 9.2 g/L at 32 h of fermentation, respectively, and shortening the process length up to 60 %. However, the final ethanol concentration is not as high as being distilled economically, since for an economic distillation, the ethanol concentration should exceed 4 % w/v. Thus, fed-batch dSSF with gradual feeding of prehydrolyzed medium was performed to enhance the final ethanol concentration and reduce the rate of inhibitor's accumulation and media's viscosity as well. In addition to the above-mentioned fermentation techniques, CBP is available in which the enzyme (cellulase) is produced with the aid of a fermentative/cellulolytic strain in an aerobic condition. After switching the condition of the vessel to the anaerobic mode, the strain is able to hydrolyze and ferment the saccharified sugars to ethanol. Despite the fact that a wide range of eukaryotes and prokaryotes have shown the ethanol production capability, most of them face with the limitations such as deficiency of sugars co-fermentation, low ethanol yield, low tolerance to chemical inhibitors (aerobic formed metabolites), intolerance to high temperatures, and low ethanol concentration (Limayem and Ricke 2012; Zerva et al. 2014). However, for instance, *Paecilomyces variotit* could successfully co-ferment the agro-industrial wastes (wheat bran, corn cob, and brewers' spent grain) to ethanol yield of 80 % of theoretical value by hydrolyzing glucose and xylose through xylanase, β-xylosidase, endoglucanase, and β-glucosidase which were produced in an aerobic mode by the strain (Zerva et al. 2014).

The most common method to separate the produced ethanol is distillation; however, membranes have been used as well in case that the final ethanol concentration is not high enough to be distilled. Fractional distillation can separate water and ethanol based on volatility. In this process, the mixture boils, and since the boiling point of ethanol is lower than water, it changes into steam sooner and is recaptured with a concentration of 95 %. Then, the water will be condensed and removed. Continuous distillation column is usually used in large industries and biorefineries in which liquid mixture is heated and flow continually. At the top and bottom of the column, volatiles and residues are separated, respectively (Limayem and Ricke 2012). Recently, Trinh et al. (2013) used pervaporative separation of bioethanol produced from the fermentation of waste newspapers. In this method, one of the components with higher affinity and diffusivity to the membrane can preferably be separated. When using membranes, its performance should be considered as well which is mostly dependent to the ethanol concentration, operational temperature, and fouling. The performance of membranes is affected by microbial cells, inorganic salts, sugars, and chemical inhibitors that exist in fermentation broth. The results showed that by using newspaper wastes, it is possible to produce 4.1 % ethanol in 48 h and separate it successfully through a hydrophobic polymeric polydimethylsiloxane (PDMS) membrane without any considerable reduction in performance (Trinh et al. 2013).

So far, some researchers have focused on finding new feedstock for bioethanol production including agricultural residues and municipal or industrial wastes, while others have tried to optimize the process conditions including pretreatment, hydrolysis, microorganisms, fermentation, and distillation. Although it is not possible to investigate all these studies in details, a short introduction of new findings is presented in the following.

Different countries can invest on production of second-generation ethanol depending on the type of wastes they have challenges with the most. For instance, the annual production of cotton is about 23 million ton. The cotton-based waste textiles are consisted of cellulose that can be separated and used for bioethanol production. Jeihanipour and Taherzadeh (2009) investigated the possibility of complete conversion of waste cotton-based textiles to ethanol. Although cotton does not contain lignin or hemicellulose, the crystallinity of its cellulose is high. Therefore, pretreatments should be performed before enzymatic hydrolysis to break down the hydrogen bonds between glucan chains of the crystalline cellulose and obtain the amorphous cellulose instead. Alkaline pretreatment of cotton linter (12 % NaOH) at 0 °C led to 86 % of ethanol theoretical yield after 4 days. In another study (Jeihanipour et al. 2010b), they studied the effect of *N*-methyl-morpholine-*N*-oxide (NMO or NMMO) on the enhancement of ethanol and biogas production from waste textiles since this pretreatment can be applied in industry. The results revealed that by using 85 % NMO (dissolution mode) at 120 °C for 2.5 h, the enzymatic hydrolysis would be increased to 100 % and ethanol amount of 460 g per each kg of cellulose.

Coffee residue wastes (CRW) contain 37–42 % fermentable sugars and is produced during the production of instant coffee. If these wastes dispose to the environment, they can cause problems since they contain toxic compounds. The annual production of coffee in 2010–2011 was estimated as 8.2 million ton. Thus, a large quantity of CRW are produced annually that can be converted to ethanol after a proper pretreatment. Choi et al. (2012) applied popping pretreatment to reduce the lignin and hemicellulose content of CRW as well as improving the degradation of its cellulose as a novel pretreatment. The popping equipment operated at optimum temperature and pressure of 150 °C and 1.47 MPa for 10 min, and then, the pressure reduced to the atmospheric condition again. By conducing the SSF after 96 h, 45.9 g ethanol obtained from 300 g CRW. They also used the same pretreatment procedure for producing ethanol from Mandarin peel (MP), Citrus unshiu, and decreased the amount of *D*-limonene, an inhibitor substance that exists in citrus peel. The SEM images showed that popping pretreatment can efficiently reduce the size of MPs and enhance the substrate surface area. It has also led to the hydrolysis of more xylose and glucose as well as the reduction of *D*-limonene to below 0.01 wt%. Further bioprocessing of hydrolyzed sugars by SHEF, the combination of SHF and vacuum evaporation, yielded 46.2 g/L ethanol in 12 h (Choi et al. 2013).

Waste money bills are used by Sheikh et al. (2013) as a new cellulosic material for bioethanol production. In Korea, 2,357 ton waste money bills are incinerated annually which have been duplicated recently. In USA, 500 million dollars has been allocated to manage waste money bills. Thus, its cellulose content can be

saccharified and fermented to ethanol. Waste money bills pulverized after soaking in 1.2 % NaCLO for 5 min, sterilizing with 70 % ethanol, and drying at 105 °C. As a pretreatment, 0.5 % H_2SO_4 was used at 121 °C for 30 min. Enzymatic hydrolysis was done at 50 °C for 72 h, and fermentation was conducted at three different conditions, i.e., anaerobic, anoxic, and anoxic with benzoic acid (0.4 mM). Each led to ethanol concentration of 1.00, 17.22, and 22.01 mg/ml, respectively. The *Saccharomyces cerevisiae* cells in acidic conditions are very permeable to weak acids while being impermeable to anions; thus, they can exhibit an adaptive tolerance in acidic conditions. This leads to accumulation of preservative anions and transient reduction in the pH level that can improve the ethanol yield. Dubey et al. (2012) also produced ethanol from waste papers through dilute acid (0.5 N H_2SO_4) treatment at 120 °C for 2 h and obtained 3.73 g/L ethanol. Their novelty took the possibility of xylose fermentation as well as glucose with the aid of *Pichia stipitis* into account. Economic feasibility and sensitivity analysis of ethanol production from various waste papers (newspaper, office paper, cardboard, and magazines) were investigated by Wang et al. (2013a) with the aid of *Zymomonas mobilis*. They suggested that separation of papers from other municipal wastes and recycling it into new papers is nearly harder than converting its cellulose to ethanol. They concluded that except magazines that contain impurities, other types of waste papers can economically be used for ethanol production if the office papers and newspapers receive only dilute acid and oxidative lime pretreatment, respectively. The maximum glucose and xylose yield was related to office papers, whereas the minimum quantity was obtained from magazines.

The possibility of ethanol production from kitchen wastes (restaurants, cafeterias, dining halls, household kitchens, and a food processing plant) was studied by Cekmecelioglu and Uncu (2013). Glass, metals, and plastics were first separated, and the rest was grounded into a composite. The waste consisted of fruit's peel, vegetables, bakery's wastes, coffee residues, beans, and cereal foods. Dilute acid and hot water used as pretreatments. However, the results revealed that none of them were as efficient as untreated wastes. When no pretreatment was done, ethanol concentration was 23.3 g/L, whereas it was obtained as 17.2 g/L under hot water treatment, which was a bit higher than the one treated with dilute acid. In another study, Matsakas et al. (2014) utilized household food wastes (HFW) for production of ethanol at high dry material (DM) content. They just performed enzymatic hydrolysis (8 h) on the HFW with the DM of 45 and 35 % which yielded ethanol concentration of 42.78 and 34.85 g/L, respectively, after 15 h of fermentation. They suggested that the solid waste, remained after fermentation, still contains cellulose that can be fermented as well if its recalcitrance being removed by hydrothermal pretreatments (microwave at 200 °C for 10 min) in the presence of acetic acid as a catalyst. This new waste led to ethanol concentration of 15.92 and 11.44 g/L at DM of 45 and 35 %, respectively.

In Mediterranean countries, there is a possibility of ethanol production from different seaweeds, duckweeds, and water hyacinth which are regarded as residues and contain considerable amounts of cellulose. Pilavtepe et al. (2012) used *Zostera marina* residues, considered as an environmental problem in Mediterranean beaches because of its bad smell, to produce ethanol. The plant was first extracted by

supercritical CO_2 (P = 250 bar, T = 80 °C) and ethanol (20 %) and then pretreated at different acid (H_2SO_4) concentrations and reaction times at 120 °C followed by an enzymatic hydrolysis. The SSF yield was obtained as 8.72 % after 24 h. In another study (Pilavtepe et al. 2013), they transformed *Posidonia oceanica* residues to bioethanol at the same conditions as their previous study. The ethanol yield in a 2-L fermenter attained as 62.3 %. Duckweeds and water hyacinth are sea plants capable of growing rapidly in wastewater to clean it and then being used as bioethanol feedstock. Physical and chemical pretreatments such as drying, boiling, steaming, sonication, dilute acid and alkaline are required to process sea plants into bioethanol (Bayrakci and Koçar 2014).

A wide range of agricultural residues have been used for ethanol production. The global production of cellulose, hemicellulose, and lignin from agricultural residues including wheat, barley, corn, rice, soybean, and sugarcane accounts for 3.7 Pg/year (Bentsen et al. 2014). In 2004, it was estimated that the potential bioethanol production from crop residue can reduce 29 % of the gasoline consumption (Kahr et al. 2013).

López-Linares et al. (2014) used rapeseed straw for bioethanol production through different bioprocesses, i.e., SHF, SSF, and PSSF. They concluded that high solid rates at SSF and PSSF caused an inhibition to microorganism, while at SHF, the theoretical yield was improved. De Bari et al. (2014) tried to convert the produced glucose and xylose to ethanol after steam explosion and acidic pretreatment of corn stover. Xylose isomerase is able to convert xylose to ketose (xylulose) which is consumable by *S. cerevisiae* through pentose phosphate pathway (PPP). However, the optimum pH and temperature for yeast and xylose isomerase are different. Thus, sequential addition of enzymes and microorganisms should be applied in order to make the conversion simultaneously possible. At first, the pH and temperature were adjusted at suitable range for cellulose hydrolysis (pH = 4.8, T = 50 °C). After 24 h, they changed the pH to 6.5 and temperature to 60 °C followed by a pH adjustment at 7 after passing another 24 h and holding it on for 7 h more. Finally, the pH and temperature were adjusted to 6.5 and 35 °C, respectively, to be proper for the addition of yeast. The maximum xylose conversion in the best case led to the most ethanol production, 70 % on the basis of glucose, xylose, and galactose.

The ethanol produced from various lignocellulosic materials needs to be evaluated to be used as a fuel in engines. The standard limitations in different countries may vary depending on the environmental issues and industrial development. However, there are some general impurities (acetaldehyde, acetone, ethyl acetate, methanol, iso/n-propyl alcohol, and isobutyl/amyl alcohol) within the lignocellulosic-based ethanol that should be reduced or omitted due to their negative effect. Styarini et al. (2013) detected some of these impurities through gas chromatography and suggested that the maximum and minimum amounts were related to isobutyl and isopropyl alcohol according to their own standard protocol.

More details about bioethanol production are presented in Chap. 5.

2.2.3 Wastes to Biobutanol

Biobutanol was first produced via microbial fermentation by *Pasteur* in 1861 and later in 1914 was industrialized when *Weizmann* discovered *Clostridium acetobutylicum*, the strain that can ferment sugars to biobutanol through a process known as acetone–butanol–ethanol (ABE) fermentation (Stoeberl et al. 2011; Kumar and Gayen 2011). However, butanol as a solvent could not compete with the petrochemical-based type due to the high costs of raw material, product inhibition, and low ABE yield, about 0.28–0.33 g/g (Kumar et al. 2012; van der Merwe et al. 2013). Recently, the production of biobutanol as a biofuel has gained more attention since the conversion of lignocellulosic wastes as a non-food cheap raw material is available. However, commercial production of butanol is still not feasible if the yield is lower than 25 % (Kumar and Gayen 2011).

Furthermore, the fuel characteristics of butanol are more interesting than the ethanol. Unlike the ethanol, butanol can blend with petrol in different ratios to be used in car engines and it has higher calorific value and hydrophobicity as well as lower freezing point and heat vaporization (Kumar et al. 2012). It is also not corrosive and has more similar features to petrol (Han et al. 2013).

Similar to ethanol, various pretreatments and enzymatic hydrolysis should be applied on lignocellulosic materials to break down the recalcitrant structure of cellulose, hemicellulose, and lignin. The fermentation processes, i.e., SHF, SSF, or other modifications, are also done the same as for ethanol (Kumar et al. 2012). However, in ABE fermentation, there are two different metabolic pathways leading to butanol production. In the first step which is known as acid genesis, acetate, butyrate, hydrogen, and CO_2 are produced when the microorganism is in its exponential growth phase which then would be converted to acetone, butanol, and ethanol in a process named as solvent-producing step (Stoeberl et al. 2011). Like ethanol fermentation when using SSF, lack of sugar at the beginning of the process may lead to lower productivity for butanol production. Cheng et al. (2012) used a novel method, sequential SHF-SSF, to overcome the problem of lack of sugar at the beginning of fermentation. They used sugarcane bagasse and rice straw as a feedstock pretreated by 2 g/L H_2O_2 and 15 g/L NaOH at 120 °C for 20 min followed by an enzymatic hydrolysis for 2 days. Then, more pretreated feedstock within mixed bacterial culture was added to the media. The enzymatic hydrolysis and fermentation continued for the next 3 days. In fact, at the second day, the system was changed into the SSF process from the SHF one. This sequential technique led to butanol production of 1.95 and 2.93 g/L for bagasse and rice straw, respectively. However, these amounts were obtained as 2.29 and 2.92 g/L when the experiment is conducted by the SHF alone.

The major problems associated with butanol production include sustainable biomass selection, low productivity, butanol inhibition, and high recovery costs (Srirangan et al. 2012; Xue et al. 2013). Lignocellulosic wastes have balanced the obstacle of biomass supply to some extent. Nevertheless, it has added a new expense by necessitating the pretreatment step. There are some new articles trying to focus on

producing butanol from different new wastes with no requirement to pretreatments. Virunanon et al. (2013) used the starchy wastewater of Tapioca factories. Raw materials included cassava pulp (CP) and cassava starch wastewater (CWW) which were hydrolyzed by enzymes for 2 h in order to be used as a carbon source for both butanol and ethanol production. When the CP was used alone, the produced butanol was extremely low (0.03 g/L) and the acetone was undetectable, whereas the obtained ethanol was as high as 8.98 g/L. However, using the CWW alone resulted in ethanol, butanol, and acetone concentration of 1.76, 0.85, and 0.25 g/L. In contrast, the combination of CP and CWW yielded more butanol, i.e., 2.51 g/L, in comparison with ethanol, i.e., 1.76 g/L, and acetone, i.e., 0.6 g/L. This increase might be related to the presence of a substance in CWW that leads the metabolic pathway to more butanol production or the fact that the more starch has released more reducing and fermentable sugars. Felled oil palm trunk was also used as a new feedstock in two different ways, sap and trunk fiber, with the aid of various types of *clostridia* strains by Komonkiat and Cheirsilp (2013). The first one could be used directly; however, the latter needed pretreatment. The mixture of sap (30 g/L sugar) and yeast extract (1 g/L) after 144 h of fermentation resulted in butanol, ethanol, and acetone production of 7.3, 1.5, and 2.1 g/L, respectively, while a potential of mixture of pretreated oil palm trunk and the nitrogen source led to butanol, ethanol, and acetone concentration of 10.0, 0.19, and 3.88 g/L after 144 h of fermentation. Chen et al. (2013) suggested that non-pretreated rice straw can be used under the non-sterile condition as well as the sterile mode if the inoculation is added at a high concentration. By using non-sterile conditions, the cost of process can significantly be reduced. The concentration of butanol was obtained as 6.6 and 6.3 g/L under non-sterile and sterile conditions within the cell concentration of 2,331 mg/L.

Apart from this, in most cases, the lignocelluloses should be pretreated to enhance the productivity. Ranjan et al. (2013) investigated the possibility of butanol production from rice straw under diluted acid (1 % w/v H_2SO_4) and agitation (200 rpm) at 120 °C and 150 psi for 15 min. The agitation caused the rapid release of sugars, and the concentration of butanol, ethanol, and acetone was obtained as 13.50, 0.82, and 6.24 g/L, respectively. Decanter cake waste, the effluent of palm oil mill, was used as a substrate for butanol production after being pretreated by 1 % HNO_3 and a 15-min agitation at 150 rpm and 121 °C for 20 min as well. Cheap nitrogen source (whey protein or ammonium sulfate) was added to it which yielded 3.42 g/L of butanol (Loyarkat et al. 2013). The improvement of ABE process was investigated by Moradi et al. (2013) through an alkaline (12 % w/v) and H_3PO_4 (85 %) treatment of rice straw which led to butanol production of 2 and 1.4 g/L, respectively. When no pretreatment was done, the butanol concentration was 0.16 g/L. Both pretreatments not only enhanced the glucan content but also reduced the xylane and lignin. Furthermore, 2-year-old willow biomass was studied for ethanol production by Han et al. (2013). Acid hydrolysis was performed by 24 N H_2SO_4 at 30 °C for 1 h, then it was diluted with boiling water and hydrolyzed for an extra hour at 105 °C. By fermenting the stem and bark of the willow biomass, butanol concentration of 4.5 and 4.3 g/L was obtained. However, during acid hydrolysis, inhibitor compounds such as acetic and formic acids and furfural

formed. Some researchers tried to find a solution for decreasing the amount of inhibitor compounds by applying membrane-based technologies. For instance, Cai et al. (2013) separated furfural through a pervaporation process. After acid hydrolysis with 0.2 % w/v acetic acid at 170 °C for 30 min, the hydrolysate was detoxified by pervaporation. The maximum efficiency of furfural separation was achieved as 94.5 %. Then, the fermentation was conducted, and the butanol was separated by the PDMS membrane as well. The concentration of produced butanol, ethanol, and acetone was 12.3, 2.5, and 6.1 g/L, respectively. Since effluent of biohydrogen-producing bioreactor contains significant amount of butyrate, Chen and Jian (2013) used it for butanol production. However, mixed culture exists in the effluent may cause inhibition for ABE production which can be separated by membranes. The butyrate was not sufficient enough as a carbon source for the fermentation; thus, saccharose was added as well. Finally, the saccharose and butyrate concentration of 25 and 3.5 g/L, respectively, resulted in butanol formation of 0.47 mol/mol. A new pretreatment procedure by electrolyzed water was performed on distiller's dried grains with solubles (DDGS) to produce butanol (Wang et al. 2013b). The DDGS is a major co-product in ethanol fermentation of corn. Electrolyzed water was combined with acidic (1 % H_2SO_4) and alkaline agents, and the results were compared to solo alkaline (2.5 % NaOH) and hot water (140 °C for 20 min) pretreatments. The maximum butanol concentration obtained via hot water and acid-electrolyzed water were, respectively, 3.64 and 3.62 g/L, whereas the solo alkaline could only lead to butanol formation of 2.09 g/L after 96 h of fermentation.

Batch, fed-batch, and continuous process are some options for the fermentation step. The first two require a long time for sterilization of bioreactor and reinoculation with a low productivity and high solvent inhibition, whereas the latter is more economical and can overcome most of these hurdles; however, its contamination risk and capital cost are high. Different techniques are available in a continuous process such as free/immobilized cell. In free cell method, due to the mechanical agitation or air-lifting capability, the cells are free to move in the fermentation broth, which improves the mass transfer as well, whereas the cells are longer alive during solvent-producing step in the immobilized cell technique, since the mechanical agitation does not exist anymore. In order to improve the free cell fermentation process, cell recycling and bleeding or continuous flash fermentation is designed. Through cell recycling, the cells would be recycled to the bioreactor via a membrane which enhances the cell concentration and productivity of butanol by helping the fermentation broth to be kept at optimum state. This method can increase the butanol productivity up to sixfold. Flash fermentation, in contrast, consisted of three interconnected units, i.e., vacuum flash vessel, cell retention system, and fermenter. It can reduce the distillation cost and is more compatible with environment (Kumar and Gayen 2011).

Another option for enhancing the productivity of butanol and reducing the solvent production or increasing the tolerance of microorganism to butanol is obtained by applying modified microorganisms through mutagenesis and evolutionary and metabolic engineering. *S. cerevisiae* and *Escherichia coli* for instance can be used instead of *Clostridia* for butanol production without solvent formation (Xue et al. 2013).

However, there are some *Clostridia* strains that can produce butanol without producing acetone. Gottumukkala et al. (2013) used *Clostridium sporgenes BE01* to produce butanol from dilute acid-treated rice straw (4 % H_2SO_4 at 121 °C for 60 min) enriched with calcium carbonate (buffering agent) and yeast extract (nitrogen source) without acetone formation. When the hydrolysate was detoxified by anion resins and enriched with mentioned additives, butanol was produced as high as 5.52 g/L, while it was obtained as 3.43 g/L when no calcium carbonate was added to control the pH and enhance the tolerance of strain to butanol.

Butanol inhibition may occur in both steps of ABE process. During the acid production, the *Clostridia* strains suffer the acidification of cytoplasm or accumulation of anions leading to its growth reduction and butanol inhibition. In addition to this, butanol is toxic for *Clostridia* as a result of its lipophilic feature. In fact, butanol can cause physiological dysfunction on the cell membrane by disrupting the phospholipid contents and altering some physicochemical properties such as preferential transport of solute, permeability of the membrane, and the amount of glucose uptake (Xue et al. 2013; Kumar and Gayen 2011).

In order to separate butanol from other solvents and water, four distillation towers are required. Since the boiling point of acetone (56.53 °C) and ethanol (78.4 °C) is lower than others, they get distilled in the first tower followed by removing water and butanol as residue. While the second tower is designed to separate the acetone and ethanol, two series of towers and a decanter are targeted to break the azeotrope point of butanol and water (Kumar et al. 2012). Separating butanol with four distillation towers is not only energy consuming but also very expensive. Therefore, it is of interest to find new economical techniques for butanol separation. Adsorption, gas stripping, liquid–liquid extraction (LLE), perstraction, pervaporation, and reverse osmosis (R.O.) are of the examples. Abdehagh et al. (2014) reviewed various separation techniques for butanol. Through an adsorption, butanol is first adsorbed on an adsorbent surface, i.e., activated carbon, resins, and zeolites, following by desorption as a result of temperature increase until a concentrated butanol solution is obtained. This method is considered as one of the energy-efficient separation processes.

Gas stripping is a simple method through which the striper gas, nitrogen, or fermentation gases such as CO_2 or H_2 flow into the fermentation broth and carry the acetone, ethanol, butanol, and water with them at their equilibrium partial pressure. The stripping gas can further be condensed and returned to the fermenter. In spite of high gas flow rate, although the cells are not damaged, foaming might happen. Due to the simplicity of the process (not requiring complex chemicals or facilities), providing a low concentrated butanol condition to prevent the inhibition and high productivity of butanol and sugar-consuming rate, the selectivity is low and the heater and condenser need high amounts of energy. In contrast, LLE is a high selective technique in which an organic water-insoluble extractant is mixed with fermentation broth to remove butanol. However, the extractant with high distribution coefficient is usually toxic for the microorganism. Thus, the extraction may occur in an external column to avoid the low mass transfer to the extractant, emulsion formation, and cell growth inhibition.

Perstraction, pervaporation, and R.O. are the examples of membrane-based methods. The latter can improve the distillation process by dewatering the fermentation broth via a semi-permeate membrane at high pressures and achieving a high concentrated final product. In addition to enhance the dewatering, the membrane would not be affected by fouling if the fermentation broth goes through an ultrafiltration first. However, the ultrafiltration membrane should be replaced regularly due to the fouling. Perstraction can overcome some of the problems associated with LLE since it uses a membrane which is in contact with both extractant and broth on each of its sides. The most important point in this method is choosing a high selective membrane to butanol and an extractant with a high distribution coefficient. However, the fouling and expensive price of the membranes can reduce its popularity. A binary or multicomponent liquid mixture is likely to be separated via pervaporation. By using this method, the components would get separated after a partial vaporization based on their diffusivity and adsorption to the membrane rather than the volatility. In fact, permeate adsorbed to the membrane firstly and diffuse through it. Finally, it would desorbed and evaporate under low pressures (Niemistö et al. 2013). Of advantages of pervaporation are high selectivity, no influence on microorganism, prevention of substrate or nutrient loss, suitable for azeotropic mixtures, and requiring low operation temperatures and energy. However, it is faced with some limitations such as low permeation flux, membrane swelling, and concentration polarization.

Despite the fact that second-generation biobutanol production is still in its infancy, the effort for its development is still in progress. As an example, Nilsson et al. (2014) have recently introduced a novel method for butanol production. Through this technique, succinic acid (SA) obtained after fermenting the CO_2 by *E. coli* would be converted to butanol by being hydrogenated via catalysis. The SA is an intermediate product in the citric acid cycle which can be produced under the anaerobic fermentation as well. By integrating the processes in which SA is produced with a catalysis process, the possibility of butanol production might be feasible.

Chapter 8 of this book has a more specific view on biobutanol production.

2.2.4 Wastes to Biogas

Anaerobic digestion (AD) consisted of four series of bioreactions (hydrolysis, acidogenesis, acetogenesis, and methanogenesis) by consortium of microorganisms through which organic materials are converted into a mixture of CH_4 and CO_2 and a trace amount of water vapor, H_2S, and NH_3, which is known as biogas. Depending on the feedstock used and the operational conditions of the digesters, the biogas content varies. However, its major component is methane. Via the first step, referred as hydrolysis or liquefaction, hydrolytic or fermentative bacteria, which are capable of extracellular enzyme secretion, hydrolyze complex organic materials such as lipids, proteins, and polysaccharides to soluble monomers or oligomers such as

amino acids, long-chained fatty acids, sugars, and glycerol. In the second stage, acidogenic bacteria convert simple and soluble compounds obtained in the previous step to CO_2, H_2, alcohol, and low molecular weight volatile fatty acids (VFAs), e.g., propionic or butyric acid. Through the third step known as acetogenesis, hydrogen-generating bacteria metabolize the alcohols and VFAs anaerobically to form acetate, H_2, and CO_2. However, hydrogen-oxidizing bacteria or homoacetogens can produce acetate form the CO_2 and H_2. Finally, two groups of methanogens produce methane. Whereas acetotrophic methanogens utilize acetate as a substrate for methane production, the hydrogenotrophic methanogens reduce CO_2 by using H_2 as an electron donor and generate methane. In fact, 70 % of methane is attained through decarboxylation of acetate, while the rest is achieved by reducing the CO_2. However, a few amounts of methane are probable to be produced from other organic substrates such as formic, propionic, and butyric acids (Surendra et al. 2014).

The produced methane is targeted to be upgraded and used as a fuel in transportation or simply burned for domestic heating and cooking. It is of interest to notice that it has the potential for supplying the heat and electricity of a plant as well as a local area. It is estimated that 1 m^3 biogas which is comprised of 60 % methane equals to the heating value of 21.5 MJ (Surendra et al. 2014). Whereas in India, 1,281 MW electricity is produced annually from the agro-waste-based biogas. In 2007, Sweden supplied 11 % for its domestic fuel need (about 38 PJ) from anaerobic digestion of organic wastes (Sarkar et al. 2012). Furthermore, the digestate remains after the process is applied as a fertilizer for agricultural purposes (Ariunbaatar et al. 2014).

The LCA studies have proved that biogas production from energy/dedicated crops is in direct competition with food on agricultural land and water requirement (Graebig et al. 2010). It is worthwhile to remind that due to the increase in population and the huge rate of waste generation in recent decades, municipalities are involved in great trouble for waste management. Currently, incineration and landfilling are of the major disposal methods for most of the wastes which not only are threatening for the environment, causing soil and water contaminations or GHG emissions, but also are not cost-effective (Zhang et al. 2014; Kothari et al. 2010). Hence, a wide range of organic wastes are introduced to be digested in an anaerobic digester as the feedstock, including sewage sludge, agricultural residues, the organic fractions of municipal solid wastes (OFMSW), animal manure, and fruit and vegetable wastes.

According to the statistics, the annual production of MSW is reported more than 2.5 billion ton/year which can yield up to 50–70 % methane. Whereas the incineration or landfilling of MSW causes the loss of fertile lands, health problem, and GHG emissions, its AD would result in heat and electricity supplementation as well as producing a transportation fuel. However, there are some uncertainties over techno-economic potentials of biogas production from MSW in an industrial scale. In conjunction with this concept, Rajendran et al. (2014) studied six different scenarios by using Aspen Plus® based on the industrial data. They suggested that the main uncertainties over the techno-economic feasibility of biogas from MSW are due to the transportation and collection of wastes and reduced operations of the

plant. Furthermore, it was concluded that not only the biogas production from MSW as fuel is a positive investment, but also the integration of produced biogas with the one from WWTPs along with its upgrading through water-scrubbing process is the most economical and profitable venture. In addition, Hung and Solli (2012) compared four scenarios for managing the organic waste with incineration and biogas production to obtain either heat and electricity or a fuel for buses in Trondheim city. They claimed that in spite of negligible climate benefit of biogas production from MSW in Trondheim, the elimination of diesel fuels from the bus's engines and its consequent influences on less photochemical oxidant production and particulate matter formation have significant benefits. Their studied scenarios showed that construction of a new biogas plant in Trondheim is the most environmental-friendly one. Recently, Afazeli et al. (2014) investigated the potential of biogas production from livestock and slaughterhouse wastes in Iran, suggesting the possible annual production of 8,600 million m^3 biogas from livestock excreta. However, this sort of waste is still not efficiently used in developing countries like Iran. By taking into account some of the advantages of digesting livestock and slaughterhouse wastes, i.e., development in industry and job creation, cutting down the zoonotic disease transmission and medical expenses as well as odor controlling and managing the hazards associated with slaughterhouses and animal husbandries, it would be more rational to bring these wastes into consideration for a more sustainable and healthier society.

In the USA, the annual production of brown grease, consisted of trapped, sewage, and black grease collected from either the restaurants or food processing industries, is estimated as 1.84 million ton whose landfilling accounted for US$110 per metric ton in 2002. Besides the high disposal costs related to landfills, soil and water contaminations should be considered as well. Hence, this sort of waste was utilized in a high rate pilot-scale anaerobic digester for biogas production by Zhang et al. (2014). In order to reduce the water consumption during the digestion, pulp and paper liquid wastewater was also added. The methane yield was obtained as 0.4–0.77 m^3 CH_4/kg $VS_{removed}$, indicating the successful possibility of reutilizing it in digesters rather than landfilling.

Alkanok et al. (2014) estimated the food waste generation by supermarkets in Turkey between 5 and 45 tons/month or 1–10 tons/week. They utilized fruit, vegetables, meat, sugar, dairy products, and flower wastes at 10 % TS, under mesophilic conditions in a batch digester to produce biogas with the highest methane yield of 0.44 L CH_4/g VS_{added}. They suggested that if the wastes are separated at source and collected in a centralized biogas plant, being supplemented by trace elements and VFAs, ammonia, pH, and C/N ratio being monitored well, the process would be more efficient and stable.

Scano et al. (2014) evaluated a full-scale power plant for biogas production from fruit and vegetable wastes (FVWs). The estimated power output for the daily produced methane (290 Nm^3/day) based on 9 ton waste/day was calculated as 42 kW within annual electrical production of 300 MWh/year. In summary, they suggested a successful and economical AD power plant from FVWs.

Liu et al. (2012) focused on biogas production and its GHG emission reduction by co-digestion of food (FW)/fruit and vegetable wastes (FVWs) mixed with dewatered sewage sludge (DSS). They considered three scenarios in a pilot-scale continuous CSTR at mesophilic temperatures (35 °C) and obtained the maximum biogas yield of 0.72 m^3/kg VS_{added}. It is believed that the main source of GHG emissions in China is related to landfill disposal of MSW, since 79 % of it was landfilled in 2009. Hence, the GHG emissions produced after biogas production from MSW with the possibility of power or bionatural gas (BNG) generation were compared with its landfill disposal. Both scenarios associated with biogas production showed a significant reduction in GHG emissions compared to landfills. It was estimated that an AD plant with a capacity of 500 ton/day can reduce the GHG emissions for power generation and BNG up to 20,800 and 95,400 ton CO_2, respectively.

Around 40 % of fibers used in waste textiles are comprised of cellulose, mainly cotton or viscose. In Sweden, 53,000 ton cellulose is wasted annually which can be used for more than 20 Nm^3 methane generation as well as 4 TWh power per year by considering a methane yield of 415 ml (at STP) per g of cellulose. Jeihanipour et al. (2013) used waste textiles in a two-stage process for a high rate biogas production. However, they have already introduced a novel process for biogas production from cellulose in blended-fibers waste textiles (Jeihanipour et al. 2010a) and enhanced its production from high-crystalline cellulose by different modes of NMO pretreatment (Jeihanipour et al. 2010b). In their recent study, they used viscose and cotton fibers blended with polyesters (unseparated cellulose fibers) in both batch one-stage (SBR) and two-stage (SBR connected to a CSTR in series) processes. In addition, either the pretreated textiles with NMMO or untreated ones were digested in a semi-continuous two-stage reactor with different organic loads. The maximum biogas yield for viscose and cotton fibers was obtained, respectively, as 55 and 31 ml/g VS/day in a batch single stage. Whereas the two-stage process did not improve the biogas yield for viscose fibers, it enhanced the methane yield up to 80 % for cotton fibers. Besides, the results of a semi-continuous reactor showed that pretreatments can increase the yield by 100 %.

In Portugal, between 70,000 and 80,000 ton/year sardines are captured of which the half is processed in canning industry. The co-digestion of waste oils obtained from sardine canning industry with pig manure at ratio of 5:95 yielded 26 m^3 CH_4/m^3 feedstock. Since the sardine oil does not contain sufficient nutrients and its alkalinity is low, pig slurry was added to supply the nutrients as well as maintaining the buffering capacity (Ferreira et al. 2012). However, Eiroa et al. (2012) evaluated biomethane production potential of solid fish wastes. The maximum methane yield was almost the same for all types of fish, 0.47 g COD-CH_4/g COD_{added}; however, it was a bit higher for mackerel (0.59 g COD-CH_4/g COD_{added}) due to its more oil content.

Of factors influencing the AD process are pH, alkalinity, temperature, and retention time. Different microorganisms existing in the digester have a distinguished optimum pH and temperature. For instance, methanogenetic bacteria usually tend to work under neutral pH, while the fermentative ones are still active at

wider ranges (4–8). During acidogenesis, the formation of VFAs would result in a sharp pH dropping (Appels et al. 2008). At low pH, glycolytic enzymes, which are sensitive to acids, lose their function and high-level dissociated acids penetrate the cell membranes of methanogens and disrupt the macromolecules. In fact, high amount of VFAs can lead to inhibition of methanogens. In order to reduce the inhibitory effect of VFAs, different strategies such as adjustment of C/N by co-digestion or other options, addition of trace elements to accelerate the growth, and separating methanogenesis and acidogenesis step through using two-stage digesters have been introduced (Xu et al. 2014).

Kafle and Kim (2013) co-digested apple wastes (AW) and swine manure (SM) in a batch, either at mesophilic (36.5 °C) or at thermophilic (55 °C) conditions, as well as a continuous process at 36–38 °C to overcome methanogen's inhibition caused by both VFA formation and high ammonia content of swine manure. Although the biogas yield obtained at thermophilic conditions was higher than the mesophilic one, the methane percentage did not differ. Besides, the mixture of AW and SM could enhance the biogas yield up to 16–48 % more than the solo SM. The continuous digestion of the mixture yielded more biogas by increasing AW addition.

Different types of digesters are designed to improve the operation conditions. Standard rate (cold) digesters are the simplest one with long digestion periods (30–60 days) which are used in small scales. Feedstock is usually neither heated nor mixed in this type. In contrast, at high rate digesters, the feedstock is either heated or mixed to establish a uniform media for microorganism leading to more stability and efficiency of the digester (Appels et al. 2008). These types of bioreactors, usually known as upflow anaerobic sludge bed (UASB), are commonly used at industrial plants. Since the biological reactions take place at the bottom of the reactor, a denser sludge bed would guarantee the success of UASBs. Besides, the application of microbial consortia granules stimulates the interspecies electron transfer and high rate methane production (Sabra et al. 2010).

Anaerobic digestion may occur via batch or continuous process in a one- or a two-stage digester. In the batch mode, the wastes are entered to the reactor and the process step by step. One-stage digesters are used in large scales, and all the reactions take place at once in the same vessel. In contrast, two different reactors are used for acidogenesis and methanogenesis steps in a two-stage digester. This helps to avoid the inhibition caused by VFAs; however, the one-stage digester is easier to design and needs lower investments (Sitorus et al. 2013).

Digesters can operate at different temperature conditions, called as mesophilic (30–38 °C) or thermophilic (50–57 °C). At high-temperature conditions, the bioreactions occur faster as well as more pathogen destruction; however, the energy requirement and odor potential are higher, and finally, poorer stability is attained (Appels et al. 2008).

Depending on the TS of feedstock, the AD is performed as liquid (L-AD) or solid-state AD (SS-AD) process. The operational TS range for L-AD is between 0.5 and 15 %, while the SS-AD is more than 15 %. Although the reaction rate is higher and retention time is shorter in L-AD, it is faced with hurdles such as floating and stratification of fibrous substances. In contrast, SS-AD is more economical due

to its lower reactor volume, energy input, and almost zero mixing energy requirement (Liew et al. 2012).

The type of biomass used in a digester plays a key role in the final yield and the selection of operational criteria. It is believed that hydrolysis step is the most rate-limiting one. However, methanogens can be rate-limiting as well if the organic materials degrade easily. In order to overcome these problems and improve the yield of biogas as well as taking economical aspects into account, pretreatments are of interest (Ariunbaatar et al. 2014). However, co-digestion of different feedstocks would help as well. Various pretreatments such as physical or mechanical, chemical, biological, and their combination have been widely used for different feedstocks. Physical or mechanical pretreatments not only disintegrate cell membranes, but also improve the contact between bacteria and feedstock by increasing the surface area, resulting in better digestion consequently (Carrère et al. 2010). Sonolysis was used as a mechanical pretreatment on sewage sludge followed by a co-digestion with kitchen wastes by Cesaro et al. (2012) to enhance the biogas yield. After running the AD process for 45 days, the biogas increased up to 24 % within the sonolized sludge in comparison with untreated sample. As a matter of fact, ultrasonication can enhance the biodegradability of sludge through both physical and chemical reactions by cavitation phenomena and free radical formation. However, the co-digestion process was also beneficial. The pretreatment was performed at 20 kHz and 750 W with a probe diameter of 13 mm for 60 min.

Thermal pretreatments in contrast disrupt the cells by providing a pressure gradient on the cell surface which leads to the release of cell components followed by series of physical and chemical reactions between them (Bougrier et al. 2006). Solid slaughterhouse wastes rendering products (SSHWRP) were sterilized at 115–145 °C to disrupt its lipid cell's membrane by Pitk et al. (2012). The methane yield was obtained in the range of 390–978 m^3 CH_4/kg VS after this dry rendering method for different waste products, revealing that the melt waste yielded the most. They concluded that dry rendering methodology could recover 4.6 times more primary energy than unsterilized SSHWRP.

In chemical methods, agents such as acids, alkaline, and oxidants are applied to treat the biomass. Although this method has been frequently used, it should be avoided for high carbohydrate content substrates due to its fast disruption effects and the subsequent accumulation of VFAs which would result in methanogen inhibition. Biological pretreatments are usually carried out on lignocellulosic biomass or sewage sludge (Ariunbaatar et al. 2014). Fungal and bacterial consortium and enzymatic techniques are of examples of biological pretreatments. While fungi strains are mostly able to degrade lignin and hemicellulose, microbial consortium is more capable of attacking cellulose and hemicellulose. Moreover, despite the fact that enzymes are almost expensive and have a minimal influence on biogas yield, there are not usually applied in full scales. Liew et al. (2012) utilized four lignocellulosic wastes (corn stover, wheat straw, yard wastes, and leaves) with TS of 22 % to produce methane under an enzymatic hydrolysis by cellulase for 72 h at 50 °C and 150 rpm in a 30-day SS-AD process. Whereas corn stover resulted in maximum methane production of 81.2 L/kg VS, wheat straw, leaves, and yard

wastes led to methane yield of 66.9, 55.4, and 40.8 L/kg VS, respectively. However, Ye et al. (2013) suggested that it is more economical to co-digest agricultural residues rather than pretreating them before the AD process. By co-digesting rice straw, kitchen wastes, and pig manure with a ratio of 1:0.4:1.6, the methane yield was 383.9 L CH_4/kg VS. Since kitchen wastes are easily degradable substrates with high moisture, carbohydrate, and lipid and protein content, its solo digestion could lead to VFA formation and pH drop causing the methanogen inhibition. Thus, the addition of pig manure that is rich in organic substances and is able to balance the C/N ratio was done to increase the methane yield.

Some researchers reported ensilaging as an effective method in comparison with enzymatic pretreatments (Zheng et al. 2014). For instance, Kafle et al. (2013) reported that by ensilaging the fish industry waste (FW) with bread wastes (BW) at 25 °C for 22 days, the methane yield would increase to 441 ml CH_4/g VS from the initial value of 306 ml CH_4/g VS. Since the FW contains a large amount of protein, its ensilaging alone would lead to butyrate formation as a result of *Clostridia* strain's dominant role in fermentation. Thus, by adding BW that mainly consisted of carbohydrates, sufficient energy for growth of lactic acid bacteria (LAB) will be provided and the fermentation would drive under the LAB influence.

More detail about biogas production is presented in Chap. 6 of this book.

2.2.5 Wastes to Biohydrogen

Hydrogen is the cleanest fuel that can be used in transportation sector since its only by-product when it is either used in fuel cells for electricity generation or heat engines as a fuel is water, which is not only a pollutant but also a favorable product (Show et al. 2011; Chong et al. 2009). Hydrogen is a colorless non-toxic gas without any odor or taste (Chong et al. 2009), which in comparison with other fuels generates more energy, about 122 kJ/g (Sekoai and Gueguim Kana 2013), having the calorific value of 143 GJ/ton (Show et al. 2011). Even though the current hydrogen application is limited to ammonia manufacture (49 %), petroleum refining (37 %), methanol production (8 %), and miscellaneous uses (6 %), it is believed that it can be a strategic bridge to the future of world's energy (Kırtay 2011). At present, there are various technologies available for hydrogen production such as chemical (steam reformation or coal gasification), electrochemical (water electrolysis), and thermochemical (biomass gasification or pyrolysis) methods (Azbar and Levin 2012; Das et al. 2014). Whereas the first two methodologies are extremely dependent on fossil fuels, turning them into energy-intensive approaches with a great potential of GHG emissions, the latter is carbon neutral since the generated CO_2 is fixed through photosynthesis by the new biomass. However, tar and char are the most undesirable by-products after thermochemical techniques (Kırtay 2011; Bartels et al. 2010). Therefore, biological methods are gaining more attention in recent decades as a new approach which has changed it into biohydrogen production. Albeit, the economic aspects of biohydrogen production are still under

question and further researches and modifications should be carried out to make it commercially established (Show et al. 2011).

Biological methodologies of biohydrogen production are categorized into four groups including direct/indirect water photolysis by cyanobacteria/green or blue algae, photofermentation of organic compounds by photosynthetic bacteria, dark fermentation of organic compounds by anaerobic bacteria, and hybrid reactors or microbial electrolysis cell (MEC), which is the integration of dark and photofermentation (Ren et al. 2009; Kırtay 2011; Hay et al. 2013).

Regardless of the higher yield provided by photolysis or photofermentation, the commercial light supplementation in photobioreactors is not economic enough to develop it into an industrial scale. Within dark fermentation, there are main challenges as well, such as low yield and hydrogen inhibition due to the accumulation of end products (propionate, lactic acid, and ethanol) (De Gioannis et al. 2013). However, it is more suitable to mature dark fermentation or hybrid systems in which organic wastes and residues even lignocellulosic materials are used as the merely source of electron and energy for hydrogen formation. Of other advantages of dark fermentation are process simplicity, less energy requirement, and more cost-effective production through reutilizing wastes and residues (Sydney et al. 2014). However, there are some key factors that should be considered for enhancing the biohydrogen production via dark fermentation, e.g., microorganism, pH, temperature, organic load, inoculum pretreatments, HRT, substrate, and trace elements.

During anaerobic fermentation, two groups of bacteria are active. While some of the bacteria can convert acetic acids to methane, others would utilize H_2 and CO_2. Therefore, the second pathway should be eliminated if the H_2 is preferable (Liu et al. 2013). As a matter of fact, the H_2 production would be enhanced if the metabolic pathway switches to more acid formation or the domination of acidogenesis step (Mohan et al. 2012). This would be obtained through inoculum pretreatment such as heat-shock or physical and chemical methods resulting in methanogen's activity reduction (Chinellato et al. 2013). However, it is not appropriate in full scales (Pan et al. 2008). Faloye et al. (2014) optimized the inoculum by using two pretreatment conditions, i.e., heat shock (pH = 8.36, T = 89 °C, t = 68 min) and autoclave (pH = 8.93, T = 121 °C, t = 15 min), which resulted in maximum hydrogen production of 0.78 mol H_2/mol glucose and 1.35 mol H_2/mol glucose, respectively. They, also conveyed a microwave pretreatment at pH 11 for 2 min on the inoculum and obtained 1.92 mol H_2/mol glucose, increased the H_2 production by 32.41 %. In addition to this, the scale-up optimization in a batch semi-pilot reactor with and without pH control resulted in 2.07 and 1.78 mol H_2/mol glucose, respectively (Faloye et al. 2014).

Depending on the consortia of bacteria, acetate or butyrate paths would be dominant in H_2 production in which 4 and 2 mol H_2 would be formed per mol of glucose, respectively (De Gioannis et al. 2013).

When the sugar concentration (organic load) is too high in the media, the pH decreases and more end products form resulting in inhibition of hydrogen-producing bacteria. This inhibition happens as a result of increase in ionic strength of the solution at low pH, which alters the metabolic pathway to solvent generation rather

than H_2 production. Indeed, non-polar dissociated acids penetrate into cell walls and release proton inside them where the internal pH is higher. This phenomenon would increase the energy requirement of cell for maintaining its pH at neutral level and consequently reduce the flux of glucose through glycolysis (Chong et al. 2009). Even at high pHs in which H_2 production is accelerated, inhibition may occur for hydrogen-producing bacteria since the rapid formation of acids can decrease the buffering capacity (Hay et al. 2013). The best approach for overcoming this hurdle is to adjust food-to-microorganism ratio (F/M) or design two-stage systems in which the first stage is used for H_2 production and the remaining organic biomass can be reconsumed by methanogens for methane generation in the second stage. This process would lead to biohythane production consisting of 10 % H_2, 30 % CO_2, and 60 % CH_4 which can be used in combustion engines as well with a higher thermal efficiency and power output (Cavinato et al. 2011). Simultaneous production of hydrogen and methane increases the total energy recovery by 100 and 30 % compared to single-stage hydrogen or methane production process, respectively (Liu et al. 2013). Cavinato et al. (2011) optimized a two-phase thermophilic biohydrogen production through reject water recirculation. They suggested that in order to prevent the methanogen activity, HRT should be low. However, it can cause a great decrease in pH, thanks to the high organic load. Therefore, instead of adding chemical agents to control pH, recirculation of the AD effluent to the dark fermentation reactor can buffer the system and maintain the pH at optimum level. The maximum hydrogen produced was 51 L H_2/kg TVS at a low organic loading rate of 16 kg TVS/m^3 d accounting for 37 % increase in H_2 generation. Chinellato et al. (2013) also obtained the maximum H_2 of 2,116 ml at organic loading rate of 30 kg TVS/m^3 by recirculating the digestate for pH control.

Pan et al. (2008) adjusted F/M ratio of food wastes at both mesophilic (35 °C) and thermophilic (50 °C) conditions instead of pretreating the inoculum to enhance the hydrogen yield. They indicated that pretreatment is an expensive approach for full-scale purposes and it is not certified that the entire of hydrogen-consuming bacteria are dead. Additionally, only the spore-forming H_2 bacteria survive after thermal treatments. The maximum hydrogen obtained at F/M ratios of 6 and 7 under mesophilic and thermophilic modes was 39 and 57 ml H_2/g VS, respectively.

The partial pressure of H_2 in the liquid phase is another crucial point that should be considered. The less the H_2 is dissolved in liquid phase, the more the H_2 is found in the form of gas. The hydrogenase enzyme can reversibly oxidize and reduce the ferredoxin. Oxidation of reduced ferredoxin will lead to oxidation of H_2 to proton which means a decrease in concentration of H_2 at gas phase. However, by nitrogen sparging, the concentration of hydrogen in liquid phase would decrease significantly (Chong et al. 2009).

The volumetric productivity is low in thermophilic mode ($T > 60$ °C), since the strains tend to grow in lower cell density in suspension media or switch their metabolic pathways to other reduced final products such as lactate, ethanol, and alanine rather than H_2. However, it can provide better conditions for H_2 production in reactors compared to mesophilic mode. Furthermore, in thermophilic mode, less

by-products are formed, the H_2 tolerance is higher, and it is economically feasible and best (Pawar and van Niel 2013).

Long HRT usually leads to inhibition of hydrogen-producing bacteria and methanogen activity. In fact, when the HRT is short, which means that dilution rate is high, washout might happen to methanogens since their specific growth rate is lower than hydrogen-producing bacteria (Cavinato et al. 2011).

Addition of some nutrients (peptone or yeast extract) and trace elements such as iron which is a key factor in hydrogenase and other enzyme generation, vitamins, and growth factor may enhance the H_2 production (Hay et al. 2013). Pan et al. (2013) investigated effect of ammonia on biohydrogen production from food waste. They concluded that at F/M of 3.9 and 8, the hydrogen yield of 77.2 and 51 ml H_2/g VS would increase to 121.4 and 60.9 ml H_2/g VS by adding 3.5 and 1.5 g/L ammonia, respectively.

So far, different wastes and residues have been used for hydrogen production as well as other biofuels. Municipal solid wastes, wastewater sludge, lignocellulosic materials, and industrial wastes are of some examples. However, some of these feedstocks should be pretreated in order to increase the yield. It is usually preferred to pretreat the lignocellulose before digesting them in an AD process since they have a heterogeneous and crystalline structure. Lignocellulosic wastes account for 220 billion ton worldwide which is equal to 60–80 billion ton crude oil. In China, the annual production of lignocellulose is estimated as 1.4 billion ton which has the potential of hydrogen production of 100 billion kg. It is stated that 10.3 kg cellulose is required to produce 1 kg H_2 equating to the energy provided by 1 gallon of gasoline (Ren et al. 2009). During 1998–2001, in Western Europe, 0.7 billion ton of agricultural and forestry residues were generated. In France, for instance, this amount was estimated as 374 million ton in 2006. Therefore, there is a great potential for using lignocellulosic wastes as a feedstock for different biofuel productions. Guo et al. (2010) reviewed hydrogen production from agricultural wastes via dark fermentation. Jia et al. (2014) applied fruit–vegetable wastes by alkali and acid pretreatments followed by enzymatic hydrolysis and obtained the maximum hydrogen production of 10.11 ml/h after acid hydrolysis. In South Africa, 2.95 and 7.88 million ton agricultural and municipal wastes are produced, respectively, of which only 35 %, mainly MSW, are recycled and the rest are either incinerated or landfilled. Sekoai and Gueguim Kana (2013) compared bean husk (BH), cornstalk (CS), and OFMSW individually and as a mixture to produce hydrogen. Each of BH and CS could hardly produce H_2 since they need pretreatments. However, in combination of same ratio of OFMSW and BH, 41.16 ml H_2/g TVS was produced, while the solo OFMSW produced 56.47 ml H_2/g TVS. Li et al. (2012) indicated that 4 million tons of agricultural waste is produced as well as 69 m^3 beverage wastewater per year which consisted of 74 % carbohydrate. They evaluated the techno-economic feasibility of hydrogen production from beverage wastewater and mushroom farm solid wastes (55 % cellulose) by considering two different pretreatment conditions for mushroom wastes with the aid of Aspen Plus software. The results showed the hydrogen production rate of 4.38 l/d/l with maximum annual

profit and return rate of 60 and 39 %, respectively, based on Aspen price. They concluded that the process is economically feasible.

In Korea, Japan, China, and Malaysia, food wastes are the major components of municipal solid wastes, about 20–54 %. In 2011, 930 tons of unconsumed food was wasted daily in Malaysia, which has been doubled over the past 3 years (Yasin et al. 2013). In Hong Kong, food wastes are estimated as 3,280 ton/year (36 % of MSW). Xiao et al. (2013) could achieve a hydrogen yield of 155.2 ml/g VS of food waste at 37 °C by adding 40 ppm $FeSO_4 \cdot 7H_2O$.

In India, 580 billion rupees of food items is wasted annually. Pasupuleti et al. (2014) studied the up-scaling of biohydrogen production in a semi-pilot biofilm reactor through optimizing organic load. They observed an inhibition at organic load as high as 66 g COD/l. However, they gained a maximum hydrogen production of 9.67 l/h. Gadhe et al. (2014) performed an ultrasonication on food waste at 20 kHz for 15 min at TS of 8 % to enhance the hydrogen yield and obtained 1,192 ml H_2. Elbeshbishy et al. (2011) also compared the single and combined effects of different pretreatments on food wastes. The maximum and minimum H_2 production was obtained as 97 and 46 ml H_2/g VS after single ultrasonication and alkaline treatment, respectively. Whereas the combination of ultrasonication and acidic pretreatment enhanced the hydrogen yield up to 118 ml/g VS, the combination of ultrasonic either with alkaline agents or with thermal methods had negative effect on hydrogen production.

Textile industries produce the most toxic wastewaters due to their need for applying different substances such as dyes, surfactants, and textile's additives. Since biological methods are not able to eliminate dye which is a structured polymer with low biodegradability, other methods including active carbon, cation exchange resin, solvent recovery, chemical catalysts, gold extraction, gas separation, and liquid adsorption are used to degrade it. The dark fermentation of textile wastewater at 37 °C, treated with active carbon and inoculated with municipal WWS having a sugar concentration of 20 g/L which was obtained after hydrolysis with amylase for 20 min at 70 °C, resulted in 1.37 mol H_2/mol reducing sugar.

Benincasa hispida or *petha* sweet is a solid waste produced in a sugar syrup process. About 30–35 ton of *petha* is produced daily which can be used for hydrogen production. Singhal and Singh (2014) pretreated the inoculum (cow dung) using microwave at 320 W for 5 min and obtained 14 mmol H_2/mol soluble sugar.

In developing countries, herbal medicines are popular and 80 % of world population tend to use it instead of chemicals. The wastewater produced after processing such kind of medicines contains a great amount of COD whose treatment is essential. Thus, it can be applied in AD process for biohydrogen production. At optimum pH and temperature of 6.5 and 50 °C, the maximum H_2 yield was 480 ml/g COD (Sivaramakrishna et al. 2014).

The liquid residue at the bottom of distillation tower of processing sugarcane to ethanol is called *vinass*. It is estimated that 12–15 L *vinass* is usually produced per one liter of ethanol. In Brazil, 25 billion liter ethanol was produced in 2012–2013, which resulted in production of 370 billion liter *vinass*. However, it is currently

used as a fertilizer, due to its low pH and high COD and potassium content that can contaminate soil and groundwater, and its application is limited. Therefore, it has the potential to be used as nutrient additives for carbon sources such as molasses and sugarcane juice for biohydrogen production. Adding *vinass* to sugarcane juice and molasses resulted in hydrogen generation of 7.14 and 3.66 mol/mol sucrose, respectively, with the aid of different microorganisms (Sydney et al. 2014).

In 2011, 80.3 million automobiles and vehicles were manufactured globally. An automobile industry with annual production rate of 400,000 cars is capable of producing 410,000 m^3 wastewater which is rich in heavy metals, grease, oil, and dye. The produced wastewater is usually treated with chemicals, and after decantation, the effluent that is called DECA can be co-digested with municipal or industrial sludge and glucose for biohythane production. However, the presence of compounds containing zinc or other metal salts can inhibit methanogens. While the methane was completely inhibited at concentration of 75 mg/L of Zn, the hydrogen was produced as 2.4 mol/mol glucose. It was reported that the addition of glucose could prevent the zinc inhibitory effect and decrease the hydrogen yield instead (Bajaj and Winter 2013).

Biohydrogen production is explained in detail in Chap. 7.

2.3 Challenges Ahead

Due to the everyday increase in worldwide energy demand and the depletion of fossil fuels as well as growing concerns on environmental issues, the global attention has been dragged on biofuels which are mainly based on biomass resources. However, other renewable types of energy such as wind, solar and geothermal are being investigated likewise. It is predicted that by 2025, the global energy demand would increase by 50 % (Ragauskas et al. 2006). The transportation sector has a great share in energy consumption, comprising the 60 % of global oil uptake (Tsita and Pilavachi 2013). In 2011, 94 % of the required energy in this section was supplied from fossil fuels, indicating that it has a great share not only in energy consumption but also on GHG emissions (Menten et al. 2013). About 15–22 % of world's GHG emissions are related to transportation vehicles of which 70 % are originated from diesel and gasoline usage on roads (Soimakallio and Koponen 2011; Chin et al. 2013). Since a 40 % increase in transportation sector is expected by 2035 (Chin et al. 2013), expanding the biofuel's portion in engines is being considered by politicians. According to RED, a directive enacted by European Union on June 2009, the application of biofuels in transportation sector in EU was about 3.5 % in 2008 which should be enhanced to 10 % by 2020, provided that its GHG emission's share be reduced by 35, 50, and 60 % until 2016, 2017, and 2018, respectively (Soimakallio and Koponen 2011). On the other hand, the US Energy Independence and Security Act depicts a 136 billion liter rise in ethanol production by 2022 from the initial amount of 41.9 billion liter in 2009, of which 79.3 billion liter must be obtained from advanced biofuels (Menten et al. 2013).

In 2009, the Europe exceeded the world on biodiesel production by achieving 65 % of its share. The global ethanol production was also 20 million gallon including the USA (54 %), Brazil (34 %), and EU (5 %) as the major manufacturers (Serra and Zilberman 2013). It is worthwhile to remind that these quantities are entirely supplied from first-generation biofuels. Even though there is a good potential for increasing the production rate of this sort of biofuels, some concerns, such as deforestation, devastation of biodiversity, and haze pollution, increase in GHG emissions, thanks to the change in land use, water shortage, loss of fertile lands, and "food versus fuel confliction" have taken it into criticism (Chin et al. 2013). In this case, advanced biofuels (second and third generations) might seem a good solution for some of these problems; however, they are somehow challenging as well.

Although 170 billion ton biomass is produced annually (Sivakumar et al. 2010), lignocellulosic materials such as straw that can balance the farmland fertility and organic carbon might be a challenge if be overharvested from fields (Lindorfer et al. 2014). Furthermore, the permanent availability of lignocellulosic feedstocks around all time of the year is under doubt.

From environmental point of view, GHG emissions of advanced biofuels should be calculated in order to prove its advantages. Many LCA studies have been carried out to figure out the GHG emissions of advanced biofuels. Recently, Menten et al. (2013) reviewed this issue by meta-regression analysis. They concluded that while the GHG emissions for third-generation biofuels produced from microalgae (G3) have the highest amount, cellulosic ethanol (G2 Ethanol) and biodiesel (G2 BtL) produced biologically and thermochemically, respectively, emitted lesser amounts. However, the measured values were higher in the USA than EU due to the selection of different approaches in the LCA study. Figure 2.3 displays a clearer comparison between the cases. Whereas fossil fuels as a reference index discharged 83.8 g CO_2/MJ in average, the G3, G2 Ethanol, and G2 BtL released 60, 19.7, and 19.5 g CO_2/MJ, respectively. Thus, the production of second-generation biofuels can reduce environmental concerns to some extent if only the proper method is applied.

According to Lee and Lavoie (2013), second-generation biofuels are supplied from homogenous, quasi-homogenous, and non-homogenous feedstocks which are mostly regarded as residues and wastes. Homogenous feedstocks like white wood

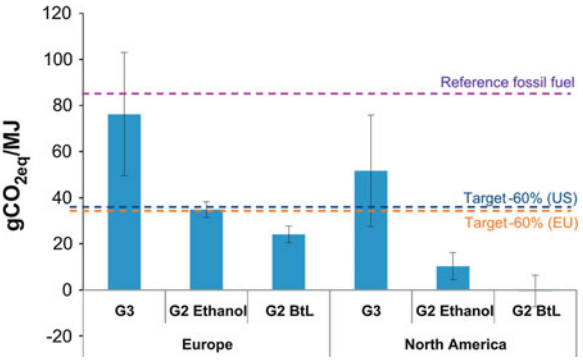

Fig. 2.3 Comparison of GHG emissions between advanced biofuels and fossil fuels in the North America and Europe (Menten et al. 2013)

chips cost about US$ 100–120/ton, while agricultural and forest residues as well as low-value feedstocks such as municipal solid wastes have lower prices of US$ 60–80/ton and US$ 0–60/ton, respectively. In spite of low price that non-food feedstocks offer, technological challenges decelerate their development. In most cases, the application of pretreatment which is usually energy intensive and energy expensive as a result of requiring chemical agents and detoxification step is critical. In addition, the price of enzymes (cellulase) usually needed for enzymatic hydrolysis of pretreated lignocelluloses during ethanol and butanol fermentation is predicted to reach US$ 0.12–0.2/L by 2015. Therefore, the amount of cellulose, hemicellulose, and lignin content of a biomass has a key role on the biofuel yield and costs. Furthermore, in order to make the process more economical, the conversion of hemicellulose or extraction of lignin has to be counted too. In North America, 45 % of the forestry biomass is made of glucan resulting in ethanol production of 313 L/ton with market and production price of US$ 0.68/L and US$ 0.3/L, respectively. Cellulosic ethanol has a value of US$ 212/ton of biomass whose biomass costs around US$ 60–80/ton. By taking the saccharification price into account as well, it would be revealed that this process is more expensive than the first-generation ones (Lee and Lavoie 2013).

Another technological challenge that put off the commercialization of second-generation biofuels is associated with genetic engineering progress in modifying high-tolerant and high-yield microorganisms. Besides, the development of more efficient and less energy-intensive pretreatment methodologies is still under investigation (Tsita and Pilavachi 2013). From another aspect, biofuel production from wastes postulates new stages of biorefineries (cluster biorefineries) where not only the wastes are collected and recycled but also value-added products are fabricated with the minimum to almost zero carbon emissions (Ragauskas et al. 2006; Chin et al. 2013). Moreover, integrated biorefineries would be more cost-effective if they are installed besides current petroleum refineries to benefit the existed infrastructures and pipelines (Yue et al. 2013). Since the spectrum of raw materials for second-generation biofuels are diverse, lack of experienced workers would also lead to some uncertainties in process yield such as causing errors in operation, production of unqualified products, and delay in order delivery (Yue et al. 2013).

Apart from mentioned technical hurdles, some social hindrances are also in the horizon. Although social acceptance is not a major obstacle, it can slow down the advancement of the process. The dispute of food versus fuel for the first-generation biofuels is a good example for lack of social acceptance in this issue. Furthermore, stakeholders and investors who have spent their money on first-generation industries are still waiting for the ball to drop, and naturally, they are less willing to start a new bargain. As a matter of fact, financial supports from governments and private investors or legislating new supportive policies could help the start-up of large scale second-generation biofuels. Additionally, local residents may prevent the establishment of new plants as a consequence of unawareness. In this case, developing ideas such as decentralized biorefineries or scattered small-scale plants which are more visible than a large plant may be restricted by more social groups (Chin et al. 2013; Yue et al. 2013).

As a more economical approach, it should be indicated that finding a new market for biofuels can also help its development. Conducting the airline and aircraft industries to utilize second-generation biofuels is a new gate for the market. Additionally, it has environmental benefits as well (Köhler et al. 2013).

In essence, it should be stated that the transition of first-generation biofuels to the newer versions does not mean to omit the primers. It is praiseworthy to mention that relying entirely on wastes and residues for continuous biofuel production is not possible since some of them are not abundant all the time. Hence, the integration of both first- and second-generation biofuels will be continued until achieving the most compatible technology to economics, environment, and society (Sims et al. 2008).

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Chapter 3
Pretreatment of Lignocellulosic Biomass

Marzieh Shafiei, Rajeev Kumar and Keikhosro Karimi

Abstract Lignocellulosic biomass is an abundant, nonfood-based, sustainable, and low-cost resource for the production of renewable fuels and chemicals. However, its inherent recalcitrance to biological conversion hinders its application for commercial production of biofuels. Therefore, a pretreatment step is applied to overcome the biomass recalcitrance prior to biological conversion. Worldwide research to improve the pretreatment efficiencies by understanding the biomass recalcitrant structure and changes made by pretreatment to the biomass structure is being carried out. In general, it is thought that the cell wall components, their distribution, and the inter- and intra-linkages between components influence the biomass recalcitrance. In this chapter, a brief introduction to the anatomy of the plant cell, cell wall, and the features that impact the biomass recalcitrance are presented. Furthermore, the most important parameters affecting the biofuel production are reviewed and some methods for their analysis are presented. Moreover, the leading pretreatment processes for the commercial production of biofuels are presented. For each pretreatment method, the process, reactions, and changes incorporated in the biomass during pretreatment as well as the efficiencies, advantages, and drawbacks are reviewed.

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3.1 Introduction

Carbon dioxide from fossil fuels combustion is one of the largest drivers of global warming (IPCC 2013). In short term, second generation of renewable fuels such as ethanol, biodiesel, and biomethane is the most applicable substitutions for transportation fossil fuels because of the similarity of their chemistry to the conventional fuels. Third generation of biofuels from algae is still in the preliminary stage and thus far has proven to be economically uncompetitive. The biofuels do not produce harmful residues and are essential for the reduction of the greenhouse gasses. Lignocellulosic biomass, the source for these fuels, is abundant, renewable, and motivates developments in agricultural and forestry industry sectors. The projected amount of biofuels is 90–170 Mt in 2030 based on different predictions (World-Energy-Council 2010; Dudley 2013; Ugarte et al. 2007). However, its predicted contribution in the transport sector does not exceed 4–5 % in 2030, mainly because of comparatively high manufacturing costs of these fuels (World-Energy-Council 2010).

The current production of fuel ethanol from sugar or starch-based feedstocks such as corn, wheat, and sugar cane competes with the food and feed and thus impact the food prices. The production of ethanol from low cost and widely available lignocellulosic raw materials such as agricultural and forestry residues does not directly compete with the food/feed and have negligible impact on their prices (World-Energy-Council 2010; Dale et al. 2014). The drawback of lignocellulosic materials, however, is their recalcitrance to biological conversion, which increases the manufacturing cost of the biofuels. The plants cells are aggregated in a compact structure. A high lignin content material called middle lamella sticks the cells together and thus physical size reduction to a certain extent is inevitable for commercial biofuel production. The accessibility of enzyme to cellulose is also hindered by the presence of lignin and hemicellulose. Therefore, a pretreatment step is applied to overcome this problem and to maximize the yield of enzymatic hydrolysis (Karimi et al. 2013; Taherzadeh and Karimi 2008; Wyman et al. 2005).

3.2 Recalcitrance Features of Lignocelluloses

Lignocellulosic materials from plants are feedstocks for the second generation of biofuel. For biofuel production, the dead plant tissue is used. Inside a dead cell, there is an empty chamber, called lumen, which is surrounded by the cell walls. Lumen is the place of cell organelles when the cell was alive. The plant cell wall, which constitutes a large proportion of the dead cell dry weight, contains enormous amounts of carbohydrates that can be converted to ethanol (Davison et al. 2013). However, these high molecular weight carbohydrates are wrapped in a compact structure which is highly resistant to biological conversion. The cell wall of most plants comprises a primary and secondary cell walls, although some cells only have primary cell wall. The primary cell wall is synthesized during the cell growth, and

when the growth is stopped, a thick cell wall deposits by layers, called secondary cell wall (Harris and Stone 2008). The secondary cell wall has three sub-layers of highly oriented cellulose microfibrils. The orientation in each layer differs from other layers. Main components of the cell walls are cellulose, hemicellulose, lignin, and small amounts of proteins, minerals, pectin, and other components. Cellulose microfibrils are surrounded and linked to a non-crystalline matrix composed of hemicelluloses, pectins, lignins, and proteins and acts as glue between the microfibrils. It is believed that the main barrier to the enzymatic hydrolysis of lignocelluloses relies on the structure of the cell wall and arrangement of these components, referred to as biomass recalcitrance (Ding and Himmel 2008; Harris and Stone 2008; Karimi et al. 2013).

Lignocellulosic biomass feedstocks for biofuels production are classified in the category of vascular seeded plants, and this category has two groups: gymnosperms (naked seed plants) and angiosperms (flowering plants). Softwoods, e.g., pine and spruce, are examples of potential biofuel feedstocks from classification of coniferous gymnosperms. Angiosperms are further classified into two groups: monocotyledons (monocots) and dicotyledons (dicots). Hardwoods, e.g., poplar and willow, are dicot angiosperms, while energy crops such as wheat straw, corn stover, grasses such as miscanthus, switchgrass, giant reed, and reed canary grass are among the monocot angiosperm feedstocks (Harris and Stone 2008).

There are dissimilarities in the cell's types and arrangements in the tissue of the gymnosperms and angiosperms. Furthermore, the compositions of the cell wall components, e.g., cellulose, hemicellulose, and lignin, as well as their amounts differ in gymnosperms and angiosperms. Thus, all of these variations significantly affect the results of pretreatment, subsequent enzymatic hydrolysis, and biofuel production (McCann and Carpita 2008). It is believed that pretreatment conditions for hardwoods, agricultural residues, and herbaceous plants are less harsh compared to the conditions for softwoods (Taherzadeh and Karimi 2008). The higher number of vessels in the hardwood and grass angiosperms which permit greater heat and mass transfer into the biomass matrix is believed to be the reason for lower recalcitrance for these feedstocks (Cochard and Tyree 1990; Hepworth et al. 2002; Kim et al. 2012). Conversely, the dominant hemicellulose in softwoods is heteromannan (hexosans), while the dominant hemicellulose polymer in the other types of plant is heteroxylan (pentosan). Fermentation of mannose (monomeric unit of mannan backbone) is possible with a large number of microorganisms, e.g., some species of *Saccharomyces cerevisiae*; however, there are very few organisms which can metabolize xylose to produce ethanol. Although genetic engineering of organisms (such as yeast) made it possible to metabolize both hexoses and pentoses, engineered strains cannot compete with the native strains in terms of productivity and ethanol tolerance yet (Cai et al. 2012).

The knowledge of chemical and biological features of plant cell wall helps in better understanding the biomass recalcitrance. Furthermore, this knowledge helps to propose a better solution to overcome this barrier by an efficient pretreatment. Therefore, a brief introduction to the plant anatomy, cell, and cell wall components is presented here.

3.2.1 Anatomy of Plant Stem

When lignocellulosic biomass is pretreated and hydrolyzed, the process is actually conducted on several types of cells with different characteristics and functions. Stem is the most used part of a plant for biofuel production, and it contributes to the highest portion of plant dry weight. Generally, there are two types of tissue systems in the plants: (1) Meristematic tissue which is able to divide and is responsible for the plant growth and (2) permanent tissue which is produced by meristem cells. These cells lose their ability to divide but they are specialized to become three main tissues in plant: dermal, vascular, and ground (fundamental) tissue (Fig. 3.1) (Boundless 2014). Dermal tissue is a thin waxy layer covering the plant and protects it against pathogens. Bark is a dermal tissue of woody plants with tough and waterproof texture for further protection of the plant. The vascular tissue (bundle) is responsible for transportation of water, minerals, and sugar along the plant. The ground tissue fills the rest of the area of the stem and supports the vascular bundle; it is also responsible for photosynthesis and storage of water and sugar (Ding and Himmel 2008).

Vascular bundles have two specialized conducting types of tissue which are adjacent to each other: Xylem for transportation of water and nutrients from root to other parts of plant, and phloem for transportation of water and organic compounds from the photosynthesis site to other plant cells. After maturity, xylem tissue is not alive, whereas the phloem conducting cells are alive. The vascular bundles in dicots and gymnosperms are organized in a ring around the stem pith (center), but the vascular bundles are placed randomly in stem in monocots. In a vascular bundle, phloem tissue is located toward the outside of stem and xylem tissue is located inward (Ding and Himmel 2008).

Three types of cells are present in xylem tissue: xylem parenchyma, tracheids, and vessel elements (Fig. 3.2). Water conduction in tracheids is done through pits of adjacent cells, where the thick, lignified secondary walls are absent (Ding and Himmel 2008).

Phloem tissue cell types are sieve cells, companion cells, phloem fibers, and phloem parenchyma. The sieve tube cells transport organic substances through the plant. The cells are aligned end-to-end and form a long sieve tube and exchange materials through perforated sieve plates which are placed at the end junction between cells. These cells are alive at maturity but their nucleus and some of their

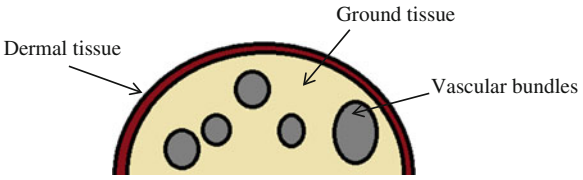

Fig. 3.1 The main tissues in plant stem [adapted from Boundless (2014)]

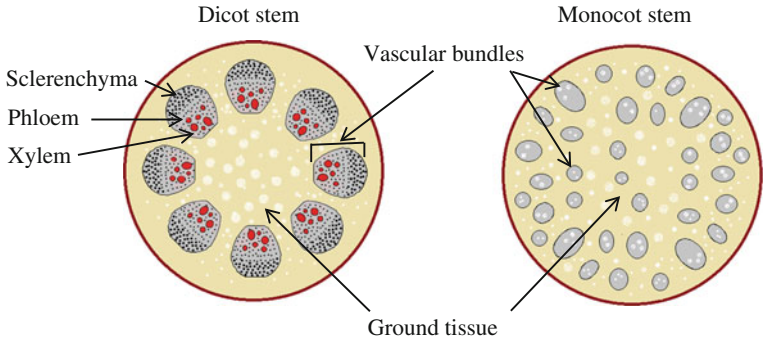

Fig. 3.2 Position of various tissues in plant stem of monocots and dicots [adapted from Boundless (2014)]

organelles are disintegrated. Therefore, the companion cells, which are placed alongside the sieve tubes, provide metabolites for them (Ding and Himmel 2008).

There are three types of cells in ground tissue: parenchyma, collenchyma, and sclerenchyma. Parenchyma cells, the most common cells in various parts of plant, are the sites of metabolic functions, e.g., photosynthesis. Collenchyma cells are usually found below the epidermis of stem and leave and are responsible for plant structural support. These cells are alive after maturity. Sclerenchyma cells also provide structural support, but they are dead after maturity. Fibers and sclereids, two types of sclerenchyma cells, have walls thickened with lignin and carbohydrate deposition (Ding and Himmel 2008).

Plants undergo two types of growth: primary and secondary. During the primary growth in summer, apical meristem cells divide rapidly and other cells are elongated. The result is the elongation of plant at root, shoots (the upper part of plant which is above the ground, e.g., stem, leaves, and flower), and tips. All plants undergo primary growth, whereas the secondary growth is the result of cell division of lateral meristem cells. Therefore, plant becomes thicker. The secondary growth is the specific growth of woody plants, while monocots lack this growth phase. The lateral meristem cells are placed in vascular cambium, between primary xylem and primary phloem, and in cork cambium (in wood) which are located below epidermis. During the secondary growth, vascular cambium produces secondary xylem and phloem between the primary tissues. Lignin deposition in the secondary xylem provides strength to stem. Cork cambium produces waxy cork (bark) cells which protect the stem from water loss and damages.

Annual rings in wood are the result of differences in the secondary xylem which is produced in different seasons (Fig. 3.3). During summer, the primary cell wall of secondary xylem is thin and the produced wood (early wood) is less dense. During winter time, the primary cell walls become thicker, and a more dense wood (late wood) is produced. During winter, the number of vessel elements decreases, while the number of tracheids increases (Novotny 2014). Harvesting time affects the digestibility of lignocellulosic materials (Bradshaw et al. 2007; Kim et al. 2011).

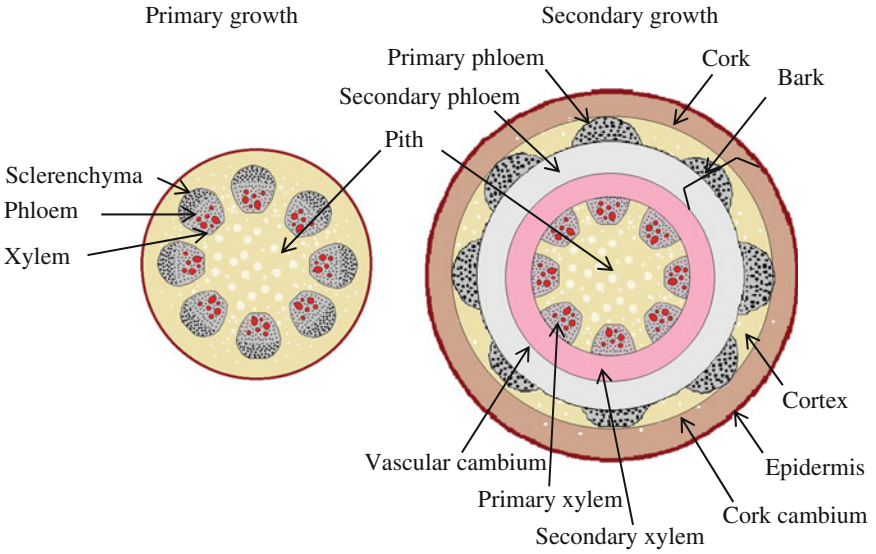

Fig. 3.3 The annual growth of stem in dicots [adapted from Boundless (2014)]

3.2.2 Plant Cells

Like all other cells, cells in plants have several parts, including plasma membrane and internal organelles as well as primary and secondary cell walls. Plant cells stick to each other by lignin rich materials called middle lamella, whereas the empty intercellular spaces are called apoplast, which help the mechanical support of plant. Places of depression in the cell wall form a pit. Usually when two cells' pits are aligned, a pit-pair is formed. Plasmodesma holes are present in the pits and connect the plasma membrane of two adjacent cells or in some cases to outer spaces. Plasmodesma holes are responsible for the movement of materials between cells. The diameter of pits can be as small as 2–5 μm in parenchyma cells to tens of microns thick in the secondary wall of xylem cells (Ding and Himmel 2006). Plant cell dimensions may vary significantly, ranging 30–200 μm in diameter and 0.3–2 mm in length (Raven et al. 1981). The internal organelles inside the cells disappear when the cell dies and an empty lumen is left inside each cell. This fact is especially seen in sclerenchyma cells in ground tissue, xylem cells, as well as sieve tube cells in vascular bundles (Charlet et al. 2010).

3.2.3 Plant Cell Wall

In a plant cell, the primary cell wall is formed at the stage of cell growth and enlargement. The secondary cell wall deposits when the cell growth has stopped.

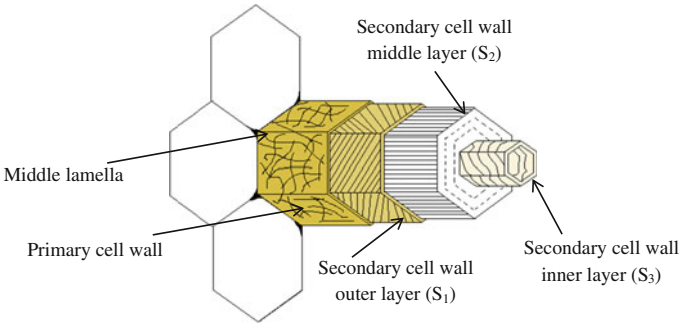

Fig. 3.4 The cell wall layers [adapted from Harris and Stone (2008)]

The secondary cell wall has three layers, S1, S2, S3, which are deposited one by one as the cell matures (Fig. 3.4). Middle S2 layer is thicker than the other two layers (Davison et al. 2013). Scanning electron microscopy (SEM), confocal scanning laser microscopy, and probe microscopy techniques, especially atomic force microscopy, are used for the study of plant cell wall before and after pretreatment (Yarbrough et al. 2009; Zhang et al. 2013; Davison et al. 2013). Beside these techniques, the chemical composition of the cell wall can be determined by TE-Raman, TE-CARS, or TE-SRS in nanometer scales (Yarbrough et al. 2009). These techniques for determination of cell wall structure and composition provide very useful data about changes of the cell wall caused by pretreatment (Chundawat et al. 2011; Davison et al. 2013).

Cellulose, hemicellulose, and lignin are the building blocks of the cell wall. Details of these compounds are presented here.

3.2.3.1 Building Blocks of Plant Cell Wall

Carbohydrates and lignin comprises of about 80 % of lignocellulosic materials (Table 3.1). Carbohydrates are among the main constituents of plant cell wall. Cellulose and hemicellulose are dominant carbohydrates in plant cell wall. Cellulose, among the most plentiful biopolymers on earth, represents 40–50 % of lignocellulosic materials by dry weight (Taherzadeh and Karimi 2008). This reveals that almost half of the dry weight of lignocellulosic materials can be converted to ethanol by typical C_6 sugar fermenting yeasts. The conversion of C_5 sugars is still an industrial challenge and requires use of mutant yeasts (C_6 and C_5 Co-fermenting microorganisms) or a two-step process (separate fermentation of C_6 and C_5 sugars). As it is discussed in the next sections, C_6 sugar residues are more abundant in hemicelluloses in the gymnosperms (softwoods) compared with angiosperms (hardwoods and grasses). Thus, theoretical ethanol yield from gymnosperms is typically higher. On the other hand, the amount of lignin in gymnosperms is typically higher than angiosperms (about 10 %). Although a lot of research is

Table 3.1 The amount of wood constituents in gymnosperms and angiosperms potentially suitable for ethanol production (Shafiei et al. 2010; Zhu et al. 2010; Sjostrom 1993; Shafiei et al. 2014b; Conde-Mejía et al. 2012; Jouanin and Lapierre 2012; Poornejad et al. 2013; Teghammar et al. 2012)

	Cellulose	Hemicellulose	Lignin
Gymnosperm wood (*softwood*)			
Spruce	40–46	21–31	27–29
Pine	40–46	18–29	25–30
Douglas fir	44	21–27	28–32
Angiosperm wood (*hardwood*)			
Oak	45	24	24
Eucalyptus	45–48	13–19	27–31
Birch	41–49	21–32	21–22
Poplar	34–44	19–22	23–25
Maple	44–46	17–23	23–24
Aspen	46–50	18	18–23
Angiosperm (*herbaceous*, *agricultural residues*)			
Switchgrass	36–43	12–25	23–28
Wheat straw	33–50	24–36	9–17
Rice straw	28–47	19–25	10–25
Corn stover	34–36	22–29	7–20.2
Sugarcane bagasse	40–41	27–38	10–20

underway in this direction (Ragauskas et al. 2014), lignin still has no commercial value in most of the processes other than burning for heat production. Fortunately, in the case of biogas production, the bacterium consortium is able to utilize both C_6 and C_5 sugars, however, similar to ethanol process lignin remains unchanged in anaerobic digestion.

3.2.3.2 Cellulose

As discussed below, cellulose has been studied at three levels: molecular, microfibril, and macrofibril.

Cellulose polymer: Cellulose is a linear polymer of D-glucose with β, 1 → 4 linkage (Fig. 3.5). In cellulose chain, two neighboring glucose molecules, entitled cellobiose, are twisted 180° relative to each other. Some researchers suggest "cellobiose" as the building block of cellulose, instead of glucose (Taherzadeh and Karimi 2008). There are three hydroxyl groups (OH) in each glucose molecule which can form both intra- and inter molecular hydrogen bonds. The hydrogen bondings in cellulose and in water are over 100 times stronger than van der Waals forces in water (Karimi et al. 2013) and they play an important role in the formation of crystalline cellulose fibers. The hydrogen bonding of O_3–HO_5 makes intra-chain

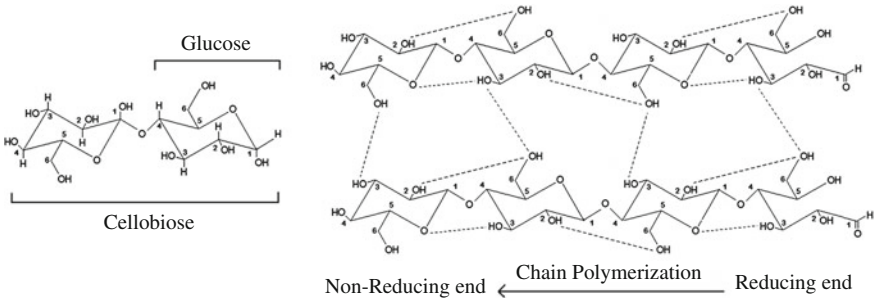

Fig. 3.5 Structure of cellulose chain and inter- and intra-molecular hydrogen bondings of cellulose I (*dashed lines*) [adapted from Festucci-Buselli et al. (2007)]

hydrogen bonding which forms the rigid and linear structure of one cellulose chain (Langan et al. 2001; Festucci-Buselli et al. 2007).

Depending on the source and isolation method of cellulose, it can appear in the crystalline, para-crystalline, and amorphous structures. The aggregation of cellulose chains forms different polymorphs of cellulose crystal: cellulose I, II, III_I, III_{II}, IV_I, and IV_{II}. Cellulose I, the main type found in the nature, has two coexisting allomorphs: cellulose, I_α and I_β, which are found in different proportions in the organisms. I_α and I_β cellulose types were obtained from algae *Glaucocystis* (I_α, triclinic) (15 biomassrec) and the tunicate *Halocynthia roretzi* (I_β, monoclinic) (16 biomassrec). However, the smaller crystal structure of native cellulose is observed (3–5 nm in diameter) in higher plants (Ding and Himmel 2006; Jarvis 2003), and they are called I_α-like chain or I_β-like chain. Annealing in weak alkaline solution (typically 0.1 N NaOH) at 260 °C (Yamamoto and Horii 1993) converts cellulose I_α to more stable I_β allomorph (Festucci-Buselli et al. 2007). The typical characteristic of cellulose I is O_2–HO_6 intra-chain hydrogen bonding; however, this bond is shorter in I_α type (Nishiyama et al. 2002).

Cellulose II is naturally produced by *Gluconacetobacter xylinum* and alga *Halicystis*. This type of cellulose is the most crystalline and thermodynamically stable form of cellulose which is commercially produced (Klemm et al. 2005). Regeneration and mercerization of cellulose I produce cellulose II. The specific characteristic of cellulose II is formation of O_6–HO_2 inter-chain hydrogen bonding, while in cellulose I, O_6–HO_3 is the dominant hydrogen bonding (Festucci-Buselli et al. 2007). The specific characteristic of cellulose II is antiparallel direction of two cellulose chains in one unit cell (Kim et al. 2013). Other types of cellulose are made by chemical or heat treatment of the native cellulose (Mohnen et al. 2008).

Cellulose fibril: In the cell wall of higher plants, two well-distinguished cellulose structures, micro- and macro-fibrils, were observed. Atomic force micrography (AFM), SEM, X-ray diffraction (XRD), nuclear magnetic resonance (NMR), and vibrational spectroscopy [infrared (IR), Raman, and sum-frequency-generation (SFG)] are the methods used to study cellulose structure (Kim et al. 2013). Theoretically, elementary fibril is the smallest aggregation of the cellulose chains in the

cell wall of higher plants. However, the exact molecular structure of elementary fibril is still unknown. Nevertheless, there are indications that it has a true crystalline core surrounded by layers of sub-crystalline and paracrystalline covers (Ding and Himmel 2008). Elementary fibrils are synthesized by a special synthase complex which is called rosette. A rosette is built by CesA proteins. Several CesA genes have been identified so far, and there are genetic evidences that a functional rosette must have at least three CesA proteins (Mohnen et al. 2008).

The early models proposed a two-layer (core/sheath) rectangular shape microfibrils, which has a 5 × 10 nm^2 cross section (Preston and Cronshaw 1958). Recently, Ding and Himmel (2006) have proposed a special model for cellulose synthesis in the primary cell wall of maize (monocot and angiosperm). In this model, several rosettes are arrayed in a honeycomb pattern in the plasma membrane. Each rosette is responsible for the synthesis of six cellulose chains and consists of at least three isoforms of CesA proteins which are arranged in a specific hexagonal geometry. As a result, the elementary fibril is formed by simultaneous cellulose synthesis and parallel bonding of 36 cellulose chains which form a hexagonal cross section. The surface of elementary fibril is highly reactive, and therefore during the synthesis, direct assembly of these elementary fibrils on the plasma membrane produces the macrofibrils. This model explains that during the cell growth and expansion of the primary cell wall, macrofibril is unwrapped and each elementary fibril is coated by hemicellulose and then deposition of lignin fills the inner spaces (Ding and Himmel 2008).

In the model by Ding and Himmel (2008) (Fig. 3.6), six cellulose chains in the core (group-C1, chains 1–6) are truly crystalline which are protected by two transitional less crystalline cellulose phases: group-C2 (chains 7–18) and group-C3 (chains 19–36). The early studies have suggested elementary fibril as a universal

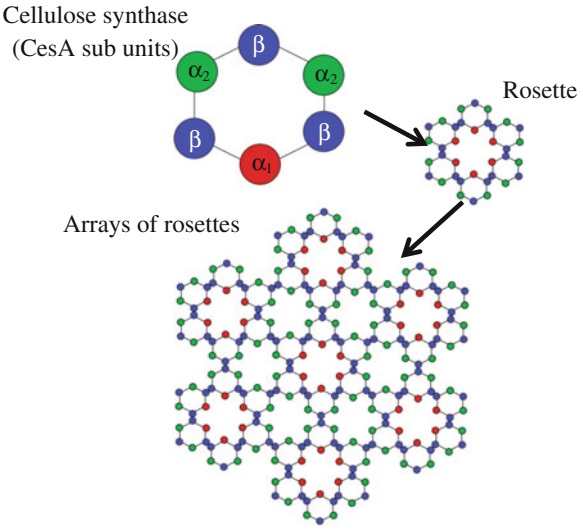

Fig. 3.6 A model for synthesis of cellulose elementary fibril, figure adapted from Ding and Himmel (2008)

and uniform size of cellulose unit, due to agreement in the observed diameter (Heyn 1969; Frey-Wyssling and Mühlethaler 1963). Recently, high gene homology in the higher plants indicates essentially similar biosynthesis pathway for cellulose crystallite could vary between 1.5 × 2 nm^2 (six cellulose chains) to 3 × 5.5 nm^2 (36 cellulose chains) (Ding and Himmel 2008).

While the elementary fibrils are quite similar, the measured diameter for cellulose microfibrils may meaningfully vary (e.g., 50–250 nm). The differences may be because of the differences in the number of elementary, post-formation modifications (covering by hemicelluloses), or the source of microfibril, e.g., the type of plant or cell wall type (Ding and Himmel 2008).

Ding and Himmel (2008) have proposed that the elementary fibril does not exist independently in the nature and they are essentially packed in the macrofibril or microfibril structure via hydrogen bonding. In the latter case, the hydrogen bonding of the surface C3 chains with the hemicelluloses may alter the semi-crystalline chains to non-crystalline structure. Therefore, the hydrolysis of oriented microfibrils must be easier than the hydrolysis of the macrofibrils.

Unlike 36 chain model for microfibrils of monocots, the observed diameter for microfibrils in the woody materials (hardwood and softwood) was 2–4 nm in both primary and secondary cell wall. However, in the secondary wall, aggregates of microfibrils with size of 20–25 (and not 36) chains with size of 14–23 nm in diameter were observed. In the primary cell wall, the degree of polymerization of cellulose is 6,000 glucose units on average, and this number increase to 14,000 in the secondary cell wall (Harris and Stone 2008).

3.2.3.3 Hemicellulose

Hemicelluloses, heteropolymers of polysaccharides and polyuronides, are synthesized and packed in the Golgi vesicles. Unlike cellulose, they vary in their sugar residue and branching chemistry (Ding and Himmel 2008). Various types of hexosans (mannan, glucosan, galactan, and rhamnan) and pentosan (xylan and arabinan) are found in polysaccharides of hemicelluloses (Karimi et al. 2013). Unlike crystalline structure of cellulose, hemicelluloses have amorphous short chain polymers (Girio et al. 2010). Therefore, acid or enzyme hydrolysis of hemicelluloses as well as their anaerobic digestion is faster and easier than cellulose (Girio et al. 2010; Keys et al. 1969). The hydrolysate of hemicelluloses may contain monomers of xylose, glucose, mannose, galactose, rhamnose, arabinose, and acetic acid. In hemicellulose of softwoods, mannose and some galactose side chains are the dominant saccharides. Conversely, dominant sugar residue in hardwood and grasses is xylose with some amounts of arabinose.

Degree of acetylation is defined as the ratio of total number of acetyl groups to the number of the monomers which can bear them. The degree of acetylation in softwood hemicellulose is lower than hardwood. Acetylation may also occur in lignin. The acetyl groups may link to aliphatic side chain of S and G units of lignin.

Lignified xylem cells of hardwood may be 1–50 % acetylated, while acetylation of xylem cells in softwood has not been reported (Pawar et al. 2013).

Hemicelluloses of primary cell wall, the most abundant and studied polyose in the primary cell wall, are xyloglucans. This polymer has a backbone of β-(1–4)-linked glucose with xylose branches. Some chains may also contain β-D-galactose and L-fucose-α-(1,2)-D-galactose, in which galactose residues are acetylated. Xyloglucans are directly linked to the surface of microfibrils, support the rigidity of microfibril, and play role in the cell wall enlargement. Glucuronoarabinoxylan is another polyose in the primary cell wall. It has a backbone of β-1,4-xylose with chains of arabinose, galactose, and glucuronic acid. Other less frequent polymers are fucosylated xyloglucan, glucans, and galactans (Ochoa-Villarreal et al. 2012; Gregory and Bolwell 1999).

Hemicelluloses of secondary cell wall, the heterogeneous polymer of secondary cell wall, are heteroxylans. Heteroxylan has a backbone of β-1,4-xylose with substitutions of (methyl)glucuronic acid, arabinose, and acetate. Heteroxylans constitute to 10–35 % of hardwood and 7–15 % of softwood. Arabino (4-*O*-methylglucurono) xylans are the most abundant hemicelluloses of grasses in both primary and secondary cell walls (Harris and Stone 2008). Glucomannans are predominant polyose (12–18 % of wood) in the gymnosperm (softwood) walls and are less frequent in the angiosperms (2–5 % of wood). The polymer backbone has non-regular residues of mannose and less amounts of glucosyl units. Some residues of glucomannans are acetylated and some amounts of galactose may also present (Ochoa-Villarreal et al. 2012; Gregory and Bolwell 1999).

Polyuronides are polymers of hexuronic acids and methoxyl, acetyl as well as free carboxylic residues with or without free polysaccharides groups (Karimi et al. 2013). Similar to the hemicelluloses, polyuronides are synthesized and packed in the Golgi vesicles. Some of polyuronides are regarded as hemicellulose. However, some types of these polymers are not considered as hemicelluloses such as water-soluble molecules, pectins, mucilages, and plant humus. Polyuronides are usually present in lignin-polysaccharide complexes (Kuznetsov 1970). Pectin is one of the well-known polyuronides mainly consists of a α-(1–4)-linked D-galacturonic acid backbone which can have branches of xylogalacturonan and apiogalacturonan. Another type of pectin has a disaccharide backbone of D-galacturonic acid and L-rhamnose (Gregory and Bolwell 1999). Chemical or biological decomposition of polyuronides is easier than polysaccharides (Karimi et al. 2013).

3.2.3.4 Lignin

Lignin is a complex polymer playing important roles in the plant cell. Lignin reinforces the strength of crystalline cellulose in the cell wall layers and middle lamellas, and thus enables the plant to grow to the highest levels. The hydrophobic character of lignin facilitates the transmission of water and nutrients. Moreover, it acts as a barrier to attack of microorganisms and pathogens (Lewis et al. 1999; Simmons et al. 2010). Lignin content varies enormously in plant species. In softwood conifers, for

example, lignin represents 25–40 % of dry weight while lignin content is 18–25 % in hardwood and it decreases to 10–20 % of the biomass dry weight in grasses and agricultural residues (Novo-Uzal et al. 2012; Wheeler 2014). Biodegradation of lignin only occurs under low levels of nitrogen (Lee 1997), and thus lignin is not degraded at normal condition of ethanol fermentation or biogas production. Thus far, lignin does not have a commercial value other than being used as heating source.

The most common monolignols (monomers of lignin) are syringyl (S), guaiacyl (G), and p-hydroxyphenyl (H) units which are derived from, sinapyl, coniferyl, and p-coumaryl alcohols. The aromatic ring in each unit is correspondingly shown by S, G, and H abbreviations. Several subunits with different side chains are present in lignins. The monolignols are radicalized after secretion and are linked with over ten types of C–C or C–O–C bonds. Lignin polymer forms a complex, branched network with the hemicellulose-covered cellulose crystals; the whole complex is sometimes called a "liquid crystal" (Vincent 1999). Besides differences in the total amount of lignin in plants, the amount of monomeric units as well as the type of linkages may vary significantly, resulting in recalcitrant biomass structure. Furthermore, the plant age, cultivation conditions, and stress affect the amount of lignin and units. In gymnosperms (softwood), main monolignol is in the form of G units besides a small proportion of H units. In angiosperms (hardwood), equal amounts of G and S units plus small amounts of H units are present (Lewis et al. 1999). It is believed that the occurrence of the G monolignols in plant cell wall reduces the accessibility of the cell. For example, S/G ratio affects the water swelling capacity of the lignocellulosic material (Ramos et al. 1992). G unit is the main monolignol type in vessel cells of angiosperms while S units are dominant in fibers (Novo-Uzal et al. 2012).

The presence of lignin is one of the key factors that hinder the degradation of plants. The degradation of less lignified cells, e.g., parenchyma cells, is much easier compared with thick xylem cells. Currently, several studies are accomplished for genetic engineering of lignin in plants with focus of biofuel production. Traditional studies focused on changes in the ratio of monolignols such as S/G ratio. The result of many of these studies enhanced hydrolysis yield but the plants were weaker and not commercially attractive (Weng et al. 2008). Instead of manipulating monolignols, new approaches are being developed to control polymerization of monolignols, apply modifications in lignin polymer structure, and research on lignin degradation enzymes (Davin et al. 2008).

3.2.3.5 Other Materials

Over 80 % of the dry weight of lignocellulosic materials is cellulose, lignin, and hemicellulose. The rest is a large number of materials which occurs in low amounts, called extraneous materials. These materials are divided into two groups: extractives and nonextractives (Fan et al. 1982). Extractive materials can be extracted by polar or nonpolar solvents. The most important extractives are resins (fats, fatty acids, resin acids, and phytosterols), terpenes (isoprene alcohols and ketones), and phenols (residue and byproducts of lignin biosynthesis) (Fan et al. 1982; Fengel and

Wegener 1984). Proteins, pectins, alkali earth carbonates, starches, silica, and oxalates are among the non-extractive materials. Various types of proteins are present in the cell wall. Structural proteins are the most important category but enzymes, proteins related to wall expansion, and signaling molecules are also present in the cell wall. Proteins account for ~2–10 % of the cell wall. Due to their low amount, the extraneous materials do not have a significant effect on the recalcitrance of the lignocellulosic materials and less investigated related to pretreatment (Karimi et al. 2013).

3.2.4 Molecular Aggregation of Polymers in the Cell Wall

After formation of the plant cell, the deposition of cellulose and pectic substances starts in the primary cell wall and middle lamella. Afterward, the deposition of arabinogalactan, xylan, and mannan starts in the primary cell wall. When the primary cell wall formation is finished and formation of S_1 layer starts, lignin deposition starts in the cell corners, middle lamella, and primary cell wall (Lewis et al. 1999). When the formation of S_2 layer is finished, the lignification of secondary cell wall starts. Xylan, mannan, and cellulose depositions continue until the completion of S_3 layer. However, lignification continues even after cell maturity. In each of the layers and locations, hydroxyphenyl (H) units are the first monolignol which deposits, and they are mostly found in the middle lamella. Subsequently, the deposition of guaiacyl (G) and then syringyl (S) monolignols starts taking place. Middle lamella contains the highest concentration of lignin (50–70 %), whereas the lowest concentration is present in the secondary wall, which is 20 % of dry matter for angiosperms and 16–19 % for gymnosperms (Harris and Stone 2008).

Lignin deposition occurs in the cell wall of all vascular bundles of plant and the cells with thick secondary cell walls. Some parenchyma cells and collenchyma cells are not lignified, but sclerenchyma cells in ground tissue (Fibers and sclereids), xylem tissue in the vascular bundles (tracheids, fibers, and vessel elements), and phloem fibers are highly lignified (Jouanin and Lapierre 2012).

The aggregation of cellulose in both micro- and macrofibrils is one of the most important features of biomass recalcitrance. High molecular weight cellulose chains are packed together by hydrogen bonding and are further covered by hemicelluloses, i.e., xyloglucans in the primary wall of both hardwoods and softwoods and galactoglucomannans and heteroxylans in the secondary wall of the respective woods. In primary cell wall, the polysaccharide matrix has less covalent interactions and more physical associations with itself and cellulose microfibrils. However, in the secondary wall, covalent interactions are present between carbohydrates, carbohydrate-lignin, and carbohydrate-proteins (Harris and Stone 2008).

Lignins are hydrophobic fillers that are deposited instead of water during lignification. As a consequence, more hydrogen bonds form between cellulose microfibril and hemicelluloses. Lignins also form covalent interaction with polysaccharide matrix and proteins (Northcote 1972; Donaldson 2001). The result is a

cross-linked matrix composed of non-cellulosic polysaccharides, lignins, and proteins. The low porosity of the matrix (in order of 20–5 nm) limits the diffusion of (hemi) cellulolytic enzymes. Furthermore, the solubilization of this reinforced matrix requires harsh condition processes, which are not only costly but may lead to the degradation of polysaccharides and formation of inhibitors.

In summary, the biomass recalcitrance to chemical and enzymatic breakdown involves several aspects:

1. Protecting dermal tissue; waxy cuticle for grasses and energy crops and bark in the woody plants.
2. The arrangement and density of the cells especially in vascular bundles and sclerenchyma cells.
3. Highly lignified middle lamella which sticks the cells together and is a barrier to enzyme or microorganism attack.
4. The negative effect of lignin and degree of lignification in primary and secondary cell wall.
5. Cross-linked matrix polymer composed of hemicelluloses and lignin which prevents penetration of water and enzymes to polysaccharides.
6. The aggregation of long cellulose chains to micro- and macrofibers.

Cellular system of plants has been developed to preserve the plant in different weather conditions and resist against microbial attacks. The recalcitrance of lignocellulosic biomass leads to low yields for biofuels, requirement of expensive enzymes, and long duration processes. For example, the cost of enzyme for starch is >100 times lower than cellulase ($0.01–$0.05/gallon-ethanol vs. $0.5–1.0/gallon-ethanol) (Stephen et al. 2012; Cullis et al. 2004; Aden et al. 2002). Required hydrolysis time for starch is 24 h, while it is 3–5 days for wood cellulose. However, pretreatment overcomes these barriers and enhances the sugar yields by several folds.

3.2.5 Parameters Affecting Pretreatment Efficiency

Pretreatment is a process to modify the structure of plant cells in order to decrease the cell wall recalcitrance. This process enhances the enzymatic or microbial degradation of lignocellulosic biomass. Traditionally, features affecting pretreatment efficiency are represented as accessible surface area, cellulose crystallinity, the protection of cellulose chains by hemicellulose and lignin, degree of acetylation of hemicellulose, the productive and non-productive binding of cellulase to biomass, and biomass swelling capacity (Wyman 1996; Taherzadeh and Karimi 2008; Hendriks and Zeeman 2009; Karimi et al. 2013). In pretreated biomass, all or most of these features are affected simultaneously, and the study of a single parameter is not practically possible. Furthermore, these features are not similar for various types of biomass. Thus, relating a single parameter to the effectiveness of hydrolysis is not possible (Karimi et al. 2013). A recent view to parameters affecting cellulose

hydrolysis categorizes all features into two groups: (1) accessibility of enzyme to appropriate substrates and (2) the effectiveness of enzymes after adsorption on the surface (Kumar and Wyman 2013). The first parameter can be further categorized to the macro- and micro-accessibility of cellulose and related to layers of hemicellulose and lignin which cover cellulose. The latter is related to cellulose structure, crystallinity, and DP (Kumar and Wyman 2013). Traditional features that affect cellulose enzymatic hydrolysis are presented here, while new point of view is briefly discussed for each factor.

3.2.5.1 Biomass and Cellulose Accessibility

Sufficient accessible surface area which provides direct physical contact of biomass with enzymes is essential for efficient hydrolysis. The external surface area of particles is related to their size and shape. The internal surface area of lignocelluloses is related to the porosity and capillary structure of the particles. Both external and internal surface areas of lignocelluloses affect the hydrolysis yield and rate (Karimi et al. 2013). Size reduction improves the external surface area of particles and improves hydrolysis efficiency (Goel and Ramachandran 1983; Dasari and Berson 2007; Kumakura 1986). For instance, final glucose yields from solvent pretreated wood chips were significantly lower than the final glucose yields from pretreated powder (Shafiei et al. 2013b, 2014b). However, this process is very energy intensive and the production of fine particles is not commercially viable. An ideal pretreatment must be efficient for large particles and should improve both internal and external surface areas in order to enhance enzymatic hydrolysis (Fan et al. 1980). Cellulose hydrolysis has two stages: a fast initial stage and a slower latter stage. In the first stage of hydrolysis, the accessible area is sharply increased because of the partial removal of cellulose and hemicellulose and production of larger pores inside the biomass (Fan et al. 1980). However, hydrolysis rate is decreased because of factors other than accessible surface area. Thus, this factor must be studied along with other parameters which affect the efficiency of hydrolysis (Karimi et al. 2013). Measurement of water swelling capacity and measurement of porosity of biomass are methods for quantifying the biomass accessibility (Chandra et al. 2009; Behrendt and Blanchette 1997).

Cellulose accessibility can be related to the biomass hydrolysis. If cellulase does not have access to the biomass, it may not reach to cellulose chains. The hydrolysis is performed in three steps: cellulase adsorption on cellulose surface, cellulose hydrolysis, and enzyme desorption to the media. The enzyme binds to cellulose via specific binding sites on the enzyme which match with cellulose surface. The accessibility of cellulose is defined as availability of the binding sites on the cellulose surface, e.g., milligrams of protein adsorbed per gram of biomass (Kumar and Wyman 2013). There are quantitative methods for measurement of cellulase adsorption such as Simons' Stain (SS) technique (Chandra et al. 2008; Esteghlalian et al. 2001). This method was used for the prediction of digestibility enhancements made by pretreatment (Chandra et al. 2008).

On one hand, cellulose macro-accessibility is hindered by the presence of lignin and hemicelluloses. On the other hand, features such as cellulose structure, crystallinity, and DP are responsible for micro-accessibility. Thus, for an example, amorphous cellulose has the highest macro- and micro-accessibility. Furthermore, when the cellulose structure is studied, the microfibrils are responsible for macro-accessibility and elementary fibrils are for the micro-accessibility of cellulase to cellulose. The micro-accessibility of cellulose is presumably enhanced by decrease in cellulose DP, increase and enlargement of nanopores within cellulose fibers, and changes in the cross-sectional radius of crystalline cellulose fibril (Kumar et al. 2009; Foston and Ragauskas 2010; Pingali et al. 2010; Wan et al. 2010; Sannigrahi et al. 2008). Some of these concepts are not examined thoroughly yet. Further investigations will provide new insights for the better understanding of pretreatment and hydrolysis processes with the aim of commercialization of the biofuel production.

3.2.5.2 Cellulose Crystallinity and Degree of Polymerization

Cellulose crystallinity and degree of polymerization are two important factors affecting hydrolysis of cellulose. Cellulose chain has crystalline and amorphous regions (Ciolacu et al. 2011). The amorphous regions of cellulose adsorb water and enzyme easier and faster than the crystalline regions and their hydrolysis is faster (by a factor of 2–25) compared to that of crystalline regions (Kumar and Wyman 2013). Cellulose crystallinity is measured as the ratio of crystalline to amorphous cellulose. Ball milling, alkali pretreatment, concentrated phosphoric acid, ionic liquid, and NMMO pretreatments are some methods in which less crystalline or amorphous cellulose is produced (Karimi et al. 2013; Shafiei et al. 2013b). In these pretreatments, reduction in crystallinity is one of the most important reasons for enhanced digestibility of cellulose (Karimi et al. 2013). Cellulose crystallinity is believed to play major role in the micro-accessibility of cellulase as well as in its effectiveness (Kumar and Wyman 2013). The mechanism is explained by the layered structure of cellulose, e.g., in cellulose elementary fibril, meaning that the outer layers of cellulose must be removed first then enzyme can reach the new active sites lying underneath (Kumar and Wyman 2013). The effect of cellulose crystallinity is even more important when the efficiency of enzyme is considered. Highly crystalline cellulose is highly hydrophobic and irreversibly binds cellulase enzyme. The bound enzymes loss up to 70 % of its activity within 10 min (Ma et al. 2008).

However, crystallinity reduction is not the sole reason for the enhanced digestibility of lignocellulosic materials by pretreatment. For instance, no change or increase in the crystallinity of cellulose after some dilute acid and lime pretreatments was reported (Karimi et al. 2013; Kim and Holtzapple 2006). Meanwhile, these pretreatments efficiently improve the hydrolysis of cellulose by other mechanisms such as hemicellulose and lignin removal.

Measurement of crystallinity for pure cellulose is performed based on well-defined methods. Some of these methods, e.g., FTIR, XRD, and solid-state NMR, are also used for measurement of cellulose crystallinity in lignocelluloses (Shafiei et al. 2014b;

Segal et al. 1959; Park et al. 2010). In some cases, the measured crystallinity was related to the initial hydrolysis rate (Chang and Holtzapple 2000). However, the presence of other constituents such as lignin and hemicellulose interferes with the cellulose crystallinity measurement, especially when FTIR and XRD methods are used (Shafiei et al. 2014b). Furthermore, the changes in cellulose DP may not be distinguished from changes in the crystallinity of biomass (Yang et al. 2011).

Reduction in cellulose DP increases the susceptibility of the cellulose chain (Kuo and Lee 2009; Zhang and Lynd 2005). The number of hydrogen bonds which must be broken in longer cellulose chains is more than that in shorter chains (Zhang and Lynd 2005). Gel-permeation chromatography (GPC) and viscometric techniques are two methods for the measurement of cellulose DP (Jahan and Mun 2009; Hubbell and Ragauskas 2010). However, these methods are more applicable for pure cellulose. The cellulose in the biomass cell wall must be separated for measurement of DP by these methods. However, the separation of cellulose without changing its structure is not possible (Hallac and Ragauskas 2011). Cellulose DP might have significant effects on micro-accessibility and enzyme effectiveness. However, because of restrictions for measurement of cellulose DP, the hypothesis needs more investigations (Kumar and Wyman 2013).

3.2.5.3 Presence of Hemicellulose

The most recent model for plant cell wall describes that cellulose chains are covered by hemicelluloses. Thus, hemicelluloses play major role in cellulose macro-accessibility. It is also believed that hemicelluloses are important in enzyme effectiveness. Humins derived from hemicelluloses may unproductively bind enzymes and limit enzyme effectiveness (Kumar and Wyman 2013; Kumar et al. 2013; Hu et al. 2012). Several studies indicated improvements in cellulose hydrolysis by xylan removal, specifically by dilute acid pretreatment (Tosun 1995; Kim et al. 2001; Um et al. 2003; Zhu et al. 2005; Canilha et al. 2011). The extent of xylan removal had significant effect on the digestibility of cellulose when dilute acid pretreatment was performed in percolation reactors (Zhu et al. 2005). For instance, addition of xylanase improved the hydrolysis yield of SO_2 exploded corn stover was by 12–22 % (Öhgren et al. 2007).

However, selective study on the effect of xylan removal is not possible since thermochemical pretreatments often change many features of biomass simultaneously. For instance, lignin removal is usually associated with hemicellulose removal and both of these features significantly enhance the cellulose macro-accessibility.

Sun et al. (2014b) investigated the physical and chemical properties of hemicellulose from *Phyllostachys pubescens* extracted by steam explosion followed by alkali or alkali/ethanol extraction. The composition of the sugars and uronic acids of the extracted hemicellulose was determined by high-performance anion exchange chromatography. This study revealed that the ratio of xylose/arabinose is lower when alkali/ethanol pretreatment is used compared with alkali extraction. This suggested that that application of ethanol along with alkali pretreatment increases

extraction of branched hemicelluloses. Molecular weights of the extracted hemicelluloses were measured by gel-permeation chromatography (GPC). The molecular weight distributions showed the extent degradation of hemicelluloses. FTIR and ^{13}C NMR analyses were used to identify the polysaccharides, their structural features and intermolecular interactions. Thus, the extracted hemicelluloses were composed of L-arabino-4-*O*-methyl-D-glucurono-D-xylan. Sun et al. (2014b) also investigated the thermal decomposition of hemicelluloses by thermogravimetric analysis (TGA).

3.2.5.4 Presence of Lignin

As mentioned earlier, lignin covers hemicelluloses which cover cellulose chains. Thus, lignin reduces cellulose macro-accessibility indirectly and prevents the enzymatic hydrolysis of cellulose. Furthermore, the hydrophobic irreversible binding of lignin to cellulase enzyme and enzyme deactivation significantly reduces enzyme effectiveness (Karimi et al. 2013; Taherzadeh and Karimi 2008; Kumar and Wyman 2013; Wyman et al. 2005). Therefore, lignin removal by some pretreatment methods, e.g., alkaline pretreatments, enhances the saccharification of cellulose. Some studies have investigated S/G ratio in lignin after pretreatment (Li et al. 2013). In addition to lignin removal, Verma and Dwivedi (2014) reviewed attempts for down regulation of lignin synthesis in genetically modified plants. However, limited studies focused on the actual changes to lignin structure by pretreatment and the changes in cross-linked bonds in lignin structure. Specifically, some pretreatment methods such as ionic liquids or NMMO do not change the lignin content, while resulting in significantly high saccharification yields. The efficiencies of these pretreatments were related to reduction of the cellulose crystallinity (Zhao et al. 2009; Dadi et al. 2006; Zhao et al. 2010). Nakayakkara et al. (2014) studied depolymerization of lignin under oxidative conditions by application of ionic liquids. The effect of ionic liquids on the structure of lignin as well as lignin carbohydrate complex during pretreatments might be other reason for enhancement of hydrolysis yields. Singh et al. (2013) studied the structural changes of ionic liquid pretreatment by TGA, differential scanning calorimetry (DSC), and size exclusion chromatography. This study showed that the pretreated technical lignin is less recalcitrant and has lower DP. The structure of ionic liquid pretreated biomass was also investigated (Singh et al. 2013).

Zheng et al. (2014) pretreated sugarcane bagasse by dilute phosphoric acid followed by steam explosion. Then ethanol was used for the extraction of lignin which contributed to 8 % of the biomass. The extracted lignin was further analyzed by non-destructive methods such as quantitative 13C, 2-D NMR, and GPC. This study showed that the basic structure of lignin was not changed; however, decrease in the amounts of β-O-4 linkages, S/G units ratio, and *p*-coumarate/ferulate ratio were observed. The enzymatic hydrolysis of ethanol extracted bagasse was 22 % higher than the non-extracted biomass.

Migration of lignin to the cell wall surfaces by chemical pulping, ammonia fiber expansion (AFEX), dilute acid, and liquid hot water pretreatments was reported (Ju et al. 2013; Chundawat et al. 2013; Yang and Wyman 2004). It is suggested that the surface lignin after chemical pulping positively improves enzyme adsorption (Ju et al. 2013). In this study, surface lignin was measured by X-ray photoelectron spectroscopy. However, in dilute acid or liquid hot water pretreatments, the solubilized lignin reacts further to insoluble products. These products precipitate on the biomass and hinder the enzymatic digestibility of cellulose. Properties of alkali extracted lignin were determined by FTIR and NMR techniques by Sun et al. (2014a). This study showed that the extracted lignin has low molecular weights, narrow polydispersities, and high contents of phenolic hydroxyl groups.

3.3 Pretreatment

Pretreatment is usually classified to physical, physico-chemical, chemical, and biological pretreatment methods. An ideal pretreatment method should meet the following requirements:

1. High efficiency, i.e., achievement of high saccharification yields in subsequent enzymatic hydrolysis at low enzyme loadings, for different types of feedstocks, as most of lignocelluloses feedstocks are mixtures of different types of materials, e.g., a mixture of hardwoods and softwoods in woody wastes or a mixture of different parts of one plant, e.g., leaves, branches, stem, and bark.
2. Minimal capital for pretreatment and auxiliary (e.g., neutralization and washing) equipment.
3. Minimal or no chemical for pretreatment. In application of expensive chemicals, e.g., ionic liquids and cellulose solvents, the possibility to reuse and recycle the chemicals with minimum make up should be considered.
4. Minimal utility (e.g., steam, cooling water, and electricity) requirements.
5. Minimum production of waste chemicals and no discharge of toxic or hazardous wastes.
6. Minimal size reduction requirements for feedstocks since milling is an energy intensive process.
7. Minimum degradation of fermentable carbohydrates.
8. Minimum production of inhibitory compounds either for enzymatic hydrolysis or fermentation.
9. Effective at high solid loading and low moisture content.
10. Facilitates recovery of lignin.
11. Minimum change in lignin properties (separation of unaltered lignin applicable for value-added chemical production).

Although no pretreatment meets all the specifications stated above, these aspects should be considered for an economically feasible process.

3.3.1 Pretreatment Reactors

Pretreatment processes are performed in batch, continuous, or semi-continuous modes of operation. Basic experiments and initial optimizations are typically performed in batch reactors. However, continuous reactors are preferred for commercial processes. Some researchers reviewed reactor types suitable for hydrolysis and pretreatment (Lee et al. 1999; Taherzadeh and Karimi 2007a; Yang and Tucker 2013; Elander 2013). Continuous operation enables faster processes with higher solid loadings, and thus requires much smaller equipment. Furthermore, fast preheating and low residence time for materials reduce the possibility of inhibitor. For some types of pretreatment, e.g., steam explosion and dilute acid explosion, continuous plug-flow reactors have been constructed and demonstrated successfully at commercial scale (Oliveira et al. 2013; Aden et al. 2002; Chen et al. 2014b; Lee et al. 1999; Taherzadeh and Karimi 2007a). If high solid loading is desired, plug-flow reactors equipped with moving paddles on the central shaft can be used (Aden et al. 2002). In plug-flow reactors, both solid and liquid move at the same velocity. Cocurrent as well as countercurrent flow of biomass and liquor is mainly provided by extruders. In extruders, a moving bed of biomass is treated by a liquid moving the same or the opposite direction (Chen et al. 2014b). Percolation reactors, which are packed-bed flow-through reactors, have been successfully evaluated in laboratory scale batch experiments. Dilute acid, ammonia, and water have been used as the pretreating liquor (Mosier et al. 2005b; Wyman et al. 2005). In such systems, the liquor is passed through a bed of biomass and removes the solubilized monomers from the media. Therefore, the residence time of solubilized sugars in the reactor is reduced while the decomposition of sugars is minimized (Yang and Wyman 2004). Shrinking bed reactor is a type of percolation reactors. In this type of reactor, a spring-loaded plunger is used for the reduction of packed-bed depth. In this way, the liquid throughput is reduced and sugar concentration is increased (Taherzadeh and Karimi 2007a).

3.3.2 Physical Pretreatments

Physical pretreatments involve changes in the structure of biomass without addition of chemicals or production of harmful residues. Mechanical comminution such as chipping, grinding or milling, extrusion, pyrolysis, irradiation, ultrasonication, and using microwaves are among the physical pretreatment methods (Taherzadeh and Karimi 2008; Agbor et al. 2014). Each of the methods is suitable for raw materials with specific properties. For instance, mechanical comminution is suggested for dry biomass, up to 10–15 % moisture content, while extrusion and colloid mills are suitable for biomass with at least 10–20 % moisture content (Zheng et al. 2014). Application of gama irradiation electron beam, ultrasonication, and microwaves is mostly suitable for wet feedstocks. Furthermore, these methods are very costly and require safety concerns (Karimi et al. 2013).

3.3.2.1 Mechanical Comminution

Process Description

Mechanical comminution is done in three steps: (1) During harvesting and pre-conditioning, coarse particles with 10–50 mm in size are produced, (2) choppers or shredders produce semi-coarse biomass with size of 10–38 mm, and (3) application of screens, diaphragms, or cyclones to the size reduction machines such as disk and ball mills to produce fine particles (0.2–2 mm). It is notable that materials with moisture content more than 25–30 % may clog screens, especially when sizes less than 6.35 mm are desired. Hammer mill and knife mill are two common machines for the production of semi-coarse particles (Vidal et al. 2011).

Mechanism

Reduction in the size of particles leads to increase in the total accessible surface area of the biomass, and thus increase in the saccharification yield (Taherzadeh and Karimi 2008; Vidal et al. 2011). In the case of pure or modified cellulose, e.g., pulped softwood, over 90 % hydrolysis yield was observed as a result of decrease in the cellulose crystallinity and degree of polymerization. However, after finest milling, the conversion rate of bonded cellulose in lignocellulosic biomass did not exceed 50 % (Vidal et al. 2011). The efficiency of pretreatment methods depends on the initial particle size as well (Taherzadeh and Karimi 2008). This suggests that mechanical comminution is necessary but not sufficient for efficient conversion (over 70 % yield) of lignocelluloses to ethanol (Vidal et al. 2011; Taherzadeh and Karimi 2008).

Efficiency of the Pretreatment

Several studies have shown that hydrolysis of smaller particles is more efficient than bigger ones. However, considering higher energy consumption to obtain finer particles, it is necessary to find the best configuration for the whole ethanol production process from size reduction to saccharification and fermentation. The "maximal particle size" is the size of particles below which no increase in pretreatment efficiency is observed (Vidal et al. 2011). A few studies focused on this concept have reported that maximal particle size depends on pretreatment type. Furthermore, biomass feedstock affects the maximal particle size, e.g., the maximal size is larger for herbaceous and grasses than the respective sizes for hardwoods and softwoods. For instance, the maximal particle size for biomass pretreated by steam explosion is 8–15 mm (Ballesteros et al. 2000, 2002; Negro et al. 2003), liquid hot water 1–15 mm (Negro et al. 2003; van Walsum et al. 1996), dilute acid 1–3 mm (Springer 1985; Yat et al. 2008; Hsu et al. 1996), and base pretreatments 0.1–2 mm (Chundawat et al. 2007; Moniruzzaman et al. 1997; Chang et al. 1997; Li et al. 2004). Thus, the larger values represented for herbacious materials while smaller values are attributed to the woody materials.

Advantages and Disadvantages

Mechanical comminution is unavoidable but not sufficient for efficient conversion of lignocelluloses to ethanol (Taherzadeh and Karimi 2008; Vidal et al. 2011). The main drawback of mechanical comminution is consumption of up to one-third of the energy requirements of biofuel production process. This concern is not often taken into account in laboratory experiments, while size reduction may become a challenge in industrial processes. The required energy for size reduction varies significantly depending on the desired particle size as well as the biomass type (Bitra et al. 2009). The required energy to obtain fine particles (<2 mm) from herbacious biomass is less than 50 kWh/t. This amount of energy is 2 % of the energy from ethanol combustion. The energy required to obtain the same particle size from woody biomass is at least twice this value (Vidal et al. 2011). When finer particles are required, the energy demand increases drastically up to five times of these values. The mill properties, type, and efficiency affect the amount of required energy as well. These factors also affect the shape of the particles and percentage of fines (the particles with smaller sizes). For example, higher percentage of fines is produced by hammer and attrition mills, whereas knife mill requires less energy (Womac et al. 2007; Bitra et al. 2009; Schell and Harwood 1994). Usually two-step size reduction requires less energy compared with one-step size reduction. Some researchers suggested that steam explosion consumes 60 % of the energy requirements of attrition milling when the same range of particle size is desired (Vidal et al. 2011). However, this method is not efficient for softwoods. Another approach to minimize the energy requirement of size reduction is to perform chemical pretreatment prior to the size reduction, named "post-chemical pretreatment size reduction." This method has technical advantages such as reduction in the mechanical energy, higher solid loading, and easier separation of pretreated materials (Zhu et al. 2010). However, post size reduction may reduce the efficiency of the chemical pretreatment, and also it is not applicable for some types of pretreatment (Taherzadeh and Karimi 2008).

In summary, mechanical comminution is an energy intensive process which requires optimization depending on the feedstock type, its moisture content, and the pretreatment type. Selection of the comminution equipment as well as pretreatment type requires extended knowledge about efficiencies of pretreatments for semi-fine particles.

3.3.2.2 Extrusion

Process Description

Extruders are used to apply heat and shear to wet biomass containing over 15–20 % moisture content (Zheng et al. 2014). Single screw extruders consist of a simple screw rotating in a barrel. This type of extruder is used for spatial mixing of materials and is not efficient for changing physical properties of the materials by application of high shear. In this regard, twin screw extruders are more efficient.

Extruders with fully intermeshing screws with co- or counter-rotating screws are useful for generation of high shear. For instance, regular flighted right- or left-handed and kneading disk screw types have been suggested for pretreatment (Kalyon and Malik 2007). Changing the physical properties is possible when the whole fluid can be forced to the tight clearance between screws or screw and barrel. This means that the separation of solid and liquid should not occur otherwise the high shear is not applied to the solid (Senturk-Ozer et al. 2011).

Mechanism

Application of high mechanical shear disrupts the biomass structure and leads to defibrillation and shortening of the fibers.

Efficiency of the Pretreatment

Application of sole extrusion is not sufficient for the efficient enhancement of enzymatic hydrolysis; however, this method has been used in continuous pretreatments in combination with other physical or chemical pretreatment methods. For instance, application of steam pressure in plug-flow reactors equipped with an extruder and explosive release is a form of continues steam explosion (Chen et al. 2014b). Combination of extrusion with chemicals such as NaOH (Carr and Doane 1984; N'Diaye et al. 1996; Senturk-Ozer et al. 2011; Vandenbossche et al. 2014; Duque et al. 2014; Zhang et al. 2012), dilute acid (Wang et al. 2014; Yoo et al. 2011; Ciesielski et al. 2014), Na_2S, and H_2O_2 was studied as well (Carr and Doane 1984). Two-stage extrusion consisting of first stage by acid/water and second stage by NaOH (Kadam et al. 2009; Senturk-Ozer et al. 2011) was also investigated.

Advantages and Disadvantages

The energy required for extrusion is lower than mechanical comminution. Another advantage of extrusion is application of high shear and rapid mixing beside application of steam and addition and removal of chemicals. Main problem with the extrusion of lignocelluloses is limitations in flowability of materials. This problem leads to separation of liquid from solid in extruder, and thus high shearing stresses are not applied to biomass. Therefore, the addition of chemicals was suggested for enhancement of flowability behavior of biomass. Chemicals such as carboxy methyl cellulose in combination with NaOH or recycled black liquor were found to be efficient for enhancement of flowability of the solids (Senturk-Ozer et al. 2011).

3.3.3 Chemical and Physicochemical Pretreatments

Chemical pretreatments involve addition of chemicals to modify the structure of lignocelluloses, e.g., alter the crystallinity of cellulose and modify and/or remove lignin or hemicellulose. In some cases, chemical pretreatments are combined with physical pretreatments such as steam explosion.

3.3.3.1 Liquid Hot Water Pretreatment (Autohydrolysis)

Process Description

Liquid hot water pretreatment (LHW) is suggested as one of the leading pretreatment methods (Wyman et al. 2005). This pretreatment is performed at elevated temperatures in the range of 150–230 °C and high pressure to maintain water in liquid form. Other names such as hydrothermolysis (Bobleter et al. 1981; Bobleter and Concin 1979), aqueous or steam/aqueous fractionation (Bouchard et al. 1991), uncatalyzed solvolysis (Mok and Antal 1992), and aquasolv (Allen et al. 1996) were also used for this type of pretreatment. This pretreatment is performed either in batch mode of operation or percolation reactors. Controlling pH by additional base materials besides buffering capacity of the biomass, e.g., buffering capacity of corn stover, is an approach to maintain solubilized carbohydrates in oligomeric state (Wyman et al. 2005). In acidic media, inhibitor compounds are produced from monomer sugars. Thus, in pH-controlled LHW pretreatment, minimum degradation occurs (Mosier et al. 2005a). Other types of reactors such as co-current and counter-current reactors were also used for LHW pretreatment (Agbor et al. 2011).

The typical pretreatment temperature for percolation-type pretreatment is 190–230 °C where the pressure is as high as 20–24 atm. Low solid loadings in range of 2–4 % and moderate reaction times of 12–24 min are used in percolation reactors. Products of LHW pretreatment in percolation reactors are a liquid with dilute soluble oligomers and monomers of hemicelluloses and lignin together with a more digestible solid which is rich in cellulose (Wyman et al. 2005).

When autohydrolysis is performed in batch mode, lower temperatures (160–190 °C) and pressures (6–14 atm) are necessary (Yang and Wyman 2004; Wyman et al. 2005). In pH-controlled pretreatment, e.g., by the addition of KOH or NaOH, the pH is maintained between 4 and 7, (Mosier et al. 2005b; Kohlmann et al. 1996; Weil et al. 1998). In this process, the reaction time is in a range similar to percolation pretreatment (10–30 min) but higher solid loading (5–30 %) is possible (Wyman et al. 2005).

Reactions and Products

Autohydrolysis is in fact the hydrolysis reaction of hemicelluloses in aqueous medium. In autohydrolysis, the extensive hydrolysis of hemicellulose to oligomers

and monomers occurs, while lignin content changes are moderate and the glucan losses are negligible. During autohydrolysis, hemicaetal linkages are cleaved, and thus acetyl groups are released in the form of acetic acid. Water also acts as an acid at elevated temperatures (Weil et al. 1997; Baugh et al. 1988; Baugh and McCarty 1988). The presence of acetic acid along with acidic nature of water is the main driver for the production of solubilized monomers and oligomers from hemicellulose (Mosier et al. 2005b; Weil et al. 1997; Zheng et al. 2014; Agbor et al. 2011). During autohydrolysis, water diffuses into the structure of lignocelluloses. Inside the material, water is present in low amounts where acetic acid is released and is not diluted. Therefore, the hydrolysis is done by concentrated acetic acid at high temperature. The acid is diluted as it is released to the media. Thus, the prolongation of batch pretreatment is not beneficial. LHW pretreatment increases the accessible surface area of the biomass by hemicellulose and lignin removal/relocation. Enhanced accessibility of enzymes to biomass increases the enzymatic digestibility of lignocelluloses (Taherzadeh and Karimi 2008).

Degradation of carbohydrates and lignin to inhibitory compounds occurs along with autohydrolysis. These compounds hinder the subsequent enzymatic hydrolysis and/or fermentation. Furan derivatives, especially furfural and 5-hydroxymethylfurfural (HMF), are produced, respectively, from degradation of pentoses and hexoses. Chiaramonti et al. (2012b) studied autohydrolysis of miscanthus (a perennial grass). This study revealed that at moderate temperatures, furfural is produced at a concentration of 0.4–0.5 g/kg, while at higher temperatures, the concentration increases to 1 g/kg. Phenolic compounds are derived from lignin decomposition and week acids such as acetic, formic, and levulinic acids, showing inhibiting effects on the subsequent processes (Palmqvist and Hahn-Hägerdal 2000a, b).

The process severity for LHW pretreatment is defined as (Overend et al. 1987):

$$R_0 = t \cdot \exp[(T - 100)/14.75] \tag{3.1}$$

where t is reaction time (minutes) and T is the hydrolysis temperature (degrees Celsius). This severity factor is used for both batch and flowthrough processes. Application of higher process severity increases the solubilization of xylo- and glucooligomers until reaching a maximum. Afterward, the prolongation of pretreatment decreases the concentration of oligomers which is due to their degradation (Caparrós et al. 2007).

Efficiency of the Pretreatment

The factors affecting pretreatment efficiency are pH, temperature, time, and mode of operation (Wyman 1996; Mosier et al. 2005a, b, c; Taherzadeh and Karimi 2008). Yang and Wyman (2004) studied the pretreatment of corn stover by batch and percolation reactors. Depending on the process severity ($2.5 < \log R_0 < 4.8$), 25–100 % xylan and 12–68 % lignin were removed by LHW pretreatment. Pretreatment by

percolation reactor resulted in about 20 % higher saccharification yield of cellulose compared with batch pretreatment at similar severity. Hydrolysis yields were in the range of 40–98 % for percolation reactor and 20–85 % for batch reactor. Lignin removal after autohydrolysis of miscanthus at moderate severity was 24–27 %, while glucan recovery was 98 % on average (Yang and Wyman 2004) (Table 3.2).

Autohydrolysis is a simple, low-cost process in which no chemical is added. However, the application of autohydrolysis as the only pretreatment method and by conventional batch reactors is not sufficient for the improvement of hydrolysis yield of glucan. In particular for softwoods, the yield of enzymatic hydrolysis does not exceed 50 % (Chiaramonti et al. 2012a).

The efficiency of LHW for sugarcane bagasse was superior to steaming pretreatment when xylan recovery was compared. The efficiency of LHW pretreatment of sugarcane bagasse was comparable to dilute acid pretreatment by Laser et al. (2002). The optimum conditions for this study were temperature higher than 220 °C, short residence times (less than 2 min), and low solid loading (less than 5 %).

Advantages and Disadvantages

The main advantages of LHW pretreatment are no cost for solvent and neutralization. Furthermore, the equipment cost is reduced since corrosion-resistant materials are not required. Compared to steam explosion and dilute acid pretreatments, less degradation of sugars and lower production of inhibitors occur in LHW pretreatment (Wyman et al. 2005; Yang and Wyman 2004; Mosier et al. 2005a, b). On the other hand, this pretreatment is more efficient than steaming (Laser et al. 2002). However, LHW pretreatment is not efficient for woody biomass (Martin et al. 2011). LHW with percolation is more efficient and produces less inhibitor compounds (Yang and Wyman 2004); however, the main disadvantage of percolation processes is low concentration of sugars in the liquid fraction, mainly because of low solid loadings (Mosier et al. 2005b).

3.3.3.2 Explosion Pretreatment

Steam Explosion

Process Description

Steam explosion, with or without addition of chemicals, is one of the most investigated pretreatment methods. This process has also been demonstrated in several pilot plants in batch or continues modes of operation, and therefore commercial equipment is available for this pretreatment (Elander 2013; Li et al. 2010c). Steam explosion was commercially used for production of fiber boards by Masonite processes in 1920s (Mosier et al. 2005b). The process involves elevation of temperature to 160–260 °C and pressure to 0.7–4.8 MPa by steaming and holding it for a certain time (30 s to

Table 3.2 Recent studies on LHW pretreatment

Raw material	Pretreatment condition	Results	References
Percolation			
Corn stover	Percolation: 5 ml/min, 2.5 Mpa, 220 °C, 30 min (shrinking bed flow-through reactor)	95 and 93.3 % enzymatic digestibility with 60 and 15 FPU cellulase per g-glucan	Kim and Lee (2005)
Corn stover	Batch: 160–220 °C, 5 % solid loading	20–85 % enzymatic digestibility	Yang and Wyman (2004)
	Percolation: 2–25 ml/min, 350–400 psig, 180–220 °C	40–98 % enzymatic digestibility	
Batch -pH controlled			
Corn fiber	160 °C, pH above 4, 20 min 18 % solid loading (8.7 % was dissolved glucan and starch)	Almost complete digestibility, 50 % fiber solubilization: carbohydrate (80 % oligomers, 20 % monomer), <1 % carbohydrate loss	Mosier et al. (2005c)
Corn stover	190 °C, pH above 4, 15 min 16 % solid loading	90 % cellulose digestibility with 15 FPU/g-glucan cellulase, 88 % ethanol yield	Mosier et al. (2005a)
Corn stover	Severity: 4.0, 10 % solid loading, 2 g NaOH/100 g substrate	~80 % ethanol yield by SSF, over 90 % decrease in degradation of hemicellulose and production of furfural than for the uncontrolled pH	Li et al. (2014)
Batch-pH not controlled			
Soybean straw	210 °C, 10 min, 10 % solid loading	71 % glucose yield in enzymatic hydrolysis, 80 % xylan removal in pretreatment	Wan et al. (2011)
Eucalyptus grandis	1st stage: 180 °C, 20 min, 2nd stage: 200 °C, 20 min 10 % solid loading	1st stage: 86.4 % xylose recovery 2nd stage: 96.63 % overall sugar recovery	Yu et al. (2010)
Reed grass	180 °C, 20 min, 5 % solid loading	79 % ethanol yield by SSF fermentation, with 40 FPU/g-biomass cellulase	Lu et al. (2013)

20 min) (Karimi et al. 2013; Agbor et al. 2011). Afterward, the materials are explosively discharged to a vessel with lower pressure, e.g., a cyclone.

Reactions and Products

Although no chemical is added to the biomass, autohydrolysis along with the mechanical forces alters the structure of biomass to enhance enzymatic hydrolysis (Chandra et al. 2007; James 1994; Saddler et al. 1993). Mechanical forces are applied when the pressure of media is suddenly released. Water is trapped inside biomass, and a portion of water is changed to vapor and suddenly expands as pressure is decreased. This process disrupts biomass to smaller particles and increases accessible areas for hydrolytic enzymes. The result of steam explosion is explosive decompression of biomass (Zheng et al. 2014), decrease in the particle size, separation of fibers, partial solubilization of hemicelluloses, removal and/or redistribution of lignin, and changes in cellulose structure (Brodeur et al. 2011). All these changes produce a more digestible biomass (Taherzadeh and Karimi 2008; Zheng et al. 2014).

Three products are derived from steam explosion pretreatment: (1) a solid containing less recalcitrant cellulose plus lignin, (2) a liquid containing solubilized hemicelluloses plus some degradation products of lignin and pentoses, e.g., furan derivatives and phenolic compounds, and (3) water vapor containing volatile compounds which are produced during pretreatment, e.g., 60–70 % of the produced furfural (Aden et al. 2002) (Fig. 3.7). In an industrial process, the vapor fraction must be treated in a wastewater treatment unit (Aden et al. 2002).

The amount of liquid fraction depends on the moisture content of substrate which directly affects the amount of steam required for heating the media. Depending on the pretreatment conditions and type of biomass, the liquid fraction contains 40–90 % of biomass hemicellulose. Fermentation of hemicelluloses is economically feasible when the sugar concentration is over 10 % (Taherzadeh and Karimi 2008; Karimi et al. 2013). On one hand, all of sugars in the liquid fraction are not assimilated by ordinary yeast cells. The ordinary yeast cells can ferment glucose and in some cases mannose and galactose. On the other hand, the economy of an industrial process depends on the credit from by-products as well as the main products (Shafiei et al. 2011, 2013a, 2014a; Aden and Foust 2009; Stephen et al. 2012). The mutant and

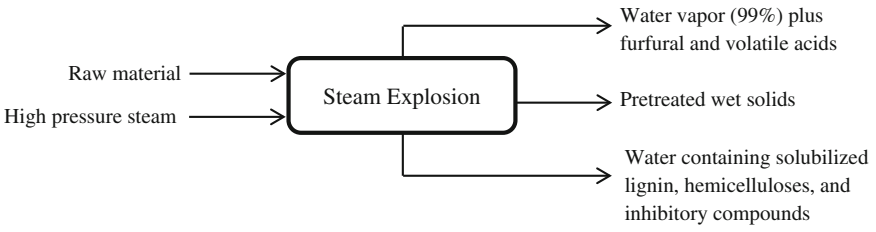

Fig. 3.7 Material flow in steam explosion pretreatment

modified strains for simultaneous hexose and pentose fermentation are available, e.g., *S. cerevisiae* 424A (LNH-ST); however, the fermentation of xylose starts when glucose is finished and is negatively affected by the presence of inhibitors such as acetic acid. Furthermore, ethanol and other fermentation metabolites significantly decrease the speed of xylose fermentation (Chundawat et al. 2013; Li et al. 2010a). Addition of a second fermentation step for the assimilation of remaining sugars may help the economy of ethanol production processes. For economic reasons, the microorganism for both steps should be essentially similar; however, the process conditions, e.g., temperature and aeration, might be different. The additional process can be fermentation with fungi which produces ethanol from both hexoses and pentoses such as *Mucor indicus*, *Mucor hiemalis*, engineered *S. cerevisiae* (Jin et al. 2010; Karimi et al. 2006a, b; Abedinifar et al. 2009; Goshadrou et al. 2011). *Zymomonas mobilis* and *Escherichia coli* are two potential bacteria for simultaneous assimilation of pentoses and hexoses. However, the performance of engineered *S. cerevisiae* is better both in laboratory and industrial scale (Aden and Foust 2009; Aden et al. 2002; Lau et al. 2010b). Consolidated bioprocessing microbes such as *C. phytofermentans* (Jin et al. 2012) may also be beneficial for simultaneous hydrolysis and fermentation of hexoses and pentoses. However, none of these solutions were found applicable in industrial scale (Taherzadeh and Karimi 2007c; Cai et al. 2012). An alternative solution is to utilize the sugars in anaerobic digestion for methane production (Shafiei et al. 2011).

The solid product from steam explosion can be washed to remove toxic compounds. However, inhibitors are present in the liquid fraction (discussed in autohydrolysis Sect. 3.3.3.1). Detoxification methods are necessary prior to hydrolysis and fermentation (Taherzadeh and Karimi 2008; Karimi et al. 2013). Alternatively, using adapted (Parawira and Tekere 2011) or mutant as well as protected microorganisms, e.g., encapsulated (Talebnia and Taherzadeh 2006), immobilized (Behera et al. 2010), or flocculated cells (Brandberg et al. 2007), may be used for the reduction of detoxification costs. Fed-batch cultivation of microorganisms is also used to reduce the inhibition effects (Taherzadeh and Karimi 2007c).

Efficiency of the Pretreatment

The efficiency of pretreatment (Table 3.3) for conversion of cellulose to glucose depends on the substrate, particle size, applied temperature, and residence time. The process severity is a function of pretreatment time and temperature. Higher severity, e.g., higher temperature and residence time, results in more solubilization of hemicellulose and better saccharification of cellulose but simultaneously promotes the production of more inhibitor compounds. The optimum temperature highly depends on biomass type and is in the range of 180–220 °C, whereas the optimum time is 2–8 min (Taherzadeh and Karimi 2008). Another concern is differences between the best conditions for maximum glucose yield and the best one for maximum hemicellulose recovery (Lloyd and Wyman 2005). Higher severity results in more digestible cellulose and simultaneously increases the degradation of sugars to inhibitors (Ruiz et al. 2008; Taherzadeh and Karimi 2007c). Thus, the

Table 3.3 Recent studies on steam explosion pretreatment

Raw material	Conditions	Result[a]	References
Lemon peel	160 °C, 5 min	~38 % fermentable sugars, ~61 % total sugars	Boluda-Aguilar and López-Gómez (2013)
Eucalyptus (hardwood)	195 °C, 5.9–34 min	18.1 g sugars/100 g DM, 94.5–99.5 % sugar yield, 91 % ethanol yield	Romaní et al. (2013)
Elephant grass	180 °C, 5 min	~50 % ethanol yield (SSF)	Eliana et al. (2014)
Japanese cedar (softwood)	~257 °C, 3 min, 45 atm	17.4 g ethanol/100 g dry material (the solid fraction was washed by methanol)	Asada et al. (2012)
Corn stover (unchipped)	150 °C, 2 min screw extrude steam explosion	89 % enzymatic hydrolysis, low formation of inhibitors	Chen et al. (2014b)
Canola Straw	180 °C, 4 min	29.40 % glucose yield, 72 h hydrolysis, 10 FPU cellulase/ L-g substrate	Garmakhany et al. (2014)
Pinewood chips	180–240 °C, 5 min	~58 % ethanol yield	Cotana et al. (2014)
Reed straw	220 °C, 5 min	36.14 % reducing sugars yield, 15.35 % glucose yield	Hu et al. (2013)

[a] The presented data are best results among several conditions

pretreatment process must be optimized to achieve optimum yields for glucose and other sugars along with minimum inhibitory effect. Steam explosion has been studied for several raw materials. The efficiency of this pretreatment is higher for agricultural residues and hardwoods than the efficiency for softwoods.

Advantages and Disadvantages

The advantages of steam explosion without addition of chemicals are given as: the requirement of relatively low capital investment, low environmental impacts, high carbohydrate recovery, moderate energy requirements (Avellar and Glasser 1998), and processes with less hazardous conditions and materials. The pretreatment can efficiently decrease particle size. The main drawbacks of steam explosion are low saccharification yields and losses of carbohydrates due to solubilization along with formation of inhibitory compounds (Taherzadeh and Karimi 2008). Higher yields are only obtained when special variations are applied. For instance, the addition of a catalyst, such as acid, base, or SO_2, is beneficial. Furthermore, special types of pretreatment reactors, e.g., percolation or extrusion reactors, have better efficiency than conventional steam explosion. These methods are discussed in the next sections.

Combination of Steam Explosion with Other Methods

The efficiency of single steam explosion as the sole pretreatment method is not enough high. A single-step steam explosion at high severity results in more digestible cellulose and simultaneously increases the degradation of sugars to inhibitors. Monomers from hemicellulose degrade to higher extent at more severe conditions (Ruiz et al. 2008). Application of two-step steam explosion has been tested to overcome this problem. The first step is performed under milder conditions with the aim of maximum xylose or mannose recovery and then the liquid fraction is separated. Afterward, the second stage steam explosion is performed at more severe conditions (over 210 °C) to enhance the digestibility of cellulose. Some studies showed that two-step steam explosion has increased the overall sugar yield, while lower enzyme loading is required and less inhibitor compounds are produced (Alvira et al. 2010). Application of SO_2 along with steam explosion is also suggested (Söderström et al. 2002). However, the study by Cotana et al. (2014) have shown that two-step steam explosion without addition of chemicals resulted in even lower ethanol yield (Table 3.4).

After acid-catalyzed steam explosion, alkaline pretreatments are the most studied pretreatments with steam explosion (Table 3.4). Significant enhancements in ethanol and glucose yields were achieved by these methods (Sun et al. 2014a; Wanderley et al. 2013; Oliveira et al. 2013; Chen et al. 2014a). Oliveria et al. (2013) studied a two-step pretreatment for ethanol production from sugarcane bagasse. Steam explosion was performed in the first step and then followed by alkaline delignification. The overall process efficiently removed about 90 % of hemicellulose and lignin and obtained 80 % glucose yield from the solid fraction. However, total cellulose losses during pretreatments were 37–43 % which is a major drawback for this pretreatment. The major solubilization of cellulose and hemicellulose occurred in the steam explosion step, and hence the fermentation of the liquid fraction might retrieve a portion of the lost carbohydrates (Oliveira et al. 2013). Combination of steam explosion with other pretreatment methods, e.g., alkaline peroxide pretreatment (Yang et al. 2010), ionic liquids (Liu and Chen 2006), and organosolv extraction (Chen and Qiu 2010), has also been studied.

Dilute Acid Pretreatment

Dilute acid pretreatment has been introduced as one of the leading pretreatment methods for the commercial production of ethanol from lignocelluloses (Wyman et al. 2005; Mosier et al. 2005b). This type of pretreatment is well studied and demonstrated in laboratory and pilot scale for several types of feedstocks. A number of reviews are available in the literature on this subject (Tsao et al. 1982; Bienkowski et al. 1984; James 1994; Hsu 1996; Jacobsen and Wyman 2000; Lee et al. 1999; Taherzadeh and Karimi 2008; Karimi et al. 2013). Sulfuric acid at concentration of 0.5–2 % is the most widely studied and the cheapest acid used for this pretreatment (Grohmann et al. 1985; Torget et al. 1992; Nguyen et al. 2000;

Table 3.4 Combination of steam explosion with other pretreatment methods

Raw material	Pretreatment		Result		References
	First step	Second step	First	Second	
Pinewood chips	Steam explosion (170 °C, 30 min)	Steam explosion (220 °C, 4.5 min)	8.89 g ethanol/32 g-cellulose in dry wood (equivalent to 33 % yield)		Cotana et al. (2014)
	–	Steam explosion (220 °C, 10 min)	10.6 g ethanol/32 g-cellulose in dry wood (equivalent to 58 % yield)		
Bamboo	Steam explosion, 2.0 MPa for 5 min	0.5 % aqueous NaOH + 60 % ethanol containing 1.5 % NaOH, solid-to-liquor ratio of 1:20 at 80 °C for 3 h	33 % glucose yield, 60 % lignin solubilization	70 % glucose yield	Sun et al. (2014a)
Sugarcane bagasse	Steam explosion (200 °C, 7 min)	1 % (w/v) NaOH (100 °C, 1 h)	73–78 % ethanol yield, fed-batch enzymatic hydrolysis		Wanderley et al. (2013)
Sugar cane straw (not grinded)	Steam explosion 180–200 °C, 5 min, industrial scale	10 kg dry solid treated with 100 L 1.5 % NaOH, 100 °C, 1 h	59–80 % glucose yield, solubilization: hemicellulose: 67–93 % glucose: ~30 % lignin: 17–0 %	85–71.5 % glucose yield solubilization: hemicellulose: 88.5–94 % glucose: 37–43 % lignin: 87–81 %	Oliveira et al. (2013)
Rice straw (15 cm long)	Impregnation with 90 g/l NH_4Cl solid/liquid: 1/1	Steam explosion 1.4 MPa, 4 min	–	62.6 %	Chen et al. (2014a)
	–	Steam explosion 1.4 MPa, 10 min	–	0.2 times lower yield	

Harris et al. 1984; Torget et al. 1991a; Lis et al. 2000; Kim and Lee 2002; Soderstrom et al. 2003; Saha et al. 2005). However, other acids such as hydrochloric acid (Israilides et al. 1978; Goldstein et al. 1983; Goldstein and Easter 1992; Titchener and Guha 1981; Higgins and Ho 1982), trifluoroacetic (Fengel et al. 1978; Fengel and Wegener 1979), and phosphoric acid (Israilides et al. 1978; Um et al. 2003) have also been investigated.

Process Description

The pretreatment is performed by dilute acid (e.g., 0.2–2 % w/w sulfuric acid) at elevated temperatures in the range of 160–220 °C (Yang and Wyman 2004; Mosier et al. 2005b) and held for a certain time ranging from seconds to minutes at pressure of 3–15 atm (Wyman et al. 2005). Different solid to liquid ratios were studied in batch or continuous operations. Dilute systems containing 10 % solid loading (Torget et al. 1990, 1991b) to higher solid to liquid ratio of 14.6:1 were studied (Karimi et al. 2006a). However, typical solid loading is 10–40 %, and solid loading over 50 % is not pumpable and seems like a wet cake rather than slurry (Wyman et al. 2005; Zhu et al. 2004, 2005). Application of concentrated acid (30–70 %) at low temperatures was also investigated. Concentrated acid, however, is not economical due to the processing cost of highly corrosive and hazardous acid besides the expenses for neutralization or recovery of acid (Taherzadeh and Karimi 2007b, 2008).

The dilute acid pretreatment process includes mixing of the substrate with acid, preheating, heating, and finally cooling down the materials. Several possibilities for operating acid-catalyzed pretreatment have been studied in laboratory and pilot scale experiments. In the conventional batch method, acid is mixed with substrate and heated either indirectly by heaters or directly by steam injection, similar to uncatalyzed steam explosion. Indirect heating prolongs warm-up time because of limitations in heat transfer, and thus promotes the production of inhibitor compounds in the overheated parts. Conversely, steam heating is a faster method which is suitable for commercial applications. However, it requires additional boiler equipment for steam production.

Continuous pretreatment was successfully studied in plug-flow reactors in laboratory and pilot scale (Elander 2013). In such processes, pre-steaming is performed in a separate reactor, and then the non-condensable gasses are separated from the solid before sending solids to the main pretreatment reactor. The motive forces for moving solid are moving paddles on the central shaft and the pressure of steam as well as plug-screw feeders (Aden et al. 2002; Elander 2013). Advantages of such system are high solid loading and short reaction times, e.g., 30 s to 2 min.

Percolation reactors have been successfully evaluated in laboratory scale. In such systems, the acidic liquor is passed through a bed of biomass and removes the solubilized monomers from the media; thus, minimizes the decomposition of sugars. Another advantage of such system is application of lower acid concentration, e.g., 0.1 %, compared with the typical method. The lower efficiency of less concentrated acid is compensated by the increase of retention time or decrease in

solid loading (Mosier et al. 2005b; Wyman et al. 2005). The solid loading is typically 2–4 % while reaction times are 12–24 min and the temperature and pressure are 190–200 °C and 2–24 atm (Wyman et al. 2005). The main parameters affecting sugar concentration are flow rate of acid, neutralization (buffering capacity of biomass), and sugar decomposition (Zhu et al. 2004; Cahela et al. 1983; Esteghlalian et al. 1997). The major drawback of this method is operating with low solid to liquid ratios and production of a dilute sugar solution. Therefore, extensive energy is required for the product recovery and flow-through reactors are not demonstrated in commercial scale (Mosier et al. 2005b).

Similar to severity factor used for liquid hot water pretreatment, a modified severity factor is used for dilute acid pretreatment (Chum et al. 1990; Yang and Wyman 2004):

$$M_0 = t \cdot A^n \cdot \exp[(T - 100)/14.75] \tag{3.2}$$

where A is concentration of acid (wt%), and n is a constant.

Reactions and Products

Two products are formed after acid-catalyzed pretreatment: A solid which is called hydrocellulose and mainly contains cellulose and lignin and a liquid. The solubilized hemicelluloses such as xylooligomers and xyloses as well as solubilized lignin and degradation products are accumulated in the liquid fraction. The products of pretreatment are at low pH and must be neutralized prior to enzymatic hydrolysis. Washing the solid fraction is sufficient for neutralization and detoxification; however, addition of a base such as NaOH or $Ca(OH)_2$ is necessary for neutralization of the liquid fraction. Overliming by $Ca(OH)_2$ is an efficient method in which neutralization and moderate detoxification are performed simultaneously (Palmqvist and Hahn-Hägerdal 2000a, b; Aden et al. 2002).

Hydrogen bonding is the major force which arranges the cellulose chains together in a crystalline structure. At elevated temperatures and acidic conditions, hydrogen bonds are loosened and acid molecules penetrate inside the cellulose structure. Acid solubilizes a part of acid-soluble lignin which accumulates in the liquid fraction (Xiang et al. 2003). Acid also cleaves glycosidic bonds in the hemicelluloses as well as glucuronosyl linkages in polyuronides (Jacobsen and Wyman 2000; Grohmann et al. 1986). The result of these reactions is cleavage of lignin carbohydrate complexes. Efficient hydrolysis of hemicelluloses, partial solubilization of lignin, as well as slight hydrolysis of cellulose occurs in acid-catalyzed pretreatment. Acid pretreatment efficiently removes acetyl esters which are bonded to hemicelluloses (Grohmann et al. 1989). The kinetic models for cellulose and hemicellulose hydrolysis reactions are investigated in several studies (Kobayashi and Sakai 1956; Shafizadeh 1963; Saeman 1945; Shen and Wyman 2011; Jacobsen and Wyman 2000).

The cleavage of bonds occurs even at mild pretreatment conditions. However, more severe conditions increase the degradation of monomeric sugars and lignin to

inhibitor compounds such as furan derivatives and phenolic compounds (Bertaud et al. 2002). Some of the inhibitor compounds that are formed are furfural, 5-hydroxymethylfurfural (HMF), and organic acids such as uronic, formic, levulinic, and acetic acid (Karimi et al. 2013). The inhibitor may partially or completely hamper enzymatic hydrolysis and/or fermentation processes. Therefore, detoxification (Palmqvist and Hahn-Hägerdal 2000a, b) or application of robust microorganisms is essential for obtaining a high-yield ethanol production (Taherzadeh and Karimi 2007a). In addition to the solubilization of hemicelluloses and lignin, a part of cellulose is also hydrolyzed to glucose. The decomposition rate of monomer sugars in dilute acid pretreatment is in the following order (Saeman 1949):

$$\text{Xylose} > \text{Arabinose} > \text{Mannose} > \text{Galactose} > \text{Glucose}$$

Although the decomposition of glucose is slower than other sugars, its recovery is lower than the expected amount (Karimi et al. 2013). In addition to decomposition, glucose monomers are lost as a result of two other reactions: the condensation of acid-soluble lignin with glucose to form a glucose-lignin complex (Xiang et al. 2003) and the condensation of glucose to oligosaccharides, i.e., glucose reversion reactions (Pilath et al. 2010).

The cellulose which remains in the solid has lower DP and is more susceptible to enzymatic hydrolysis. However, the changes in the crystallinity of cellulose depend on the substrate and applied pretreatment conditions. Acid can solubilize the amorphous cellulose and increase the porosity of the remaining cellulose at mild conditions. Thus the crystallinity changes to a certain level (Kumar et al. 2009). Decrease in the crystallinity of cotton (Mugnolo et al. 1988) was reported after acid-catalyzed pretreatment. However, other studies reported increase in the crystallinity after the pretreatment (Fengel and Wegener 1984). In addition to solubilization of the amorphous portion of cellulose, lignin, or hemicellulose (Kumar and Wyman 2013), recrystallization may also occur in sever pretreatment conditions (Karimi et al. 2013).

Condensation, fragmentation, and re-polymerization of lignin may occur in acid-catalyzed pretreatment (Yang and Wyman 2004; Fengel and Wegener 1984) which leads to redistribution of lignin. Condensation of lignin to the biomass surface must be avoided since the modified lignin has affinity to irreversibly bind to cellulase and reduces the efficiency of cellulase enzyme (Yang and Wyman 2004). In batch dilute acid pretreatment, the production of solubilization of lignin and the production of insoluble condensation are more than the batch LHW pretreatment. When flow-through reactors are used, the solubilized lignin is removed by the liquor, and thus prevents the condensation of lignin inside the reactor (Yang and Wyman 2004).

Efficiency of the Pretreatment

In acid-catalyzed pretreatment, higher solubilization of sugars is achieved compared to un-catalyzed steam explosion. The efficiency of the process depends on the:

1. Acid type and concentration
2. Applied temperature
3. The temperature profile for the whole process (preheating, heating, and cooling)
4. Time of exposure of the materials to acid, whether presoaking was used or acid was introduced before or after preheating
5. Properties of the feedstock, e.g., type, size, and moisture content
6. The neutralizing effect of buffering components in the biomass (Zhu et al. 2004, 2005)
7. Extent of xylan removal (Zhu et al. 2005)

Examples of acid-catalyzed pretreatments for different types of feedstocks are presented in Table 3.5. After acid-catalyzed pretreatment, 80–100 % of hemicellulose sugars are solubilized. Galactan, arabinan, and mannan are almost completely solubilized, while the solubilization of xylose is slightly lower (Torget et al. 1990). Lignin solubilization may vary from 4 to 26 % while 8–25 % glucan solubilization may occur. The solid fraction is usually washed thoroughly before enzymatic hydrolysis. In addition to pretreatment conditions, the digestibility of cellulose in the solid fraction highly depends on the substrate and its properties. Most of the studies reported efficient hydrolysis yields for agricultural residues after dilute acid pretreatment. The efficiencies reported for hardwood and softwood are not in similar orders and yields range from a few percent to 94 % yields. The yields from dilute acid pretreated softwood did not reach 70 % (Zhu and Pan 2010) unless other modifications are employed such as addition of Na_2SO_3 (Shuai et al. 2010). The differences in the yields may be due to operation conditions of the pretreatment, such as substrate preparation method, solid loadings, efficiency of enzymes in the presence of inhibitors, as well as configuration of the pretreatment reactor. For instance, lower yields were obtained when indirect heating was used in batch operations (Li et al. 2013).

Application of percolation reactors reduces the retention time of monomeric sugars inside the reactor and thus less degradation may occur. Successful attempts for using high solid loading were performed by Zhu et al. (2004, 2005). The studies resulted in high xylose concentration (6–7 %) in liquid fraction as well as and high digestibility of glucan and xylan. In the these studies, low degradation of sugars was the result of preheating and drying of the biomass before the main pretreatment as well as fast cooling of the pretreated biomass by nitrogen quenching. The extent of xylan removal had significant effect on the digestibility of cellulose. The enzymatic digestibility of cellulose pretreated by percolation reactor is 10–20 % higher than conventional batch reactors at the same severity. Higher hemicellulose and lignin removal as well as preventing from degradation of sugars and condensation of lignin derivatives are the reasons for superior efficiency of percolation reactors (Yang and Wyman 2004).

Table 3.5 Some studies on dilute acid pretreatment

Raw material	Catalyst	Condition	Result	References
Spruce (softwood, 40 mesh)	1 % H_2SO_4	180 °C, 30 min, liquor to solid weight ratio—5:1	55 % hydrolysis yield in 48 h, 15 FPU cellulase/g-cellulose 77.7 % cellulose recovery 56.7 total sugar recovery	Shuai et al. (2010)
	1 % H_2SO_4 + 1.8 % Na_2SO_3 wt%	180 °C, 30 min, liquor to solid weight 5:1	91 % hydrolysis yield in 24 h, 15 FPU cellulase/g-cellulose 92.5 % cellulose recovery 87.9 % total sugar recovery, 35 % less inhibitors	
Silver maple, sycamore, Black locust (hardwood, 2 mm milled)	0.45–0.5 % v/v H_2SO_4, acid is added after preheating the mixture is mixed continuous	160 °C, 10 min, 10 % solid loading	88–94 % glucose yield after 48 h hydrolysis, 42 IFPU/gm of cellulose; solubilization[a]: 13–17 % glucan, 6–16 % lignin, Xylan, 97–98 %. Very low inhibitor production	Torget et al. (1991b)
Corn cobs		160 °C, 5 min, 10 % solid loading	90–100 % glucose yield after 48 h hydrolysis, 42 IFPU/gm of cellulose; solubilization: 14–25 %[b] glucan, 26–9 % lignin, 93–100 %, xylan	
Corn stover				
Pine	Pre-impregnation, 4 h + 1.2 % w/w H_2SO_4	160 °C, 20 min 3 % solid loading, Batch experiment in test tubes	Low sugars yields	Li et al. (2013)
Eucalyptus			Low sugar yields	
Switchgrass			48 % hydrolysis yield in 24 h	
Rice straw	Pre-impregnation by 0.5 % w/w H_2SO_4 (20 h)	Steam added to reach 15 bar, kept for 10 min Solid/liquid: 600 g/41 g	74 % ethanol yield in SSF by *Rhizopus oryzae*, 15 FPU/DM cellulase	Karimi et al. (2006a)
Poplar hybrids Sweetgum	(30 min pre-steamed) 0.45–0.5 % v/v H_2SO_4, 2 mm milled samples, acid is added after preheating; the mixture was stirred continuously	160 °C, 5–10 min, 10 % solid loading	90–100 % cellulose digestibility; solubilization: 96–98 % xylan, 9–16 % glucan, 17–18 % lignin	Torget et al. (1990)
Weeping lovegrass, switchgrass		160 °C, 5–10 min, 10 % solid loading	85–90 % cellulose digestibility; Solubilization: 92–93 % xylan, 18 % glucan, 4–12 % lignin	
Legume (herbaceous)		160 °C, 5–10 min, 10 % solid loading	70 % cellulose digestibility; Solubilization: 85 % xylan, 8 % glucan, 3 % lignin	
Corn stover	(Percolation) 1 % w/w H_2SO_4, 1 g of liquid/(g dry feedstock*min	160–180 °C, 10 ml/min percolation, at end: quenching using nitrogen-gas	70–75 % recovery of xylose (6–7 % concentration, 10 % decomposition), ~95 % glucose yield in 48 h, 15 FPU/g-glucan cellulase	Zhu et al. (2004)
Corn stover				

(continued)

Table 3.5 (continued)

Raw material	Catalyst	Condition	Result	References
	1 % w/w H_2SO_4, 1 g of liquid/(g dry feedstock*min)	Percolation 160–180 °C, 10 ml/min, at end: nitrogen-gas-through quenching	92 % hydrolysis, 5 FPU enzyme, 98 % glucan and 94 % xylan recovery	Zhu et al. (2005)
Corn stover	0.1 % w/w H_2SO_4	160–220 °C, 2–25 ml/min percolation	35–87 % hydrolysis, 60 FPU/g-glucan spezyme CP and novozyme 188	Yang and Wyman (2004)
		Batch: 160–220 °C, 5 % solid loading	55–98 % hydrolysis, 60 FPU/g-glucan spezyme CP and novozyme 188	

[a] Other components are polyoses are galactan, arabinan, manan, and acetyl groups which are 100 % hydrolyzed
[b] The first number represents corn cob, and the second value is for corn stover

Advantages and Disadvantages

The conventional pretreatment is a relatively inexpensive and simple. The method is well demonstrated in commercial scale and is known as one of the leading pretreatment methods which results in acceptable saccharification yields.

Although the pretreatment process is advantageous compared to many other pretreatments, most of the problems arise after the pretreatment process. One of the main drawbacks of acid-catalyzed pretreatment is considerable losses of the carbohydrates. Furthermore, the production of inhibitory compounds hinders subsequent enzymatic hydrolysis and/or fermentation. Additional processes are essential for detoxification and neutralization of the products, which increase the costs of ethanol production process. Although sulfuric acid is inexpensive, the disposal costs of gypsum are added to the cost of acid (Hinman et al. 1992; Hsu 1996; McMillan 1994; Grohmann et al. 1985, 1986; Torget et al. 1991b). The affinity of lignin to cellulase increases the enzyme requirements as well as hydrolysis time (Wyman et al. 2005). Furthermore, the presence of modified lignin and inhibitors dictates application of low substrate concentrations in the enzymatic hydrolysis (Ramos et al. 1992). Lower solid to liquid ratio increases the capital as operating costs for recovery of ethanol. Another main drawback of acid-catalyzed pretreatments is the corrosive media which requires expensive acid-resistant equipment. Major changes in the lignin properties by acid pretreatment may also be a problem in value-added products production from lignin. Although dilute acid pretreatment is regarded as one of the leading pretreatment methods, it has many drawbacks. Some of these drawbacks are diminished when percolation reactors are used; however, operating percolation reactors in commercial scale is not demonstrated yet (Wyman et al. 2005; Mosier et al. 2005b; Yang and Wyman 2004; Karimi et al. 2013). Another approach to overcome some of these drawbacks is combination of dilute acid pretreatment with other pretreatment methods which will be discussed in the next sections.

Acid-catalyzed Steam Explosion

Application of steam explosion along with acid has been widely studied over the years for different purposes. The method was used for production of furfural. For this purpose, furfural is transferred to the vapor fraction by sudden depressurizing and recovered by distillation (Root et al. 1959; Zeitsch 2000; Zhu and Pan 2010). Dilute acid pretreatment with explosion was also studied for ethanol production prior to concentrated acid hydrolysis (Mosier et al. 2005b).

Process Description

Dilute acid steam explosion is mostly used for pretreatment of woody biomass. The pretreatment process includes mixing the substrate with sulfuric acid in liquid form or SO_2 in vapor phase, steam heating, and explosive discharge of the materials. Typically, 0–5 % acid or SO_2 is added to biomass and pretreatment is performed at

190–210 °C for hardwood and 200–220 °C for softwood (Zhu and Pan 2010). Application of steam facilitates rapid heating of the media. Furthermore, the substrate is not diluted by excess water since about 90 % of the vapor used for heating is vaporized in the explosion step. Several possibilities for operating acid-catalyzed explosion have been studied in laboratory or pilot scale experiments. The equipment for continuous pretreatment in commercial scales is also available (Aden et al. 2002; Aden and Foust 2009; Elander 2013).

Reactions and Products

The basic reactions in acid-catalyzed steam explosion are similar to acid-catalyzed pretreatment. However, the explosive discharge of materials at the end of process disrupts the biomass structure to particles with lower size, and thus increases the specific surface area of the biomass (McMillan 1992; Brownell et al. 1986; Shimizu 1988; Ballesteros et al. 2000). Physical disruption of material after acid pretreatment should be more efficient compared with un-catalyzed steam explosion, due to higher efficiency of the pretreatment. However, explosion showed no effect on the enzyme accessibility and enzymatic hydrolysis in some investigations (Morjanoff and Gray 1987; Brownell et al. 1986). High severities as well as small particle sizes might contribute to insignificant effect of explosion (Karimi et al. 2013).

Some studies suggest that the application of acid increases the inhibitor compounds (Mosier et al. 2005c; Palmqvist and Hahn-Hägerdal 2000a, b), whereas others claim that less inhibitor compounds are produced when acid is used (Mosier et al. 2005b; Sun and Cheng 2002; Brodeur et al. 2011). However, both of these claims can be true under specific conditions: When similar temperature and residence time are used for acid-catalyzed steam explosion and un-catalyzed steam explosion, more inhibitor compounds are produced in the acid-catalyzed pretreatment. However, in such conditions, higher glucose yields are obtained with the addition of acid. When sugar yield similar to un-catalyzed steam explosion is desired, then acid acts as a catalyst and reduces the temperature and/or residence time. Thus, less inhibitor are produced under these conditions by acid-catalyzed steam explosion.

Two more mechanisms may also contribute to the generation of less inhibitory compounds; in acid-catalyzed pretreatment, materials are cooled by a cooling media or just left to cool down. Whereas in acid-catalyzed steam explosion, explosive discharge of materials is used instead, thus, the pretreatment reactions are suddenly ceased, and the exposure of sugar monomers to high severity is reduced. Therefore, less inhibitor compounds are produced after completion of the pretreatment. Furthermore, a part of furans and volatile acids are evaporated after explosion.

Efficiency of the Pretreatment

The efficiency of this pretreatment (Table 3.6) is widely discussed by Zhu and Pan (2010). High efficiencies were obtained by acid-catalyzed steam explosion for hardwood (>80 %); however, the results were not sufficiently high for softwood

Table 3.6 Some of recent studies of acid-catalyzed steam explosion pretreatment

Raw material	Catalyst	Conditions	Result	References
Orange peel	0.5 % v/v H_2SO_4	180 °C, 2.5 min	~73 % pectin solubilization	Santi et al. (2014)
			~56 % glucose yield	
			0.495 g g^{-1} ethanol yield	
Olive tree pruning	1 wt% H_3PO_4	195 °C, 10 min	~70 % ethanol from detoxified liquor (SSF)	Negro et al. (2014)
		175 °C, 10 min	~80 % from solid part (SSF)	
Rice straw ≤ 2 cm	Presoaked in 1.3 wt% H_2SO_4	Continuous 185 °C, 2 min	~73 % total (glucose + xylose) yield	Chen et al. (2013)
Rice straw (2 cm length)	Impregnation, 2 % H_2SO_4, followed by steam explosion	165 °C, 2 min, 15 % solid loading	~78 % total saccharification yield (glucose + xylose /of total biomass) 20 FPU/g-cellulose	Chen et al. (2011)
		180 °C, 20 min; 6L reactor		
	Impregnation, 3 % H_2SO_4	180 °C, 2 min, 15 % solid loading	49.6 % total saccharification yield, (glucose + xylose /of total biomass) 20 FPU/g-cellulose	
Corn stover (Italian and American)	Impregnation by 3 % SO_2	170 °C, 5 min	60–69 % glucose yield[a], 53–63 % xylose yield, 7–0 % glucan loss	Öhgren et al. (2007)
	Impregnation by 3 % SO_2	190 °C, 5 min	89–87 % glucose yield, 76–70 % xylose yield, 9–10 % glucan loss	
	Steam explosion 190 °C, 5 min		71–74 % glucose yield, 67 % xylose yield, 2–6 % glucan loss	

[a] In the whole study, addition of xylanase in enzymatic hydrolysis improves the enzymatic hydrolysis of glucose 12–22 %. Best glucose yields are 103–108 % for 3 % SO_2, 190 °C pretreatments using extra xylanase

(<70 %). SO_2 is more commonly used for softwood compared with sulfuric acid (Zhu and Pan 2010).

Advantages and Disadvantages

Explosive discharge of the materials after acid-catalyzed pretreatment reduces the particle size of biomass. This advantage seems most beneficial in industrial scale processes where energy and equipment costs for size reduction are considerable. In some acid-catalyzed pretreatments, materials are cooled by a coolant media such as water or nitrogen quenching. These methods are not economically feasible in industrial scale processes. Discharge of the materials to a blowdown vessel with ambient pressure facilitates rapid temperature reduction to boiling point of water. Furthermore, the explosive discharge facilitates fast emptying of the pretreatment reactor. This is equivalent to less residence time of material in the reactor and smaller pretreatment equipment. The vapor product removes a part of inhibitor compounds which is advantageous for the subsequent hydrolysis and fermentation processes, and since a part of water is evaporated, higher xylose concentrations are obtained compared with simple dilute acid pretreatment (Zhu and Pan 2010).

The disadvantage of explosive acid-catalyzed pretreatment is additional costs of the blowdown vessel. The vapor product is highly corrosive and must be cooled and treated in the wastewater treatment.

Combination of Dilute Acid Pretreatments

Similar to single-step steam explosion, dilute acid pretreatment, suffer from differences between optimum conditions for recovery of hemicellulose monomers and best condition for maximum digestibility of cellulose. Two-step acid pretreatment successfully improved the xylose recovery as well as glucose yield (Table 3.7). However, the results for corn stover (Mosier et al. 2005b) were more promising compared with softwood (Söderström et al. 2003). The most important point of two-step acid hydrolysis is to separate the liquor before second pretreatment and if this separation is not performed, e.g., in the study by Diedericks et al. (2013), the enhancements in saccharification are marginal. Using alkali pretreatment along with acid pretreatment resulted in production of a highly digestible material which was mainly consisted of cellulose. However, the produced cellulose-rich material may be utilized for other industries to produce higher value-added products, e.g., pulp and paper industry. The operating and capital costs regarding second step of pretreatment must be evaluated to ensure the economic feasibility of an additional pretreatment step.

Table 3.7 Application of dilute acid pretreatment in combination with other pretreatments

Raw material	Pretreatment		Result		References
	First method	Second method	First	Second	
Corn stover	140–174 °C, 10–20 min, H_2SO_4	170–204 °C, 10–20 min, H_2SO_4	Up to 90 % cellulose digestibility, solubilization: 83–100 % hemicellulose (95–80 % monomer), 26–53 % lignin		Mosier et al. (2005b)
	0.0735, 0.4015, and 0.735 wt% H_2SO_4 in percolation reactors				
Sawdust from softwood (of which 62 % was hexoses)	Impregnation in 0.5 % w/w H_2SO_4, steam treated 180 °C for 10 min (Batch)	Re-impregnated in 1–2 % w/w H_2SO_4, 200 °C for 2 min (batch)	Glucan recovery: 81 % in solid, 12 % in liquid (87 % monomer)	77 % sugar yield, cellulase: 15 FPU/g DM, 65 % total ethanol yield by SSF	Söderström et al. (2003)
			Mannan recovery: 12 % in solid, 88 % in liquid (88 % monomers), 94 % fermentation yield for un-detoxified liquid		
Sugarcane bagasse (dry, milled)	Impregnation, 45-55 mmol/L H_2SO_4, 170 °C, 14 min, 5 % solid loading	45–55 mmol/L H_2SO_4, 187–220 °C, 8.6–25.4 min	78 % sugar recovery (glucose + xylose) The whole product of first stage is used in the second stage	4.8 % increase in total sugar recovery, 36.1 % reduction in furfural production	Diedericks et al. (2013)
Switchgrass	0.078 wt% H_2SO_4, 125–165 °C, 4.0 mL/min percolation for 10 min	0.078 wt% H_2SO_4, 155–195 °C, 4.0 mL/min percolation for 10 min	Solubilization: 85 % hemicellulose, 35 % lignin	Total solubilization: 100 % hemicellulose, 40–48 % lignin 16–35 % glucan	Wu and Lee (1997)
	Two stage: 0.078 wt% H_2SO_4, 4.0 mL/min percolation, 10 min each stage, 140 °C, followed by 170 °C	10 wt% ammonia, 170 °C, 4.0 mL/min percolation, 20 min	Solubilization: 100 % hemicellulose 37 % lignin ~17 % glucan 65–90 % glucan hydrolysis in 72 h by 20–60 IFPU/g-glucan enzyme	Total solubilization: 100 % hemicellulose 82 % lignin 25 % glucan 70–95 % glucan hydrolysis in 72 h by 20–60 IFPU/g-glucan enzyme	

3.3.3.3 Alkali Pretreatments

In alkali pretreatment, alkaline solutions such as NaOH, $Ca(OH)_2$, or lime are used for modification of lignocelluloses. The first pretreatment experiment of straw by NaOH for enhanced digestibility by ruminants was reported by Millett et al. (1976). Alkaline reagents were used in pulp and paper industries as well as mercerization processes in textile industries (Takai and Colvin 1978; Karimi et al. 2013).

Pretreatment with alkaline solutions, especially NaOH, modifies or removes lignin and hemicellulose. The pretreatment increases the porosity and intra-channel sizes of biomass (McMillan 1992). The pretreatment lowers the cellulose DP and increases the swelling capacity (Fengel and Wegener 1984). The swelling capacity is less improved by ammonia compared with sodium hydroxide. Reviews on alkali pretreatment, especially using NaOH, have been presented by Karimi et al. (2013), Chundawat et al. (2013), and Ramirez et al. (2013). However, most of the bases are too expensive and their recovery and reuse is not applicable (Hsu 1996). In this regard, application of ammonia and lime has been suggested (Mosier et al. 2005b; Wyman et al. 2005).

Ammonia Fiber Expansion (AFEX)/Ammonia Fiber Extrusion (FIBEX)

AFEX pretreatment is introduced as one of the leading pretreatment methods for commercialization (Wyman et al. 2005). Pressurized ammonia was used for fibrillation and plasticization of wood in a process named ammonia explosion pulping. In 1982, concentrated (>30 % NH_4OH) and pressurized ammonia was used for the first time as a pretreatment to enhance enzymatic digestibility of lignocelluloses (Dale and Moreira 1982). Concentrated or even anhydrous ammonia has been used in the most studies; however, recently aqueous ammonia with concentration of less than 15 % NH_4OH is also applied in processes with or without percolation (Chundawat et al. 2013).

Process Description

Ammonia is a highly volatile substance. Reversible dissolution of ammonia (NH_3) in water forms ammonium and hydroxyl ions (NH_4^+ and OH^-). The most important point in pretreatment with ammonia is to provide sufficient contact between biomass and ammonia, which is facilitated in pressurized reactors. Then after completion of the pretreatment the pressure is suddenly reduced. The major fraction of ammonia is vaporizes after depressurizing of the materials. Therefore, theoretically it can be recovered and reused again by more than 98 % (Chundawat et al. 2013). The recovery of ammonia is possible through compression or cooling and quenching processes (Bals et al. 2011). The economies of these two processes were studied in full biorefinery models and using cooling system was found to be less expensive (Eggeman and Elander 2005; Laser et al. 2009). AFEX pretreatment has been tested in several types of reactors.

- Conventional batch reactors: In the conventional method, liquid ammonia with concentration of 0.3–2 g NH_3/g dry biomass is used for pretreatment of moist material (0.1–2 g water/g dry biomass). The mixture is kept at elevated temperature and pressure, e.g., 40–180 °C and 15–20 atm, for a period of time, e.g., 5–60 min, and then the pressure is reduced immediately (Kole et al. 2012; Taherzadeh and Karimi 2008; Chundawat et al. 2013). Unlike steam explosion, no liquid slurry is produced after pretreatment and only two products in solid and vapor form are generated (Mosier et al. 2005b). In laboratory experiments, ammonia is not recovered (Chundawat et al. 2011).
- Plug-flow reactor (PF-AFEX): Plug-flow reactors are tubular reactors with or without conveyor screws. In a biorefinery scale, screws facilitate better contact of the biomass with NH_4OH. The pretreatment may be performed in co- or counter-current modes of operation, and after pretreatment, the materials are explosively released to a blowdown tank. A prototype plug-flow reactor (without internal screw) was demonstrated successfully for AFEX pretreatment of pumpable slurries by MBI International (http://www.mbi.org) (Chundawat et al. 2013). Continuous operation of ammonia expansion is called Fiber extrusion (FIBEX) (Wyman et al. 2005).
- Packed-bed reactor (PB-AFEX): For moist biomass with high solid content, e.g., corn stover or straw, the biomass can be treated in stationary packed beds. Gaseous ammonia passes over the biomass which is loaded in series of reactors in a cyclical fashion. This type of operation was demonstrated well by MBI International (Chundawat et al. 2013).
- Fluidized gaseous reactors (FG-AFEX) and extractive reactors (E-AFEX) are two other types of reactors for AFEX pretreatment. The former one uses hot gaseous ammonia (with or without nitrogen or steam), and the latter one uses concentrated or anhydrous ammonia (with or without organic solvent). The preliminary experiments for these two types of reactors are underway.

Reactions and Products

The composition of biomass does not change significantly by AFEX pretreatment. However, regardless of the reactor type, ammonolysis (amide-forming) and hydrolysis (acid-forming) reactions change the structures of lignin and carbohydrates (Chundawat et al. 2010). These reactions cleave the lignin carbohydrate complexes, which are the main barriers for enzymes. For instance, arabinose side chains of xylan are cross-linked through diferulates with lignin and AFEX pretreatment cleaves these bonds (Chundawat et al. 2010). After completion of the pretreatment, ammonia which was penetrated through the cell rapidly rushes out of the media. This rapid escape of materials produces large pores (>10 nm in diameter) and increases accessible surface area of biomass (Wyman et al. 2005). Meanwhile, the cross-linked structure is loosened and ammonia carries a portion of lignin and hemicellulose to outer layer of the cell wall as well as cell corners (Campbell et al. 2013). Modification of acetyl and uronic esters of hardwood by ammonolysis has

been reported during super critical or subcritical ammonia pretreatment (Weimer et al. 1986; Wang et al. 1964). AFEX pretreatment lowers the DP of hemicelluloses. The reactions for hemicellulose depolymerization are catalyzed by ammonium hydroxide. The conventional AFEX pretreatment does not change the cellulose crystallinity; however, in the absence of water, reduction of cellulose crystallinity was reported as well (Balan et al. 2009; Chundawat et al. 2013).

Inhibitory compounds such as furans and HMF are produced in AFEX pretreatment in low amounts. Lignin fragments and phenolic compounds may remain in the solid. Production of organic acids, e.g., lactic acid and succinic acid, occurs in AFEX pretreatment, however at a scale 100–1,000 times lower than NaOH pretreatment (Chundawat et al. 2010). It is claimed that washing of solid after AFEX pretreatment is not necessary; furthermore, high solid loading in the enzymatic hydrolysis is possible with unwashed-treated material (Balan et al. 2009; Teymouri et al. 2005).

Efficiency of the Pretreatment

AFEX pretreatment is most efficient for materials with lower amounts of lignin, e.g., agricultural residues, and is less efficient for softwood and hardwood. The efficiency of the pretreatment depends on the ammonia loading, solid loading, temperature, time and blowdown pressure, as well as the reactor (Taherzadeh and Karimi 2008). Some of the studies for AFEX pretreatment are presented in Table 3.8.

Advantages and Disadvantages

The pretreatment is efficient especially for agricultural residues. Ammonia is less corrosive compared to acid-catalyzed pretreatments and thus the equipment costs are lower compared with equipment for dilute acid pretreatment. Almost complete recovery of ammonia is possible. The residual amounts of ammonia in the solid increase the nitrogen content of the biomass which has positive effect on fermentation (Dale et al. 1985; Lau and Dale 2009). The only product of AFEX pretreatment is a solid fraction which can be used in subsequent pretreatments without neutralization or detoxification. Among leading pretreatment methods, AFEX pretreatment with anhydrous ammonia can be performed with highest solid loading (60–90 %) (Wyman et al. 2005).

AFEX is not efficient for high lignin biomass, especially softwood. Ammonia is a toxic, flammable, and volatile substance. Operation of AFEX pretreatment requires technical consideration for a hazardous process, which increases the operating as well as capital costs. The recovery and reuse of ammonia are necessary because of its cost as well as environmental aspects (Wyman 1996; Sun and Cheng 2002; Eggeman and Elander 2005). If fermentation of xylose is desired, addition of xylanase or hemicellulase is necessary.

Table 3.8 Some recent studies of AFEX pretreatment

Raw material	Conditions	Result	References
Switchgrass	100 °C, 5 min, 1 g ammonia/g dry biomass, 80 % moisture in biomass	93 % glucan yield	Alizadeh et al. (2005)
Reed canary grass	100 °C, 60 % moisture, vegetative growth stage: 1.2 kg ammonia/kg of DM	168 h hydrolysis, 15 FPU cellulase/g-glucan	Bradshaw et al. (2007)
		86 % glucose, 78 % xylose (growth stage)	
	Seed stage: 0.8 kg ammonia/1 kg of DM	89 % glucose, 81 % xylose (seed stage)	
Bagasse and cane leaf residue	100–140 °C, 45–60 min, 1–2 g ammonia/g dry biomass, 150 % moisture in biomass	85 % cellulose hydrolysis	Krishnan et al. (2010)
		95–98 % hemicellulose hydrolysis	
Empty palm fruit bunch fiber	135 °C, 45 min, 1 g ammonia/g dry biomass, 1 g moisture/g dry biomass, post pretreatment size reduction	~90 % hydrolysis yield in 72 h with enzymes: Accellerase, Multifect Xylanase and Multifect Pectinase (84, 31, and 24 μL/g-biomass)	Lau et al. (2010a)
Miscanthus x giganteus	160 °C, 5 min, 2 g ammonia/g dry biomass, 233 % moisture in dry biomass; post pretreatment size reduction	96 % glucose, 81 % xylose, 168 h hydrolysis, 1 % glucan loading, 15 FPU cellulase/g-glucan + β-glucosidase + xylanase	Murnen et al. (2007)
Rice straw	140 °C, 30 min 1 g ammonia/g dry biomass 80 % moisture	80.6 % glucan yield (12 % oligomer) and 89.6 % (37 % oligomer) xylan yield in 72 h enzymatic hydrolysis with spezyme CP 15 FPU/g-glucan, multifect xylanase and multifect pectinase (2.67, and 3.65 mg protein/g-glucan), 6 % glucan loading	Zhong et al. (2009)
Corn stover	130 °C, 10 min, 1 g ammonia/g dry biomass, 0.7 g water/g dry biomass	Glucan and xylan hydrolysis yields: 87.78 % and 90.64 % novozyme 50013 and 50010:15 FPU and 64 pNPGU /g-glucan	Zhao et al. (2014)
Moso bamboo	1-batch AFEX 150 °C, 10–30 min, 2–5 g ammonia/g dry biomass, 0.8 or 2 (w/w) water	64.8–72.7 % sugar hydrolysis yield, 15 FPU/g-glucan cellulase	Shao et al. (2013)
	2-post pretreatment size reduction		
	1-presoaking with 0.7–1.9 g H_2O_2 (30 wt% solution)/g-biomass, 10 min	83.4–92.1 % sugar hydrolysis yield, 15 FPU/g-glucan cellulase	
	2-150 °C, 10–30 min, 2–5 g ammonia/g dry biomass, 0.8 or 2 (w/w) water		

Ammonia Recycled Percolation (ARP) and Soaking in Aqueous Ammonia

Process Description

Ammonia recycled percolation (ARP) is performed with aqueous ammonia (5–15 %) at elevated temperatures (150–180 °C). The biomass is loaded in a packed-bed reactor and the aqueous ammonia is passed through it at flow rate of 1–5 ml/min and for 10–90 min (Kim et al. 2003, 2006; Kim and Lee 2005; Ramirez et al. 2013). In addition to reaction time, about 30 min for preheating is required too (Ramirez et al. 2013). Shrinking bed flow-through reactors were also applied for ARP pretreatment (Kim and Lee 2005). The pretreatment pressure is usually as high as 9–17 atm which is facilitated by nitrogen back pressure. Solid loading of 15–30 % can be used in ARP pretreatment (Wyman et al. 2005). The liquid input is in the range of 2.0–4.7 ml/g-biomass and optimum retention time is 10–12 min (Gupta and Lee 2009; Kim et al. 2003). After ARP pretreatment, the treated solid is extensively washed. The liquid fraction is boiled to recover ammonia in the vapor form and recover precipitated lignin in solid form (Gupta and Lee 2010).

Soaking aqueous ammonia (SAA) is performed by 15 % ammonium hydroxide in conventional oven. Pretreatment is performed either at moderate temperatures (25–60 °C) for long pretreatment times, e.g., 12 h to several days or at higher temperatures (60–120 °C) for shorter pretreatment times, e.g., 1–24 h (Table 3.9). Addition of H_2O_2 improves delignification; however, hydrogen peroxide is unstable and expensive (Ramirez et al. 2013).

Reactions and Products

The biomass undergoes swelling in contact with aqueous ammonia. The pretreatment depolymerizes lignin and cleaves lignin-hemicellulose bonds, which are mainly C–C, C=O, and C–O bonds. Thus, guaiacyl lignin is more affected by ammonia than syringyl type lignin (Gupta and Lee 2009). Most of the delignification occurs in the first 20 min (Gupta and Lee 2009; Kim et al. 2003). Negligible degradation of carbohydrates occurs during ammonia pretreatments. The liquor passing biomass removes solubilized lignin and hemicellulose. The solubilized hemicelluloses are mostly oligomers. Lignin removal remarkably affects enzymatic hydrolysis of the pretreated materials (Wyman et al. 2005; Kim and Lee 2005; Kim et al. 2003). The liquor also removes lignin derivatives which are potential inhibitors for fermentation (Palmqvist and Hahn-Hägerdal 2000a, b). The crystalline structure of cellulose is not affected significantly by aqueous ammonia (Kim and Lee 2005). However, the accessibility of enzymes to carbohydrates is improved by hydrolysis of cross-linked glucuronic acid esters. Release of uronic acid ester groups in hemicellulose in the form of amides by ammonolysis was also reported (Ramirez et al. 2013).

Table 3.9 Some recent studies for pretreatment using ammonia

Raw material	Pretreatment condition (water/ammonia/feed) kg:kg	Result	References
ARP			
Corn Stover	15 % wt ammonia, 5 ml/min: 15 g solid in 50–450 ml solution, 2.3 Mpa 170 °C, 90 min	Solubilization: 70–85 % lignin, 40–60 % hemicellulose (oligomers); 99 and 92 % hydrolysis yield with cellulase at 60 and 10 FPU/g-glucan cellulase, respectively	Kim et al. (2003)
Corn Stover, (Shrinking bed flow-through reactor)	15 % w/w ammonia, 5 ml/min, 2.3 Mpa 170 °C, 10–90 min	93 % enzymatic digestibility with 10 FPU/g-glucan cellulase, solubilization: 70–85 % lignin, 50–60, 92 % cellulose recovery	Kim and Lee (2005)
	Low liquid ARP: 15 % w/w ammonia, 5 ml/min, 2.3 Mpa, (2.7:0.47:1), 170 °C, 10 min	88.5 % enzymatic digestibility with 15 FPU/g-glucan cellulase, solubilization: 73.4 % lignin, 84 % ethanol yield	
Corn Stover	Two stages: 1-water percolation: 5 ml/min, 2.5 Mpa 190 °C, 30 min	1st stage: 83–86 % monomer xylose recovered 2nd stage: 75–81 % lignin removal, 87–89 % glucose yield with 15 FPU/g-glucan cellulase (only one stage LHW pretreatment at 170–210 °C resulted in 72–87 % hydrolysis yields)	Kim and Lee (2006)
	2-ARP: 15 % w/w ammonia, 5 ml/min, 2.5 Mpa 170 °C, 60 min		
Waste oak/Virgin oak	Preimpregnation, ARP: 15 % ammonia 18 g-biomass, 5 ml/min 130/170 °C	Over 85 % glucan hydrolysis with 60 FPU/g-glucan cellulase, ~60–85 % delignification, ~90 % glucan remained in the solid	Kim et al. (2008a)
SAA			
Bagasse	28 % v/v NH_4OH mixed with water (0.5:8) Liquid to solid ratio: 8.5:1 0.9–1.1 MPa, 160 °C, 1 h	Solubilization: 55 % lignin, 30 % hemicellulose, 9 % cellulose. 87 % glucose yield, 60 FPU/g-glucan Spezyme CP, 23 g ethanol/100 g dry biomass	Aita et al. (2011)
Switchgrass	15 % aqueous NH_3 10 % solid loading w/v 160 °C, 1 h	Recovery: 34 of 36 % glucan, 13 of 21 % xylan, 1.5 of 3 % arabinan, 8.6 of 23 % lignin. Yield: 65 % glucose, 75 % xylose 15 FPU/g-glucan spezyme CP	Garlock et al. (2011)
Oil palm empty fruit bunches	21 % w/w aqueous NH_3 60 °C, 12 h	19.5 % glucose yield with 15FPU after 96 h hydrolysis, 65.6 % ethanol yield in 168 h SSF, 5 % solid loading	Jung et al. (2011a)

(continued)

Table 3.9 (continued)

Raw material	Pretreatment condition (water/ammonia/feed) kg:kg	Result	References
Oil palm trunks	7 % aqueous NH_3 liquid to solid ratio 12:1 80 °C, 8 h	95.4 % hydrolysis yield (compared to 11 % untreated) with 60 FPU cellulase in 96 h. 78.3 % ethanol yield	Jung et al. (2011b)
Barley hull	15 % ammonia liquid to solids ratio: 12:1 75 °C, 24–72 h	83 % glucose yield and 63 % xylose yield with 15 FPU/g-glucan cellulase. Solubilization: 50–66 % lignin, 65–76 % xylan	Kim et al. (2008b)
Rice straw	21 % ammonia liquid to solids ratio: 6:1 69 °C, 10 h	71.1 % glucose yield in enzymatic hydrolysis	Ko et al. (2009)
Corn Stover	15 % ammonia, Liquid to solids ratio:11:1 60 °C, 8–24 h	84 % ethanol yield from both glucose and xylose. Recovery: 98–99 % glucan, 90–80 % xylan	Li et al. (2010b)
Corn Stover	30–50 % ammonia, liquid to solid ratio: 5:1 30 °C, 4 week	86.5 % glucan digestibility with 15 FPU cellulase/g-glucan, 71–73 % ethanol yield	Li and Kim (2011)

Efficiency of the Pretreatment

The factors affecting pretreatment efficiency are ammonia concentration, time, temperature, and amount of liquid throughput (Ramirez et al. 2013). ARP pretreatment efficiently removes 70–85 % of lignin and 40–60 % of hemicellulose. After completion of pretreatment, 85–99 % of highly digestible cellulose is recovered in the solid fraction. High yields (Table 3.9) for enzymatic hydrolysis of agricultural residues and hardwood were observed even by application of lower amounts of cellulase enzyme (Iyer et al. 1996; Kim et al. 2000). However, no study was found for pretreatment of softwood by aqueous ammonia. Pre-impregnation of biomass by ammonia improves the results of ARP, especially for woody biomass (Kim 2004). ARP pretreatment in combination with hot water percolation and dilute sulfuric acid pretreatment was used for fractionation of biomass to lignin, hemicellulose, and cellulose-rich fibers. The fibers showed high digestibility as well (Kim and Lee 2006, 2005; Wu and Lee 1997). Efficiency of SAA pretreatment is lower than ARP (Ramirez et al. 2013).

Advantages and Disadvantages

The conditions are less severe in aqueous ammonia pretreatments compared with AFEX pretreatment. The SAA and ARP pretreatments are most efficient for agricultural residues and hardwood. Xylanase activity of most of the available cellulase enzymes is sufficient for hydrolysis of xylan in the pretreated materials. Long pretreatment time for SAA is the major disadvantage of this pretreatment. This method requires high amounts of water for washing compared to other pretreatment methods (Garlock et al. 2011). Using high amounts of liquid is one of the drawbacks of percolation reactors. In ARP pretreatment, the back pressure of nitrogen is essential to maintain ammonia in liquid form (Ramirez et al. 2013). In industrial scale, operating process under nitrogen pressure requires additional operating costs.

Lime

Process Description

Lime pretreatment was used to improve in vitro digestibility of crop residues used for animal feed. Furthermore, it was used for hydrolysis of protein-rich animal wastes, such as feather. The hydrolyzed protein was used as a high valuable animal feed (Coward-Kelly et al. 2006; Chang et al. 1997).

In lime pretreatment, biomass slurry (5–15 g H_2O/g dry biomass) is pretreated by calcium hydroxide (>0.1 g $Ca(OH)_2$/g dry biomass). Long-term lime pretreatment is performed at mild temperatures (25–60 °C) for several days to weeks (Sierra et al. 2010). This type of pretreatment is suggested for treating biomass piles, in which no specific pretreatment reactor is required. Percolation of air as an inexpensive oxidizing agent is possible in long-term lime pretreatment (Wyman et al. 2005).

Shorter residence time (minutes to hours) and higher temperatures (100–180 °C) are applied in short-term lime pretreatment. In this pretreatment, the pretreatment efficiency is boosted by additional oxidizing agent such as oxygen at partial pressure of 1–15 atm or hydrogen peroxide (Sierra et al. 2009; Agbor et al. 2011; Wyman et al. 2005).

After completion, neutralization by carbon dioxide is required to reduce alkalinity of the biomass. The insoluble calcium carbonate is separated after hydrolysis or fermentation. The carbonate can then be converted to lime using established lime Kiln technology (Chang et al. 1998).

Reactions and Products

Partial solubilization of lignin and hemicelluloses as well as complete removal of acetyl groups occur in lime pretreatment. Enzyme adsorption of lime pretreated biomass is higher when it is compared with AFEX pretreated biomass (Chang et al. 1997; Wyman et al. 2005). Overliming, which is a method for detoxification of dilute acid hydrolyzate, reduces the non-productive binding of enzyme to biomass and thus the required amount of enzyme is decreased.

Lime is a week alkaline agent and has partial solubility in water (<0.2 %). Solubility of calcium hydroxide decreases as the temperature is increased. When lime is exposed to air, it reacts with carbon dioxide, and thus percolating air through biomass piles as an oxidizing agent requires addition of excess lime (Table 3.10).

Efficiency of the Pretreatment

Lime pretreatment solubilizes about 33 % of lignin and 26 % of xylan. Such modifications are sufficient for efficient hydrolysis of herbaceous materials with low-lignin content (Chang et al. 1997; Gandi et al. 1997; Kaar and Holtzapple 2000; Wyman et al. 2005). However, for biomass with higher lignin content such as poplar (hardwood), efficient enhancements are provided by combining effects of alkali and oxygen (Chang et al. 2001b). Application of oxidative lime pretreatment removed approximately 80 % of lignin from poplar wood, furthermore, the remaining cellulose was highly digestible (Chang et al. 2001a; Wyman et al. 2005). The efficiency of lime pretreatment for softwood is not reported yet.

Advantages and Disadvantages

Lime has the advantage of lower cost and higher safety compared with other alkaline reagents (Chang et al. 1997; Playne 1984). The carbon dioxide required for neutralization of lime is produced in fermentation and no additional cost is required for neutralizing agent. The produced calcium carbonate is not soluble in water and its separation is easier than other salts. Furthermore, the salt recovered is easily converted to lime in a well-established industrial process.

Table 3.10 Some recent studies for lime pretreatment

Raw material	Pretreatment condition	Result	References
Corn stover	0.5 g lime/g-raw biomass, 55 °C, 4 week	91.3 % Glucose yield, 51.8 % xylose yield, 15 FPU/g-cellulose, 87.5 % lignin removal	Kim and Holtzapple (2005)
Corn stover	0.075 g lime/g dry biomass, 5 g H_2O/g dry biomass, 120 °C, 4 h	Hydrolysis yields: 60–88 % cellulose, 47–87 % xylan, in 72 h with 10–25 FPU cellulase/g dry biomass	Kaar and Holtzapple (2000)
Bagasse (12–60 mesh)	0.4 g lime/g-biomass, 70 °C, 36 h	70.7 % total sugar yield, 3.42 FPU/g dry biomass cellulase	Rabelo et al. (2008)
Bagasse	0.12 g lime/g-biomass, 57 °C, 4 week	64.3 % total sugar yield (HPLC)	Cotlear and Benigno (2004)
Poplar (hardwood)	0.1 g lime/g-biomass, 150 °C, 6 h, 15 bar O_2	77 % total sugar yield, 5 FPU cellulase/g dry biomass	Chang et al. (2001b)
	0.1 g lime/g-biomass, 240 °C, 30 min	44 % total sugar yield, 5 FPU cellulase/g dry biomass	
Corn stover	1st stage: 1 % wt HCl, 120 °C, 30 min 2nd stage: 0.1 g lime/g-biomass, 60 °C, 12 h	1st stage: 97 % xylose recovery 2nd stage: 78 and 86 % glucose yield with 5 and 10 FPU cellulase/g of substrate	Zu et al. (2014)

One of the disadvantages of lime pretreatment is slower reactions compared with other alkaline pretreatments. Addition of oxidizing agents may lead to non-selective degradation reactions. Losses of carbohydrates as well as production of inhibitor aromatic compounds may occur as well (Hendriks and Zeeman 2009).

3.4 Concluding Remarks

There are still large differences between laboratory scale ethanol production and commercial scale processes when lignocellulosic materials are used. Challenges and bottle necks are different for each scale. Numerous laboratory scale experiments were conducted along with several pilot scale demonstrations. However, the full commercialization of ethanol production from lignocelluloses still cannot compete with first generation of biofuels and gasoline. In recent years, the biomass structures as well as pretreatment methods are better understood. The recent knowledge about biomass structure and leading pretreatment methods was discussed in this chapter.

The recalcitrance features of lignocelluloses which hinder their enzymatic hydrolysis were reviewed in this chapter. The most important features such as cellulose structure and complicated aggregation of the cell wall building blocks are responsible for the macro- and micro-accessibility of the cellulose. For instance,

covering layers of hemicellulose and lignin prevent direct contact of the enzyme with cellulose. The importance of accessibility is clearer when hydrolysis yield of un-pretreated biomass especially softwood does not exceed 10 %, while amorphous pure cellulose is almost completely hydrolyzed. Therefore, pretreatments are used to enhance the cellulose accessibility as well as to increase the enzyme efficiency. The efficiency of enzyme is improved when the number of active binding sites of cellulose to enzyme is increased, e.g., by decrease in crystallinity or DP. Thermochemical pretreatments which are discussed in this chapter such as dilute acid, AFEX, LHW are considered as leading pretreatment methods because of their potential applicability in industrial scale. Most of the discussed pretreatments improve the macro-accessibility of cellulose. However, they do not significantly improve the micro-accessibility of cellulose. Pretreatment by cellulose solvents such as concentrated phosphoric acid, ionic liquids, and NMMO enhances both macro and micro-accessibility of cellulose, without requirement for neutralization. However, with even high recovery of the expensive solvent, the process is still facing economic challenges.

Dilute acid pretreatment seems more efficient for most types of biomass, while AFEX is more suitable for agricultural residues. Similarly, LHW pretreatment is more efficient for low-lignin content biomass. Dilute acid pretreatment solubilizes major parts of hemicellulose along with partial delignification. This pretreatment also reduces the cellulose DP. The drawbacks of dilute acid pretreatment such as inhibitor production and sugar degradation are minimized by optimized pretreatment condition and by application of special types of reactors, e.g., plug reactors or percolation processes. However, neutralization and disposal of the salts are still major problems for dilute acid pretreatment. In commercial scale, the ability of process to handle multiple types of feedstock is important. Operation of liquid hot water and dilute acid pretreatments is possible in a single commercial process. For instance, if a less recalcitrant material is used, e.g., corn stover, the pretreatment is performed by water, and for more recalcitrant materials, e.g., wood, acid can be added to the system.

AFEX pretreatment enhances biomass digestibility. This pretreatment cleaves lignin carbohydrate complexes, redistributes lignin and hemicelluloses, reduces the cellulose DP, and increases the accessible surface area of the biomass. However, ammonia must be recycled and reused to reduce chemical costs, and, furthermore, operation of AFEX pretreatment requires higher safety considerations compared with dilute acid or liquid hot water pretreatment.

Steam explosion and lime pretreatment are not relatively highly efficient; however, these are relatively inexpensive and simple. Therefore, the combination of these pretreatments with other methods (before or after main pretreatment) seems interesting options for further enhancement of biomass digestibility.

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Chapter 4
Enzymatic Hydrolysis Technologies for the Production of Biofuels

Anahita Eckard

Abstract One of the major challenges in second-generation biofuel production is economical conversion of lignocellulosic biomass to fermentable sugars. The most commercially feasible method for conversion of biomass to fermentable sugars has been the combination of thermochemical and enzymatic hydrolysis treatment of biomass. Nevertheless, even with the most efficient pretreatment method, the use of cellulolytic enzymes accounts for more than half of the sugar production cost. As a result of the high cost of commercial hydrolytic enzyme and the low digestibility of pretreated biomass with minimal enzyme use, sugar production costs are not economical for commercial production of some fermentation products. One of the primary reasons for low digestibility of biomass at minimal enzyme doses is the limited enzyme accessibility to cellulose due to the presence of lignin. Other reasons include (1) the change in reactivity of cellulose during hydrolysis that occurs when amphiphilic substance is depleted and (2) deactivation of enzyme by sugars, sugar degradation products, and both soluble and insoluble lignin. With current pretreatment technologies, commercially relevant methods must be developed that would improve the performance of enzymes and make the application of lower enzyme doses feasible. An attractive solution is to generate on-site enzymes to allow for the utilization of cheap abundant protein activity in-house. This chapter reviews the cellulolytic enzyme system, the mechanism of action, rate-limiting factors of enzymatic hydrolysis, on-site enzyme production, and recovery of enzyme activity by enzyme recycling.

4.1 Introduction

Due to inexpensive prices of petroleum and natural gas in twentieth century, the US petrochemical industry and oil refining was thrived. However, gradually with the increase in price and demand of fossil fuel, many of the petroleum-driven chemical production has been moved to countries with lower production cost and the

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depletion of fossil fuel in near future started to become of a greater concern (Holtzapple et al. 1990). Clearly, cheaper and abundant resources are necessary to support the production of chemicals and fuel. Among the available alternatives, lignocellulosic biomass such as woody materials and agricultural residues is the most abundant and fast regenerated renewable resources with more than 200 million ton of production annually (Zhang et al. 2008) that can be converted to value-added products.

Lignocellulosic biomass is composed of three major components in which cellulose makes the majority followed by hemicellulose and lignin. Cellulose composed of glucose units linked to each other in linear fashion with β-1, 4 glucosidic bonds. Cellulose has amorphous and crystalline structure. Studies have shown that crystalline cellulose is in flat form, twofold helical conformation; however, minor differences in formation of cellulose chain within the crystalline structure result in 7 crystalline polymorphs identified as Iα, I_{β}, II, III_{I}, III_{II}, IV_{I}, and IV_{II} that are found to vary from each other in solubility, melting point, density or crystal shape, etc. (Kadla and Gilbert 2000). While cellulose is highly insoluble in water, conformational studies shown that other than the bottom and top of cellulose chains that are hydrophobic, the side chains are hydrophilic and able of forming hydrogen bonds. Cellulose and starch polymers are similar to each other as regards that they both are composed of glucose units; however, in opposite of cellulose, the glucose units in starch are connected with α-1, 4 bonds.

When it comes to digestibility (breaking the polymer of carbohydrate to sugar monomers) of these two polymers, starch requires 100 times lesser enzyme than cellulose to be converted to glucose. Part of the reason for the low digestibility of cellulose compared to starch was linked to β-bonds in crystalline structure that was more difficult to depolymerize than the α-bonds in amorphous starch. Also, there is a shieling effect imposed by remnant lignin and hemicellulose after pretreatment that limit enzyme accessibility while induce its deactivation and that is why cellulosic biomass requires more severe processing condition such as pretreatment before enzymatic hydrolysis compared to starch feedstocks such as sugarcane or corn. If raw lignocellulosic biomass is exposed to cellulolytic enzymes, the yield of sugar recovery can be found to be minimal. This is because at this point the structures of carbohydrate polymers are not sufficiently accessible for efficient enzyme's catalytic reaction. Therefore, pretreatments, a process during which biomass is exposed to heat and/or chemicals for certain period of time is necessary to enable the enzyme's accessibility to carbohydrate polymers. From a commercial perspective, a desirable pretreatment process should have reasonable capital cost and operating cost, use chemicals that are not highly corrosive and hazardous, and finally would only generate inhibitors in reasonable level that would allow the resulting sugars to be fermented without the need of detoxification. Details of leading pretreatment techniques are presented in Chap. 3. A sample typical process steps in which biomass is converted to value-added product such as alcohols is demonstrated in Fig. 4.1.

Once the cellulose structure has been opened and hemicellulose polymer (mainly C5) was mainly degraded, the solids composed of mainly cellulose and lignin are

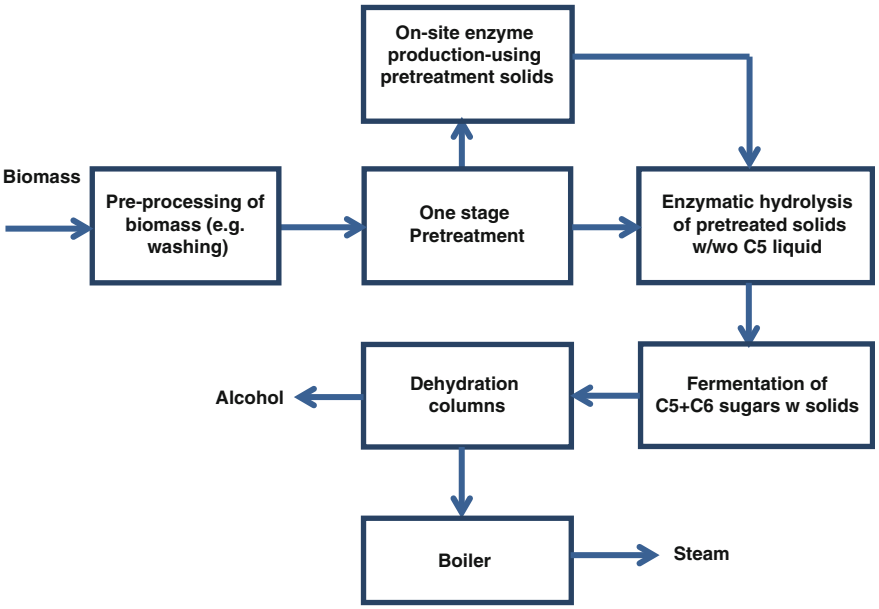

Fig. 4.1 Simplified process flow diagram of lignocellulosic biomass conversion to alcohols

enzymatically hydrolyzed. As a result of the enzyme catalysis on cellobiose or oligomers reacting with water, a single molecule of glucose can be released according to the following reaction. As can be observed, as a result of this reaction, each unit of glucose experiences a mass gain of 11.1 % when released from longer chain, meaning that the mass of glucan with 162 gr converts to 180 gr, when a water molecule is added to glucan:

$$(C_6H_{10}O_5)_n + nH_2O \rightarrow nC_6H_{12}O_6 \tag{4.1}$$

Cellulolytic enzymes act very specific by only catalyzing the addition of water to chain of glucan and as a result in opposite of acid hydrolysis, very minimal by-products are generated. Similar to cellulose, hemicellulose is hydrolyzed by water to individual sugars. Since hemicellulose is composed of both hexoses and pentoses, part of the stoichiometry of hemicellulose (glucan, mannan, galactan) hydrolysis is similar to that of cellulose (Eq. 4.1), and an 11.1 % mass gain is experienced by each molecule of hexose sugars. For xylan and Arabian hydrolysis, a mass gain of 13.6 % is experienced, when molecular weight of the sugar molecule increases from 132 to 150 gr according to the following reaction:

$$(C_5H_8O_4)_n + nH_2O \rightarrow nC_5H_{10}O_5 \tag{4.2}$$

In a typical efficient conversion of biomass, hemicellulose hydrolysis is maximized during pretreatment at temperatures of 100–180 °C, in the absence or

presence of acid as catalyst. By tweaking the process features, a modest inhibitors concentration can be obtained at maximum hemicellulose recovery (~80–90 % conversion); however, at the same time, the outcome of fermentability is very much dependent to temperature profile of pretreatment rather other factors. Then, during enzymatic hydrolysis >95 % of cellulose and <10 % of hemicellulose get exposed to enzymes in the form of polymer or oligomers for conversion to sugar monomers.

After all, the enzymatic hydrolysis has been recognized as the bottleneck of the conversion process since the high price of hydrolytic enzyme has made the sugar production cost from inexpensive and widely available lignocelluloses a challenging task for commercialization. In order to be able to convert the cellulosic fibers to fermentable sugars with high yield that could justify the economics of biomass conversion to alcohols, a large volume of a concentrated enzyme cocktail with cellulase, cellobiose, xylanase, and xylobiose activities is required. At a glance, it may appear that application of up to 5 % (w/w) of concentrated enzyme solution on pretreated biomass is not a large amount; however, cost estimations demonstrate that this amount of enzyme can accounts for more than half of the sugar production cost.

Let's take an example: A typical cellulase loading of 15 FPU/g cellulose would equate to ~30 g of enzyme per liter of ethanol produced. Assuming a liter of ethanol is sold for $0.94; thus, to reduce the cost of enzyme share to <10 % of product cost, it is essential for the enzyme cost to be reduced to less than $2/kg protein, or strategies must be developed to substantially reduce enzyme dosage rates (Himmel et al. 1999; Wingren et al. 2005; Wyman 2007).

One of the strategies for solving the enzyme cost problem is to generate on-site enzyme that uses the existing process streams, and by this way, the cost of enzyme would be reduced significantly. However, the ability to produce a high amount of extracellular enzyme proteins is only the characteristic of certain fungi that have been genetically modified. One of the most studied cellulase generating fungi is *Trichoderma reesei*. From the publically available strain (e.g., RUT C-30), the highest amount of generated extracellular enzyme protein of up to 24 FPU/ml has been reported, while the commercial enzyme cocktails (combination of different activities) contain more than 180 FPU/ml of enzyme activity. For an industrial scale biomass conversion, a high solid loading hydrolysis tank is necessary, otherwise the size of the tank and volume of the water utilized would be larger by several order of magnitude. This setting demands the utilization of an enzyme rich solution that can be produced only from modified commercially owned fungi strains, else increasing the volume of enzyme solution (due to low protein activity) added to biomass could reduce the total solid loading in hydrolysis tank that would consequently increase the capital cost drastically.

In this chapter, cellulolytic enzyme production and enzymatic hydrolysis of pretreated biomass are reviewed.

4.2 Cellulase Classification

Proteins are divided to subclasses based on the nature of reactions they catalyze by International Union of Biochemistry and Molecular Biology (IUBMB). This method uses numbering code system (EC-numbers) to specify their enzymatic activity. In this classification, *T. reesei* cellulases belong to the hydrolases and are found in group EC3 with CBHs (EC 3.2.1.91), EGs (EC 3.2.1.4) and β-glucosidases (EC 3.2.1.21). However, this system relies on the biochemical description of a protein; therefore, to enable the prediction and classification of new enzymes, a sequence-based categorization was established that assigns sequences to a range of families according to their amino acid similarities. In Cazy database, carbohydrate-active enzymes are categorized into various families such as glycoside hydrolases (GHs) that hydrolyze or re-arrange glycosidic bonds or glycosyltransferase (GTs) that form glycosidic bonds. GHs can be classified as inverting or retaining enzymes. In an inverting enzyme, the result of hydrolysis of β-glucosidic bond is a product with α-configuration, while in retaining enzyme, the product has β-configuration. GHs with retaining mechanism have found commonly to carry out transglycosylation property as well meaning that when they are in high concentrations, they can regenerate oligosaccharides from the hydrolytic reaction products. Cellulases are within several families of GHs, and fascinatingly both CBH and GH are found within the same family.

It is interesting to note that the three-dimensional structure of many members of GHs studied so far shown to share similar overall protein folding and reaction mechanism, e.g., inverting or retaining (Seiboth et al. 1997).

4.3 Cellulase System and Mechanism of Hydrolysis of Cellulose and Hemicellulose

Cellulase system can be partitioned into two categories of secreted or cell-bound cellulases. They are categorized based on their mode of action and structural properties. In opposite of some bacteria, the cellulase multienzymes of fungi are not gathered in large cellulosome complex, but the different fungal enzymes are generated independently and their combined impact on the cellulose causes the decomposition of this polymer.

The fungal cellulolytic system can be divided into three major enzyme classes composed of **endo-glucanases (EGs) (1-4, β-D glycanohydrolases; E.C.3.2.14)** that either cleaves the insoluble cellulose fibrils internally at amorphous sites in random fashion and creates new ends or works on soluble 1,4-β-glucan substrates. The activity of this enzyme was commonly measured by detecting the reducing groups released from carboxymethylcellulose (CMC) or reduction in viscosity (Whitney et al. 1999). Some of the EGs have two domain structures.

Exo-glucanases include (1-4, β-D glycanohydrolases; cellobiohydrolases (CBH); E.C.3.2.1.74) that liberate D-glucose from 1, 4-β-D-glucans while hydrolyze D-cellobiose slowly, and 1, 4-β-D-glucan cellobiohydrolases (EC 3.2.1.91) that generates D-cellobiose from 1, 4-β-glucans. Despite this overall rough model, some CBHs have been found even to cleave the cellulose internally (Seiboth et al. 2011). *T. reesei,* the main cellulase producing fungi has two cellobiohydrolases, CEL6A and CEL7A, which are composed of two separate domains. A catalytic domain and a cellulose-binding domain that are linked via a flexible linker (Seiboth et al. 2011). Cellulose-binding domain belongs to carbohydrate-binding modules (CBMs) and is responsible for making a stable anchor for the attachment of enzyme to cellulose. X-ray crystallography has shown a wedge-shaped structure for CBMs with one face being hydrophilic and another one more hydrophobic. Three tyrosines form a regular flat surface in the CBM of CEL6A that play a role in binding to cellulose (Rouvinen et al. 1990; Divne et al. 1994) (Fig. 4.2).

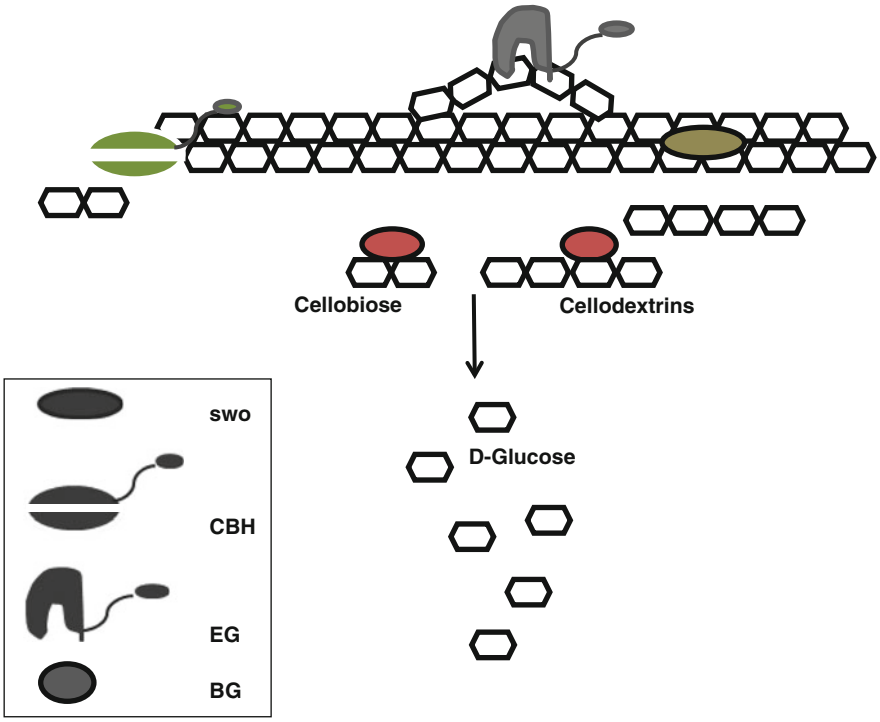

Fig. 4.2 Digestion of cellulose to monomeric sugars is the result of the action of several enzyme mono-components, in which CBHs and some of the EGs are composed of two parts which includes a large catalytic domain and a carbohydrate-binding module (CBM). CBHs, EGs, and BGLs break the cellulose synergistically to glucose. The non-enzymatic protein of swollenin (SWO) was found to aid in the degradation of cellulose by disrupting the crystalline structure and thus improving the accessibility of cellulose for enzymatic proteins (Adopted from Seiboth et al. 2011)

Overall, the CBMs of cellulases are specific for binding to the surface of crystalline cellulose but not on soluble substrates (Bayer et al. 1998). But are CBMs necessary for the catalytic activity of CBHs? It was found that the average velocity (ca. 3.5 nm/s) of CEL7A along the cellulose remained un-changed in CBM-deleted enzyme compared to the intact one, suggesting that the CBMs are not necessary for the movement of CEL7A, and the catalytic domain seems to be enough for the sliding of this enzyme on the substrate (Igarashi et al. 2009).

β-glucosidases (BG) (β-glucoside glucohydrolysase, EC 3.2.1.21) hydrolyzes the soluble cellodextrin and cellobiose to glucose monomers which is the digestible form for majority of fermenting microorganisms.

It is important to note that the majority of hydrolysis process happens simultaneously (Beguin and Aubert 1994; Tomme et al. 1995), and there is a synergism between different GHs family. It was first demonstrated by Gilligan and Reese (1954) that the amount of the reducing sugars generated from cellulose with the filtrate of combined fractions of fungal culture was larger than the sum of the sugar amounts generated by the individual fractions of these enzymes. Also, repeatedly the cross-synergism between endo- and exo-acting from different fungi has been shown (Selby 1969; Coughlan et al. 1987; Baker et al. 1994).

The action of cellulases from glycosyl hydrolase families 6, 7, and 9 is in possessive manner on cellulose, meaning that these enzymes do not become disengaged from single cellodextrin substrate until that cellulose chain is completely hydrolyzed or the enzyme becomes denatured (Wyman et al. 2005). For instance, it was found by small angle X-ray scattering (Receveur et al. 2002) that the movement of cellulase of *T. reesei* CEL7A on cellulose is caterpillar like. This is due to the fact that this enzyme has two domains connected to each other by an amino acid linker peptide (made of 30 amino acid) leading to creation of a maximum extension between the two mass of binding and catalytic modules that cover four glucose units. Despite that the binding of enzyme to cellulose is the first step in hydrolysis and crucial, cellulases that carry a fused cellulose-binding module (CBM) or an attached one by a linker peptide appear to be highly susceptible to loss on cellulose (Linder et al. 1995). This is because the highly small and reactive binding module of the cellulases may result in nonproductive adsorption to cellulose and other sites.

4.4 Impact of Supplementary Enzymes on Hydrolysis of Biomass

Similar to what is explained for the cellulase system, the conversion of biomass is dependent on the complex of few enzyme's mono-components. In addition to endo- and exo-glucanase, the action of beta-glucosidase is necessary for the conversion of cellobiose to monomeric sugars. Furthermore, both xylan and pectin can prevent from cellulase accessibility to cellulose which as a result, supplementation of xylanase to cellulase seems helpful for improvement of the overall digestibility of

biomass. It was found that with xylanase supplementation, the hydrolysis of glucan and xylan was improved by 42.5 and 43.6 % for acid treated hybrid poplar. This improvement was even higher for ammonia recycles percolation (ARP)-treated corn stover for which the digestibility of glucan and xylan was increased by 4.79 and 10.74 %, respectively (Qing and Wyman 2011). In addition to xylanase, pectin causes steric hindrance for the contact of cellulase to cellulose and hemicellulose and therefore reduces the rate of cellulose and hemicellulose hydrolysis. According to Zheng et al. (2009), addition of pectinase improved the hydrolysis of both glucose and xylose by 7.5 and 29.3 %, respectively.

4.5 Impact of Pretreatment on Efficacy of Enzymatic Digestibility of Biomass

There are few key factors that are known to affect the digestibility of biomass during enzymatic hydrolysis. Optimum solubilization of hemicelluloses during pretreatment process results in an increase of accessible pore volume and the specific surface area of cellulose and eliminates the barrier of enzyme accessibility to cellulose (Stone and Scallan 1969; Saddler et al. 1982; Brownell and Saddler 1987; Puls and Schuseil 1993; Mooney et al. 1998). This is in agreement with Chang and Holtzapple (2000) that showed the maximum acetyl removal of biomass can significantly improve the digestibility of biomass regardless of moderate lignin and high crystalline content of the biomass (Chang and Holtzapple 2000). It was found that only a limited swelling can take place when there is close association between lignin and cellulose during enzymatic hydrolysis (Mooney et al. 1998). According to Chang and Holtzapple (2000), lignin and crystallinity index of biomass is the greatest driver for enzymatic digestibility of biomass such that high digestibilities were obtained when only one of these two factors was low (Fig. 4.3). A prediction model developed by analysis of different pretreated samples suggested

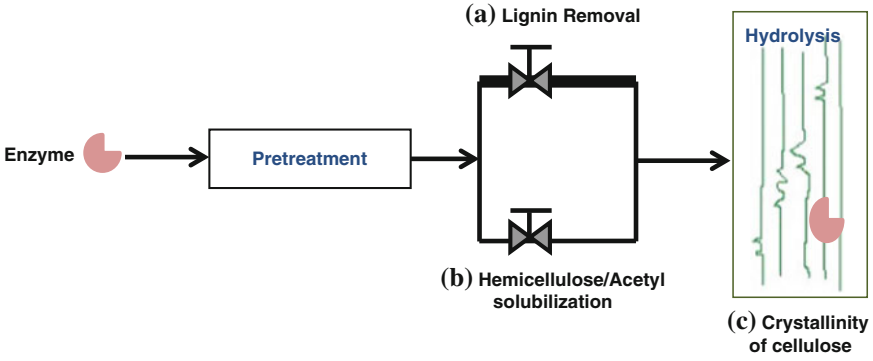

Fig. 4.3 Simplified schematic of key factors impacting the enzymatic digestibility of biomass

that if the crystallinity and lignin content of the biomass is reduced to <30 and 15 %, respectively, the biomass becomes hypothetically nearly 100 % digestible meaning that minimum amount of enzyme (e.g., >5 FPU) can be sufficient for hydrolysis (Chang and Holtzapple 2000). Lignin is well known to decrease the efficacy of enzymatic hydrolysis for numerous reasons. (i) Lignin forms a shield for cellulose against of the chemical, physical, or microbial degradation and limits the significant swelling of the cell wall, thus restricting the accessibility of cellulose to the enzyme (Sutcliffe and Saddler 1986); (ii) Lignin also irreversibly attracts a large fraction of the cellulase, making it unavailable for enzymatic hydrolysis of cellulose (Lu et al. 2002; Uribe and Sanpedro 2003; Eckard et al. 2013b). A sample schematic diagram of key factors affecting digestibility of biomass is shown in Fig. 4.3. In this figure, if assuming the lignin and hemicellulose or acetyl groups as two critical valves on the path of pipes that transfer the enzymes to cellulose, then it is important to open at least the valve on the larger pipe (A-lignin) to allow the enzymes flow to biomass. However, opening the thinner pipe (B-hemicellulose solubilization) also helps in improving the enzyme flow (accessibility) to biomass, especially when lignin removal is minimal. These two factors are highly dependent to severity and type of pretreatment method. A simple example of a scenario with two closed valves is the enzymatic hydrolysis of raw biomass without pretreatment. The third key factor is reduction in biomass crystallinity that was shown to be the second most important factor in biomass digestibility after lignin removal (Chang and Holtzapple 2000). Consideration of these three factors can help to reduce the enzyme dose significantly.

4.6 Rate-Limiting Factors in Enzymatic Hydrolysis Reaction

Despite of a generally known concept that the hydrolysis of beta bonds in a crystalline structure is far more difficult to depolymerize than the alpha bonds in amorphous starch (Wyman et al. 2005), still the hydrolysis of cellulose is rate limited due to many other reasons. It has been clearly shown that the rate of the enzymatic hydrolysis per adsorbed enzyme (specific rate) was decreased significantly as the hydrolysis proceeded (Wyman et al. 2005). In spite of many hypotheses, still the clear-cut reason for the decline in the rate of hydrolysis is not well understood (Ooshima et al. 1991; Kurakake et al. 1995; Mansfield et al. 1999; Zhang et al. 1999).

Declined hydrolysis rate was primarily postulated to be related to the depletion of the amorphous cellulose (the reactive substance), as a result, reduced reactivity of cellulose particles with the progress of cellulosic substrates conversion is one of the key reasons to reduced hydrolysis rate (Nutor et al. 1991; South et al. 1995). It was found that when enzyme concentrations were increased, the specific hydrolysis rate remained unchanged; however, when the substrate concentrations were increased,

the specific hydrolysis rate was further reduced, both observations suggesting that the reduced hydrolysis rate is highly impacted by substrate conversion to a non-reactive form over time in addition to enzyme deactivations (Wyman et al. 2005).

Other studies suggest that the falloff is also related to enzyme inactivation with several other factors that are further explained below. The nature of this deactivation can be the deformation of major enzyme's substructure and/or unavailability of enzyme in solution for catalytic activity due to irreversible adsorption (Castanon and Wilke 1981; Parke et al. 1992). Several factors such as thermal effects, shearing force, high surface tension, and interfacial or air–liquid contacts have been shown to adversely impact the enzyme activity (Kim et al. 1982; Aymard and Belarbi 2000). This irreversible adsorption was assessed in several studies, using direct measurement of adsorbed enzyme on biomass at 4 °C incubation (no hydrolysis) with nitrogen combustion analyzer (Yang and Wyman 2006; Kumar and Wyman 2008). It was found that the irreversible adsorption of enzyme was not only limited to insoluble lignin, but also to pure cellulose (Eckard et al. 2011).

High concentration of several molecules showed inhibitory effects to enzymatic hydrolysis. This include glucose and cellobiose (Holtzapple et al. 1990), monomer sugars of hemicellulose (i.e., xylose, mannose, galactose) (Xiao et al. 2004; Qing et al. 2010), xylo-oligomers (Qing et al. 2010), soluble lignin, and lignin degradation products, polymeric phenol tannic acids, and to less extent the monomeric phenolic compounds (Ximenes et al. 2011).

In aqueous pretreatment liquors, a release of 2–5 g/l of lignin in solution is expected as phenols (Ximenes et al. 2011). It was found that the digestibility of pure cellulose (Avicel) that was hydrolyzed with 15 FPU of cellulose was dropped by 10–23 % by addition of 20 % lignin (w/w). This seemed to vary strongly based on the source of the lignin (Cantarella et al. 2014). According to this result, phenolic compounds (at 10 mM) demonstrated 1–5 % more inhibition than non-phenolic ones due to impact of hydroxyl groups on enzyme activity. This result also suggests that the phenolic's hydroxyl groups may play more severe role than physical barrier and non-specific adsorption in enzymatic hydrolysis of cellulose.

According to the study of Cantarella et al. (2014), the stability of endo-glucanase was impacted by all the phenolics (1–2 g/l) through reduction in half-life of the enzyme and the reaction rate. Out of the phenolic compounds tested, the most inactivating ones were vanillin, hydroxybenzaldehyde, and protocatechuic acid (at 1 and 2 g/l). This inactivation was irreversible meaning that after the removal of phenolics, the inactivation still lasted. Similarly, the exposure to syringaldehyde (at 1 and 2 g/l) reduced the enzyme activity, and after syringaldehyde removal, the activity of enzyme was not recovered. Other phenolics such as p-coumaric acid when increased from 1 to 2 g/l, the enzyme reaction rate was reduced by fourfold, but the inactivation was ceased upon removal of the compound. Similarly, when hydroxybenzoic acid was tested, the inactivation was increased by 5 times upon the increase of concentration from 1 to 2 g/l; however, the enzyme activity was recovered when hydroxybenzoic acid was removed from contact with enzyme (Cantarella et al. 2014).

4.7 Cellulase Generating Microbes

When it comes to the industrial cellulase producing fungus, the commercially used strains were mainly derived from an isolate which was collected on the Solomon Islands during World War II (Reese and Mendels 1984; El-Gogary et al. 1990) and have been mutated to an enhanced cellulase producing form from *T. reesei*, (the anamorph or asexual reproductive stage of the *Hypocrea jecorina*). Cellulolytic enzyme secretion from *T. reesei* includes eight EGs (CEL5A, CEL5B, CEL7B, CEL12A, CEL45A, CEL61A, CEL61B, and CEL74A), two CBHs (CEL6A and CEL7A), and seven β-glucosidases (CEL1A, CEL1B, CEL3A, CEL3B, CEL3C, CEL3D, and CEL3E). The low pectin degrading content of *T. reesei* shows that the enzymes from this microbe are the most suitable ones for degrading the dead plant rather than attacking living intact plants.

Montenecourt and Eveleigh developed two lines of mutants that resulted in RUT C-30 (the hypercellulolytic strains) and RL-P37 (El-Gogary et al. 1990) (Fig. 4.4). Today, *H. jecorina* RUT C-30 is the most frequently used strain for cellulase production in laboratory research (Domingues et al. 2000; Shin et al. 2000; Bailey and Tahtiharju 2003; Collen et al. 2005; Levasseur et al. 2006; Zhang et al. 2007).

It is important to understand that the genetic modifications of *T. reesei* RUT C-30 is a truncation in the cre1 gene, the carbon catabolite repressor, and β-glucosidase II encoding gene (Geysens et al. 2005). As a result, in the presence of sugar monomers, still enzyme secretion can be observed at *T. reesei* RUT C-30, as far as the inducer is present, but the titer of enzyme production is minimal and does not exceed, e.g., 24 FPU/ml of fermentation broth (Seiboth et al. 2011). However, as a result of strain improvement, the capacity of cellulolytic enzyme protein secretion in the industrial strains has reached to 100 g/l (El-Gogary et al. 1990) with up to 60 % of the cellulase been composed of CEL7A (CBHI) and 20 % of CEL6A (CBHII) (Seiboth et al. 2011).

Trichoderma spp. have great skill in dealing with a vast variety of environments such as tropical forest as well as the dark and sterile environment of a biotechnological reactors and flask. When it comes to identification, other than pigmentation,

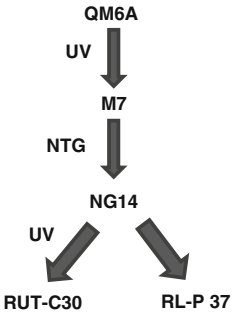

Fig. 4.4 Pedigree of strain *T. ressei* RUT C30 and its relationship to the wild-type isolate QM6A. Classical mutations were introduced by UV light (UV) and nitrosoguanidine (NTG) (Adopted from Seidl et al. 2008)

species identification within the genus is difficult because of the narrow range of variation of the simplified morphology in *Trichoderma* (Gams and Bissett 1998). *T. reesei* generates a wide range of pigments from bright greenish-yellow to reddish, with some being also colorless. Similarly, conidial pigmentation varies from colorless to various green shades and sometimes gray or brown.

4.8 Mechanism of Enzyme Production by *T. reesei*

The secretion of cellulolytic enzymes from *T. reesei* is subjected to multiple levels of control in which most of the regulation occur at the level of transcription. The role of the cellulolytic enzymes are to break the cellulose; therefore, for expression of the enzyme producing genes in fungal cells, cellulose and/or soluble oligomers (as carbon source and for energy production) need to be provided to fungal cells.

Understanding the correct molecular basis of cellulolytic enzyme productions and how *T. reesei* sense cellulose initiates enzyme secretion is crucial for a successful fermentation process. As it was described earlier, metabolite adjustments are more important in enzyme generation than the gene manipulation. Fungal cells do not receive signal from the presence of cellulose itself, they need a soluble inducer that can be up taken inside the fungal cell and that creates signal for secretion of enzyme. In the absence of no soluble inducer (only available cellulose), a basal enzyme is produced which attacks to the insoluble cellulose that generates inducer (dimmers of sugars) or inducer precursors (sugar oligomers) that can be taken up to generate enzyme (Carle-Urioste et al. 1997).

Other than the above mechanism, ready inducers can be provided to fungi from the beginning and throughout the fermentation. Among the inducers, Sophorose is one of the most efficient cellulase-inducing sugars and is produced via transglycosylation of cellulose by initial cellulase enzyme secreted from *T. reesei* (Gritzali and Brown 1979; Vaheri et al. 1979). Other than the cellulose-derived inducer, lactose (dimer) and L-sorbose (sugar monomer) are the other inducers and by far lactose has been recognized as a more soluble and cheaper source of inducer compared to other disaccharide inducers such as cellobiose and sophorose (Kubicek et al. 2009; Seiboth et al. 2007).

In *T. reesei,* expression of a majority of cellulases does not occur during the growth on glucose; however, as is known, increase of microbial population also requires providing mono sugars to fungi. This causes a lag between the times required for generation of microbial population and the time spent on enzyme generation. As a result, one of the first efforts for improvement of cellulase generation in *T. reesei* was to eliminate the effect of carbon catabolite repression. From this effort, a publically available strain of RUT C-30 with strong cellulose generating capability was produced that has truncation in CREA-1 gene and is carbon catabolite depressed (Ilmen et al. 1996; Seidl et al. 2008). However, despite of the effort, still the utilization of glucose or other carbon sources showed to result in low

cellulase yield; this was also proved by deleting CREA-1 gene from wild type. These results showed that even the carbon catabolite depressed strains are still inducer dependent and carbon catabolite sensitive (Nakari-Setälä et al. 2009).

4.9 Strategies for Improvement of Enzyme Activity

Improvement of enzyme activity for commercialization of biomass to sugars would require either maintaining the activity of enzyme for a longer period of time or reusing the enzyme by recycling. As it was mentioned in previous sections, a natural drop in hydrolysis rate is expected due to change in cellulose reactivity; however, fraction of the enzyme activity that has been lost to different soluble and insoluble inhibitory compounds can be recovered when the inhibitory effect is eliminated (Cantarella et al. 2014).

Several methods have been proposed for the reduction of enzyme utilization that includes enzyme immobilization (Yang et al. 2009; Pavani and Basil 2010), Enzyme recycling (Steele et al. 2005; Qi et al. 2011), washing off the biomass with sodium chloride solution to release the adsorbed enzyme (Yang and Wyman 2006), and application of surfactants, lipids, or metal ions prior to application of enzymes to prevent from their irreversible adsorption (Yang and Wyman 2006; Zhang et al. 2008; Borjesson et al. 2007).

4.10 Enzyme Recycling, an Approach for Reduction of Process Cost

After an effective pretreatment, lignocellulosic biomass can be fairly digestible to fermentable sugars; however, the enzymatic hydrolysis process is still slow and the enzyme requirement remains high (up to 15–30 FPU/g glucan) from a commercially desirable standpoint (Ferreira et al. 2000; Hambrid et al. 2011; Eckard et al. 2013b).

During hydrolysis, enzymes are partitioned between solid and liquid phase. Several studies in the past evaluated the enzyme recycling with recycles of solid residues as well as the liquor from hydrolysis (Lee et al. 1995; Tu et al. 2009, 2007; Eckard et al. 2013a). Most of these studies were conducted using low total solid of 2–5 % and supplementation of beta-glucosidase in new hydrolysis cycle (Lee et al. 1995; Tu et al. 2007, 2009). The results specifically shown that recycling is much less effective with pretreatment methods that do not remove lignin and is more efficient when pretreated materials contained less lignin (Tu et al. 2007, 2009). This is because lignin unproductively adsorbs a large fraction of the cellulase, making it unavailable for enzymatic hydrolysis of cellulose (Uribe and Sampedro 2003; Taherzadeh and Karimi 2009).

For recycling of free enzyme from liquid phase, a re-adsorption technique was first proposed by Sinistyn et al. (1986) as an alternative to costly ultrafiltration techniques. In this method, the slurry of hydrolysate (liquid after enzymatic hydrolysis of biomass) or fermentation broth is centrifuged or filtered, and the supernatant containing soluble enzymes is incubated with fresh biomass for 2 h to allow the adsorption of free enzymes from cycle 1 onto fresh biomass. After a second separation step, the insoluble solids of biomass are then re-solubilized in fresh buffer and additional β-glucosidase is added for another cycle (2nd) of hydrolysis or fermentation (Tu et al. 2007, 2009). It has been suggested that the re-adsorption technique with two separation steps prevents the accumulation of lignin degradation by-products and sugars to toxic levels (Tu et al. 2007; Palmqvist et al. 1996; Xue et al. 2012). However, it should be noted that an additional separation is costly and due to the advantage of the SSF process that assimilates the inhibitors and sugars generated, the additional re-adsorption step might not be necessary (Eckard et al. 2013a).

As it was mentioned earlier, non-specific adsorption of enzymes to lignin and maybe crystalline cellulose limits the efficacy of enzyme recycling greatly. One of the strategies for improvement of this problem is the use of amphiphiles prior or during the enzymatic hydrolysis that prevent irreversible adsorption of enzyme on substrate and free enzymes can be then recycled for hydrolysis of fresh substrate (Tu et al. 2007; Eckard et al. 2013a). The ability of amphiphiles to adhere to nonproductive sites of lignocelluloses such as lignin and prevent irreversible enzyme adsorption has been clearly demonstrated before. According to Errikson et al. (2002), the adsorption of cellobiohydrolase decreased by 60–70 % onto steam-exploded lodgepole pine (SELP) using Tween 20. Likewise, desorption of cellulases (cellobiohydrolase and endo-glucanase) into the liquid phase was improved from 46 to 73 % during the hydrolysis of SELP when Tween 80 was used as an enzyme stabilizer (Tu et al. 2007). This property of amphiphiles can be exploited for recycling of cellulase if the revenue made from the additional amount of sugar and ethanol is higher than the cost paid for the amphiphiles. Moreover, when surfactant was applied to ethanol pretreated-lodgepole pine (EPLP), it increased the free enzyme levels of cellulase from 71 % of the original amount to 96 %. Similarly, in another study, it was found that the efficiency of enzyme recycling (using re-adsorption technique) was significantly higher in lower lignin content substrates, compared to feedstock's such as SELP that contained higher lignin levels (Tu et al. 2007). Differences in enzyme recycling also may be related to the source of enzymes. For instance, under similar experimental conditions (similar substrate and surfactant), enzymes from *T. reesei* were successfully recycled for 4 cycles, while penicillium-derived enzyme was only recycled once successfully (Tu et al. 2007).

According to Eckard et al. (2013a), after two recycling of fermentation liquor containing enzymes, the ethanol yield was improved by 80 and 130 % with the aid of Tween 20 and liquid casein micelles, respectively. polymeric micelles (PMs) of PEG–Tween and PEG–casein improved enzyme recycling further, such that the ethanol yield was improved by 50 and 108 % beyond that obtained with only

Tween and casein, respectively. Amphiphiles of acid casein were also found to improve the sugar recovery and fermentability of dilute acid, lime, alkali, and extrusion pretreated corn stover by up to 31 and 33 %, respectively. Neither of Tween 20, nor the accumulated sugars showed toxicity to microbial or enzyme activity (Eckard et al. 2012).

Several mechanism of action have been suggested to describe how surfactants enhance the cellulose activity and enzymatic hydrolysis of pure cellulose or lignocellulosic biomass: (1) Surfactants can extract hydrophobic degradation products from lignin and hemicellulose by forming emulsions, thereby enhancing the removal of lignin and increasing the access of feedstock's reaction sites to the cellulolytic enzyme (Kaar et al. 1998; Tu et al. 2007; Seo et al. 2011); (2) Surfactants lessen irreversible, nonproductive adsorption of cellulase to nonproductive sites of biomass (e.g., crystalline cellulose and lignin), which allows the enzyme to be available in solution and have higher activity (Castanon et al. 1981; Errikson et al. 2002; Parke et al. 1992); (3) Improved electrostatic interaction between surfactant monomer or micelles and enzyme causes an enhanced enzyme activity by activating a certain amino acid in the enzyme or reforming enzyme secondary structure, specially the α-helixes (Eckard et al. 2013a, 2014); (4) Surfactants protect enzyme from thermal deactivation after extended incubation period (Kim et al. 1982) and denaturation by reducing the surface tension and viscosity of liquid that in turn diminishes the contact of enzyme with air–liquid interface (Kim et al. 1982). Overall, in a solution of surfactants, enzymatic reactions occur either inside the surfactant micelle core or at the interface of the micelles or monomers and the pseudo-phase of the liquid, depending on the enzyme hydrophobicity (Biasutti et al. 2008). In spite of the above hypotheses, a mechanism that can consistently explain how surfactants improve enzymatic hydrolysis has yet to be developed.

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Chapter 5
Bioethanol from Lignocellulosic Wastes: Current Status and Future Prospects

Farid Talebnia

Abstract Ethanol is the most dominant type of biofuel that can be used either as a neat fuel or in the blended form with gasoline. Sugars, starches, lignocellulosic materials, and more recently algal biomass are the main raw materials used for ethanol production. Among these raw materials, lignocellulosic feedstocks are abundant and available at low price that make them suitable and potentially inexpensive feedstocks for the sustainable production of fuel ethanol. However, unlike sugar- and grain-based ethanol (first generation), technology for cellulosic ethanol production (second generation) is more sophisticated and needs further development. Biochemical processing and thermochemical processing are two main conversion pathways of bioethanol from lignocellulosic biomass. This chapter covers different aspects of ethanol production including global market situation, various raw materials, status, and recent advances of the applied technologies, future perspective, and environmental merits of fuel ethanol. In addition, some of the publicly announced pilot and demonstration cellulosic-based ethanol plants constructed worldwide, along with information on type of raw material, applied technology, capacity, products, and future plan, are presented. Finally, technical challenges encountered in development of the biomass-based ethanol are presented and potential approaches to overcome these technical barriers and to improve the economy of the whole process such as process integration and multiple product lines using biorefinery concept are presented and discussed in detail.

Nomenclature

MSW	Municipal Solid Waste
ML/d	Million Liters per Day
t/yr	Ton per Year
L/ha	Liters per Hectare
MG/yr	Million Gallon per Year

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5.1 Introduction

Energy security plays a critical role in the economy of both developed and developing countries. Today, the major energy demand is supplied from conventional fossil fuels such as oil, coal, and natural gas. As global demand for energy continues to grow, the current price of fossil fuels is not likely to decrease. In addition, utilization of fossil fuels over the last few decades has drastically increased the level of greenhouse gasses (GHGs) in the atmosphere of our planet. The negative impacts of fossil fuel on the environment and consequent global warming, rising demand for energy, inevitable depletion of the world's energy supply, and the unstable oil market have renewed the interest of society in searching for alternative fuels (De Fraiture et al. 2008; Himmel et al. 2007). The alternative fuels are expected to be cost-effective, produced from renewable feedstocks which are available worldwide, and must contribute to GHG reduction targets (Hahn-Hägerdal et al. 2006; Wei et al. 2013).

Production of fuel ethanol from biomass seems to be an interesting alternative to traditional fossil fuel. Bioethanol can be utilized as a sole fuel in cars with dedicated engines or in fuel blends as an octane booster. Ethanol is currently produced from sugars, starches, and cellulosic materials. The first two groups of raw materials are presently the main resources for ethanol production. However, concomitant growth in demand for food and feed, similar to energy, can make them potentially less competitive and perhaps expensive feedstocks in the near future, leaving the cellulosic materials as a sole potential feedstock for ethanol production (Talebnia et al. 2010; Taherzadeh and Karimi 2007b). Cellulosic materials obtained from wood and agricultural residuals, municipal solid wastes, and energy crops represent the most abundant global source of biomass (Lin and Tanaka 2006). These facts have motivated extensive research toward developing efficient technologies for conversion of lignocelluloses into sugar monomers to be used as feedstocks for fermentation to ethanol.

5.2 An Overview on Bioethanol Production

5.2.1 Global Market

Ethanol production worldwide has drastically increased since the oil crises in 1970, and today, it is the most dominant biofuel (Nguyen 2012). In 2012, three quarters of global biofuels market was for bioethanol, with the remaining quarter consisting of biodiesel sales. Global bioethanol production showed an upward trend over the last 25 years with a sharp increase from 2000 to 2010. This increase is mainly correlated with fuel ethanol since development in two other major sectors of the ethanol market, i.e., industry and beverage, is less dynamic. The industrial alcohol market showed a rather modest rate of growth similar to the increase in gross domestic

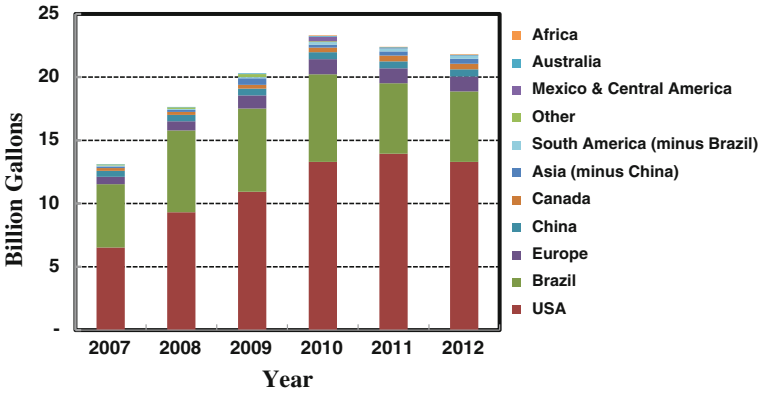

Fig. 5.1 Global ethanol production by the major producers (Alternative Fuels Data Centre 2014)

product (GDP) in many countries. The market for beverage alcohol in most developed countries is stagnating or even declining, due to increased health awareness (Talebnia 2008).

Global ethanol production by country or region, from 2007 to 2012, is represented in Fig. 5.1. Together, the USA and Brazil dominate the bioethanol market, accounting for 85 % of the world's ethanol production and 82 % of global consumption. This makes the global fuel ethanol market very reliant on the markets of these two countries (Alternative Fuels Data Centre 2014). Brazil, as one of the main producers in the world, produces ethanol mainly from sugarcane. Because of Brazil's optimal climate, two seasons of sugarcane growth can be achieved, providing a great potential of producing both sugar and bioethanol at lower prices. Originally, the Brazilian alcohol industry was an offshoot of the sugar industry, but after world oil prices soared in 1974, the country started the world's first major program (so-called Proalcool) for the production of renewable fuels in 1975. Brazilian ethanol is most likely the cheapest in the world, with an estimated production cost in the range of $0.19–0.21 per liter in 2005 (Otero et al. 2007). The huge availability of raw material, i.e., sugarcane, has made Brazil the most cost-effective ethanol producer in the world. Brazilian gasoline has a legal alcohol content requirement ranging from 20 to 25 % according to renewable fuel standards. Most vehicles are being run on E20 or E22, but sales of flex-fuel vehicles capable of operating on E85 blends are strong (Licht 2006; Mabee 2007).

Bioethanol manufacturing in the USA was initiated in the 1980s, and its fuel ethanol market is the fastest growing market in the world. Presently, the United States is the world's largest producer of ethanol, having produced over 13 billion gallons in 2012 alone. The main raw material for the US bioethanol industry is corn and to a lesser extent wheat. Most bioethanol production capacity is located in the Midwest, where corn is found in abundance. The recent policy developments in the USA stem from the Energy Policy Act of 2005, H.R. 6, which created a nationwide

renewable fuel standard (RFS) that increased the use of biofuels to 28.4 billion L/yr by 2012 (Mabee 2007; Wang et al. 2011).

Production of ethanol from sugarcane is in close competition with the sugar and grain markets. As shown in Fig. 5.1, global ethanol production peaked in 2010 (23.3 billion gallons) and then slightly decreased. The reason is that Brazil's bioethanol market has been in decline since 2009 and the US market has stagnated since 2010. In the USA, the biggest issues are the "blend wall" and the poor corn harvest of 2012. In Brazil, a combination of successive poor sugarcane harvests, strong international sugar prices, and a smaller domestic price spread between ethanol and gasoline caused a growing number of motorists choosing to use gas rather than ethanol for their flex-fuel vehicles in the last four years (Alternative Fuels Data Centre 2014).

Outside the Americas, in Europe, with the policy of increasing the share of biofuels in the transportation sector, the production will rise strongly. Total bioethanol production in European Union (EU) showed an increase by more than twofold from 2007 to 2012 (Fig. 5.1). Major fuel ethanol producers in the EU are Germany, France, Spain, and Sweden. While France was a dominant producer for a long time, ethanol production has been increased in several EU countries after 2000. Since 2009, the EU policy has moved away from supporting biofuels produced from food feedstocks such as rapeseed oil and grains. This may slow market growth for biofuels, but at the same time, it can motivate industries for producing bioethanol from non-food feedstocks such as cellulosic-based materials and wastes (EurObserv'ER 2014).

The largest producer of ethanol in Asia is China with 555 MG (2.1 billion liters) fuel ethanol production in 2012. Ethanol has been under development in China for some time to be used as a vehicle fuel. China is planning to promote ethanol-based fuel and to create a new market for its surplus grain with the aim of reduction of oil consumption. About 80 % of bioethanol production in China is grain based, mainly derived from corn, cassava, and rice (Dufey 2006). Compared to 1.30 billion liters (Licht 2006) fuel ethanol production in 2005, China increased its production by 62 % in this time period. The world's largest ethanol plant, "Jilin Tianhe," with an initial capacity of 600,000 t/yr (2.5 ML/d) is located in China. However, since 2008, China's food/feed price inflation has forced the government to tighten its control on the grain processing sector (including ethanol) with the result of lower financial support for grain-based ethanol production. Since then, government subsidies were cut for fuel ethanol production to all designated plants and 10 mandated provinces (Junyang 2012). These facts can probably explain the lower China's fuel ethanol capacity that was projected to be 2.43 billion liters in 2012.

Overall, the forecast for the global biofuels market over the next decade is of a single-digit growth rate, as consumption in the USA and Brazil for bioethanol and consumption in the EU for biodiesel experience only modest gains. Less political support for biofuels in most of the key regional markets for both bioethanol and biodiesel is going to slow growth of the global biofuels market through the next decade and result in a total of 41.7 billion gallons sold in 2022, a compound annual growth rate (CAGR) of just 4.0 % between 2013 and 2022 (World Biofuels Market 2014).

5.2.2 Bioethanol as a Fuel

Sustainable alternative fuels are nowadays in focus due to global warming caused by excessive emissions of CO_2, rising prices of fossil fuels and unstable world oil market. Ethanol, also known as ethyl alcohol, can be used as a substitute for, as well as additive to, the traditional fossil fuels (Talebnia et al. 2005). Ethanol with the chemical formula C_2H_5OH is a flammable, clear, colorless, biodegradable, and slightly toxic chemical compound with acceptable odor. It can be produced either from petrochemical feedstocks by the acid-catalyzed hydration of ethene, or from biomass feedstocks through fermentation. On a global scale, synthetic ethanol accounts for about 5 % of total production, while the rest is produced from fermentation of biomass—mainly sugar crops, i.e., cane and beet, and of grains (mainly corn) (Talebnia 2008; Licht 2006).

Ethanol as a neat fuel or even in the blended form with gasoline has a long history as automotive fuel. In 1860, German inventor Nicholas Otto used ethanol as a fuel in an early prototype of an internal combustion engine (ICE) because it was widely available throughout Europe for use in spirit lamps. A few years later, Henry Ford built his first automobile with an engine that could run on ethanol. In 1908, Ford unveiled his Model T engine equipped with carburetors that could be adjusted to use alcohol, gasoline, or a mixture of both fuels (Solomon et al. 2007). Ethyl alcohol as "the fuel of the future" was presented by him for the first time. In 1925, he told the New York Times: "The fuel of the future is going to come from fruit like that sumac out by the road, or from apples, weeds, sawdust—almost anything." However, fossil fuels were predominantly used for automobile transportation throughout the last century, obviously due to their lower production cost. As an automotive fuel, hydrous ethanol can be used as a substitute for gasoline in dedicated engines. Anhydrous ethanol, on the other hand, is an effective octane booster when mixed in blends of 5–30 % (by volume) with no engine modification requirement (Seshaiah 2010; Licht 2006).

Using bioethanol as a fuel offers several advantages when compared with fossil fuels such as gasoline. Bioethanol has a higher octane number and anti-knock index (AKI) (109 and 99.5, for ethanol and gasoline, respectively), broader flammability limits, higher flame speeds, and higher heats of vaporization than gasoline. These properties allow for a higher compression ratio, shorter burn time, and leaner burn engine, leading to theoretical efficiency advantages over gasoline in an ICE. Disadvantages of bioethanol include its corrosiveness, lower vapor pressure (making cold starts difficult), miscibility with water, and its lower energy density than gasoline (Balat 2009). Energy contents of bioethanol and gasoline are 21.1 and 32.6 MJ/L, respectively. This means that for the same energy content as one liter of gasoline, 1.6 liters of ethanol is needed. This is not necessarily a disadvantage of bioethanol because the lower energy content, to a great extent, is compensated by higher yield of fuel conversion to useful work because alcohol-fueled engines can be made substantially more energy efficient. This difference in efficiency can partially or totally balance out the energy density difference, depending on the

particular engines being compared. For instance, it has been reported that addition of 10 % ethanol to gasoline (E10) will result in a blend with an energy content equivalent to 97 % of the energy content of the base gasoline (Rehnlund and AE 2005).

5.2.3 Raw Materials for Ethanol Production

Ethanol can be theoretically produced from any material containing carbohydrates. This will lead us to three main groups of raw materials in nature, namely sugary, starchy, and non-food cellulosic materials. Nowadays, vast majority of ethanol is produced from sugar and starch; the former is mainly produced from sugarcane, beet, sweet sorghum, etc., and the latter comes from grains and root crops such as corn, wheat, and cassava. Technology for ethanol production from these materials is mature, and ethanol produced is referred to as first-generation ethanol (Fig. 5.2). Taking into account the rapid expansion of human populations and the fact that the first-generation bioethanol utilizes food crops, raise a question that for how long and to what extent they can be used as raw materials for biofuels production. Additionally, this issue can have strong impact on the price of current ethanol feedstocks. Therefore, non-food materials will gain priority and remain the only viable candidate to serve as renewable feedstock for ethanol production.

Lignocellulosic materials such as agricultural and forest residues, crops, and herbaceous materials in large quantities are available in many countries with

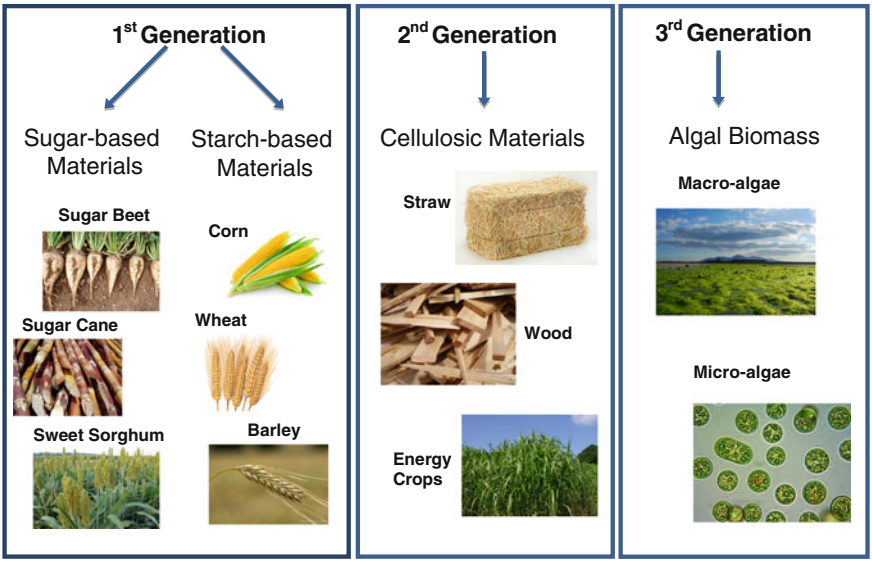

Fig. 5.2 Classification of raw materials used for bioethanol production

various climatic conditions, making them suitable and potentially cheap feedstocks for sustainable production of fuel ethanol. The global production of plant biomass, with over 90 % lignocellulose content, is estimated to be about 200 × 10^9 t/yr, where about 8–20 × 10^9 tons of primary biomass remains potentially accessible annually (Lin and Tanaka 2006). Over the last few decades, extensive attention has been devoted to research on the conversion of lignocellulosic materials to ethanol (Prasad et al. 2007). Lignocelluloses are complex mixtures of carbohydrate polymers, namely cellulose and hemicellulose, lignin, and a small amount of compounds known as extractives. Production of ethanol from lignocellulosic materials, referred to as the second-generation ethanol (Fig. 5.2), has the advantage of abundant and diverse raw material availability compared to the current utilized resources. Besides, cellulosic ethanol can contribute to the reduced GHG emissions. However, due to structural complexity of lignocellulosic materials, technology for the second-generation ethanol production requires more processing steps to make sugar monomers available to the microorganisms for fermentation.

Another class of non-food raw materials for biofuel production including bioethanol is algal biomass which has a very distinctive growth yield as compared with classical lignocellulosic biomass (Brennan and Owende 2010). Algae include a wide variety of photosynthetic organisms living in many diverse environments and present in all existing ecosystems on Earth (Wei et al. 2013). Cultivation of algae at seawater or industrial wastewater has been suggested as an interesting alternative solution for biofuels production. The biofuels produced from algal biomass are considered as the third-generation biofuels (Fig. 5.2). There are two different types of algae: microalgae and macroalgae.

Microalgae or phytoplankton are being widely investigated for producing food supplements and biofuels due to their high photosynthetic efficiency (up to 5 % compared with below 1 % for terrestrial plants) and their ability to produce lipids, polysaccharides, proteins, and carotenoids. Therefore, microalgae can be converted to biodiesel, bioethanol, biooil, biohydrogen, and biomethane via thermochemical and/or biochemical methods (Demirbas 2011; Rösch et al. 2012; Dragone et al. 2010). Although the production of biofuels from microalgae usually relies on their lipid content, but certain species of microalgae are capable of producing high levels of carbohydrates instead of lipids as reserve polymers. These species are suitable candidates for bioethanol production since the carbohydrates content can be extracted to produce fermentable sugars. It has been estimated that approximately 5000–15,000 gallon/acre/yr (46,760–140,290 L/ha) ethanol can be produced from microalgae. This yield is several orders of magnitude larger than yields obtained for other feedstocks (Nguyen 2012).

Macroalgae, also known as seaweeds, are classified into three groups: green, brown, and red. They do not generally contain lipids and are being considered for the natural sugars and other carbohydrates, which can be fermented to produce either biogas or alcohol-based fuels (Burton et al. 2009). Macroalgae could be a promising feedstock for ethanol production because it contains little to no lignin to interfere with the hydrolysis of polysaccharides. Depending on the various types of

Table 5.1 Chemical composition of potential raw materials for ethanol production

Raw material	Cellulose	Hemicellulose	Lignin
Hardwood stem	40–50	24–40	18–25
Softwood stem	45–50	25–35	25–35
Nut shells	25–30	25–30	30–40
Corn cobs	45	35	15
Grasses	25–40	35–50	10–30
Wheat straw	33–40	20–25	15–20
Rice straw	40	18	5.5
Leaves	15–20	80–85	0
Switch grass	30–50	10–40	5–20
Cotton, flax	80–95	5–20	0
Primary wastewater solids	8–15	NA	24–29
Paper	85–99	0	0–15
Newspaper	40–55	25–40	18–30
Algae (green)	20–40	20–50	0

Adapted from DEMİRBAŞ (2005), McKendry (2002), Prasad et al. (2007), Sun and Cheng (2002), Balat (2009)

seaweeds, carbohydrate composition such as cellulose, starch, agar, carrageenan, mannitol, laminarin, and alginate might be available in their structure (Wei et al. 2013).

Overall, there are several advantages in using algae biomass for bioethanol production compared with those utilized in the first- and even in the second-generation bioethanol. Unlike corn and sugarcane, algae biomass does not compete directly with foods and does not require agricultural land or use of freshwater to be cultivated. Algae biomasses usually have faster growth rates than terrestrial crops and consume a high level of CO_2 during their growth, which makes them environmentally attractive as a CO_2 sink (Limayem and Ricke 2012). Furthermore, the ethanol yield of algae per growing area is much higher than those from biomasses used in the first- and second-generation ethanol (Rodolfi et al. 2009; Nguyen 2012). The compositional structure of some raw materials used in the second- and third-generation bioethanol is shown in Table 5.1.

5.2.4 Environmental Impacts

Over the last 150 years, human activities have caused a dramatic increase in the emission of a number of GHGs such as CO_2, which has led to changes in the equilibrium of the earth's atmosphere (Galbe and Zacchi 2002). As a result, desire for utilization of renewable and environmentally benign fuels is increasing. Fuel ethanol is suggested as a sustainable fuel which can be produced from renewable

resources and led to maintain or even reduce the level of GHGs. The net emissions of CO_2 are reported to be close to zero, since the CO_2 released from the ethanol-producing plant and vehicle's fuel combustion is recaptured as a nutrient during the growth of ethanol feedstocks (i.e., crops and plants). Ethanol in blend with gasoline increases octane and provides oxygen to promote more complete combustion. Addition of ethanol or derivative such as methyl tertiary butyl ether (MTBE) to gasoline as an oxygenate reduces tailpipe emissions of CO and unburned hydrocarbons, which can contribute to improving the urban air quality. Unlike MTBE, which is not readily biodegradable and is known as a groundwater pollutant, ethanol is a water-soluble and biodegradable compound and therefore less pernicious to the environment, groundwater, and soil (Wyman 1999). However, because in addition to solar energy, other energy inputs, often in the form of fossil fuel, are required in the manufacturing and marketing of biofuel such as ethanol, the entire process is not likely to be completely carbon neutral (Granda et al. 2007). Thus, the "greenness" of a biofuel like ethanol is highly dependent upon the efficiency of all stages in the process from raw material to the end use of product and its avoided use of fossil fuels.

A large number of life cycle assessments (LCA) performed to estimate the environmental merit of bioethanol show contradictory results (von Blottnitz and Curran 2007; Niven 2005; van der Voet et al. 2010; González-García et al. 2012). This could be related to variation in several important aspects of the LCA methodology used for a biofuel system assessment including the definition of the system boundary, the choice of functional unit, the choice of allocation methods, the treatment of biogenic carbon, the selection of impact categories, the choice of reference system, and the effect of biomass removal from soils (Cherubini and Strømman 2011; Wiloso et al. 2012). These factors can strongly affect the outcome of a LCA assessment, and hence, any comparison among bioethanol systems must be based on the defined LCA framework and identified system components. For instance, the system boundary can be defined so that the agricultural chain and bioethanol production chain are included, but different and more realistic results can be expected by inclusion of bioethanol use chain into an LCA assessment. Bioethanol use chain, in turn, refers to the combustion of the fuel blend (ethanol/gasoline) with different proportion in vehicle internal engine. Therefore, tailpipe emissions are the most important factor to be considered here. Besides, in order to get an overview of the environmental impact of bioethanol, emissions other than CO_2 including nitrogen oxides (NO_x), carbon monoxide (CO), sulfur oxides (SO_x), total particulate matter (TPM), and volatile organic compounds (VOCs) such as aldehydes, alcohols, ethers, esters, and other organics need to be considered. It has been reported that although ethanol (used as E85) generally generates less emissions in tailpipe (Wu et al. 2004), life-cycle analysis covering entire routes from crop to wheel may give opposite results. Even though the GHG emissions are lowered when ethanol from different routes versus gasoline is used, it may result in high increases of TPM, NO_x, and SO_x emissions.

In addition, LCA assessment of the first- and second-generation ethanol may show different results as well. While corn ethanol is claimed to have either negative

energy content or slightly positive value with little GHG saving, environmental benefits of cellulosic ethanol cannot be refuted (Farrell et al. 2006; Granda et al. 2007). Second-generation ethanol using various lignocellulosic feedstocks can achieve more than 50 % GHG emission reduction compared with gasoline which is much higher than that from corn-based ethanol. This value, however, can vary depending on the type of raw materials, ethanol production route, and assessment method (Mu et al. 2010). Overall, although diverse approaches in terms of LCA methodology, system definition, and level of technology have been applied to study the bioethanol systems, the conclusion to favor the second-generation bioethanol is quite robust for the two most studied impact categories, i.e., net energy output and global warming (Wiloso et al. 2012).

5.3 Conversion Pathways of Bioethanol from Lignocellulosic Feedstocks

Biofuels production form lignocellulosic materials might be accomplished through two major pathways which are biochemical and thermochemical pathways. Through the first route, the biomass is fractionated to its main structural components, i.e., cellulose, hemicellulose, and lignin, and the carbohydrate polymers are then broken down into monomeric sugars. Therefore, this route mainly includes pretreatment and enzymatic hydrolysis of cellulose followed by fermentation of liberated sugars from both cellulose and hemicellulose to ethanol. The latter which is an emerging technology includes heat treatment of biomass at different temperatures in presence or absence of an oxidizing agent. In this process, the biomass is converted to three main fractions: biochar (solid), biooil (liquid), and a gas mixture known as syngas. The produced gas mixture can be then converted to biofuels through either raw syngas fermentation or Fisher–Tropsch catalysis (Xu et al. 2011; Lee and Lavoie 2013) (Fig. 5.3).

5.3.1 Biological Route

The technology of ethanol production through biological route consists of several steps and varies depending on the type of raw materials used. It becomes more sophisticated as the raw materials turn from sugars to starches and cellulosic materials. While sugars can be directly fermented to ethanol, starchy materials require a preliminary hydrolysis step to liberate the sugars that can then be fermented to ethanol. Unlike starch which is readily hydrolyzed enzymatically, the specific structure of cellulose favors the ordering of the polymer chains into tightly packed and highly crystalline structure which is water insoluble and resistant to depolymerization. Thus, an additional pretreatment step is necessary to disrupt this

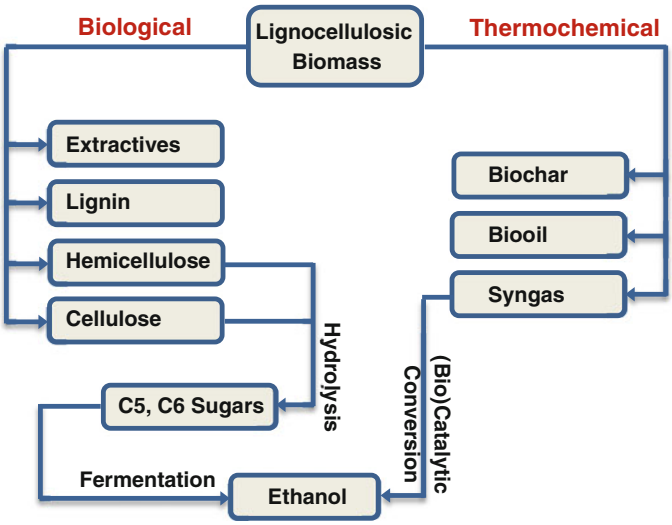

Fig. 5.3 Block diagram of lignocellulosic biomass conversion routes to ethanol

structure. Overall, for ethanol production from cellulosic feedstocks, four major unit operations are required: pretreatment, hydrolysis, fermentation, and separation/purification, although some of them can be combined in more advanced setup as described later (Talebnia et al. 2010).

5.3.1.1 Pretreatment

One of the main problems in application of cellulosic feedstocks as raw materials for ethanol production is resistance against enzymatic depolymerization. The carbohydrate polymers in lignocellulose are tightly bound to lignin mainly by hydrogen bonds as well as by some covalent bonds which make it a recalcitrant and inaccessible substrate for hydrolysis agents. The objective of pretreatment is therefore to increase the surface area and porosity of the substrate, reduce the crystallinity of cellulose, and disrupt the heterogeneous structure of cellulosic materials. This process makes the carbohydrate polymers accessible for depolymerization. The pretreatment results in an improved rate and yield of liberated sugars in hydrolysis step (Hendriks and Zeeman 2009). A number of pretreatment methods have been developed and applied for cellulosic biomasses (Jeihanipour et al. 2010; Talebnia et al. 2007; Talebnia and Taherzadeh 2012; Geddes et al. 2011). The overall efficiency of the pretreatment process is correlated with a good balance between low inhibitors formation and high substrate digestibility. The pretreatments are roughly classified into physical, physicochemical, chemical, and biological processes. The applied methods usually use combination of different

principles, such as mechanical together with thermal and chemical effects, in order to achieve high sugar release efficiencies, low toxicants production, and low energy consumption (Talebnia et al. 2010).

5.3.1.2 Hydrolysis

Hydrolysis of cellulosic materials includes the processing steps that convert the carbohydrate polymers, e.g., cellulose and hemicellulose, into monomeric sugars. Cleavage of these polymers can be catalyzed enzymatically by cellulases or chemically by acids such as sulfuric acid (Mosier et al. 2005). The factors that have been identified to affect the hydrolysis of cellulosic biomass include porosity or accessible surface area, cellulose fiber crystallinity, and the content of lignin and hemicellulose (Prasad et al. 2007).

Enzymatic Hydrolysis

Hydrolysis of cellulosic materials can be catalyzed by a class of enzymes known as cellulases. These enzymes are mainly produced by fungi, bacteria, and protozoans that catalyze the cellulolysis or hydrolysis of cellulose. At least three major groups of enzymes including exoglucanase, endoglucanase, and β-glucosidase are involved in depolymerization of cellulose to glucose. β-glucosidase catalyzes cleavage of cellobiose, which plays a significant role in the hydrolysis process, since cellobiose is an end product inhibitor of many cellulases including both exo- and endoglucanases (Galbe and Zacchi 2002; Lee 1997; Rabinovich et al. 2002; Sun and Cheng 2002). β-Glucosidase, in turn, is inhibited by glucose and, therefore, enzymatic hydrolysis is sensitive to the substrate concentration (Nikzad et al. 2012). In addition to substrate concentration, pretreatment of cellulosic materials and hydrolyzing conditions such as temperature and pH are among factors influencing the efficiency of enzymatic hydrolysis. Most cellulase enzymes show an optimum activity at temperatures and pH in the range of 45–55 °C and 4–5, respectively (Galbe and Zacchi 2002; Duff and Murray 1996).

Acid Hydrolysis

Interest in wood hydrolysis dates back to 1819, when Braconnot discovered cellulose could be dissolved in concentrated acid solutions and converted to sugar. Acid hydrolysis can be performed with various types of acids including sulfuric, sulfurous, hydrochloric, phosphoric, nitric acid, etc. Acid hydrolysis is subdivided into concentrated and dilute acid hydrolysis. Through the concentrated acid hydrolysis, the biomass is treated with high concentration of acids at near-ambient temperatures, which results in high yield of sugars. However, this process has drawbacks including high acid and energy consumption, equipment corrosion, and

longer reaction time (Galbe and Zacchi 2002; Harris et al. 1945; Taherzadeh and Karimi 2007a). Dilute acid hydrolysis, on the other hand, uses low-concentration acids and high temperatures. High temperature is required to attain acceptable rates of cellulose conversion to glucose. Despite low acid consumption and short reaction time in dilute acid hydrolysis, application of high temperatures in this method accelerates the rate of hemicellulose sugar decomposition and increases equipment corrosion (Galbe and Zacchi 2002; Taherzadeh and Karimi 2007a). Decomposition of sugars not only lowers the ultimate yield of sugars in dilute acid process, but also produces a number of by-products that show severe inhibiting effects on the subsequent fermentation step (Klinke et al. 2004; Luo et al. 2002).

The main inhibiting compounds are classified in three groups: furans (furfural and HMF), phenolic compounds, and carboxylic acids (Clark and Mackie 1984). The simple pathway of side reactions from lignin and liberated sugars (both pentoses and hexoses) to their corresponding inhibitory chemical compounds is presented in Fig. 5.4. The furan derivatives can be further converted to formic acid and levulinic acid. Acetyl group in hemicellulose through the hydrolysis can result in acetic acid in the resultant hydrolyzate. These weak acids with relatively high pK_a at the higher concentrations have negative impact on the cell viability, even though their toxicity is pH dependent. Undissociated forms of these acids can penetrate through the plasma membrane and dissociate in the cytoplasm and disturb neutral intracellular pH (Pampulha and Loureirodias 1989; Taherzadeh et al. 1997; Verduyn et al. 1990).

Furfural and hydroxymethylfurfural (HMF) are known as the two strongest inhibitor compounds present in the hydrolyzate. Higher concentrations of furfural and HMF resulted in less fermentability of the hydrolyzates. Furfural is metabolized by *Saccharomyces cerevisiae* and reduced to corresponding alcohol (furfuryl

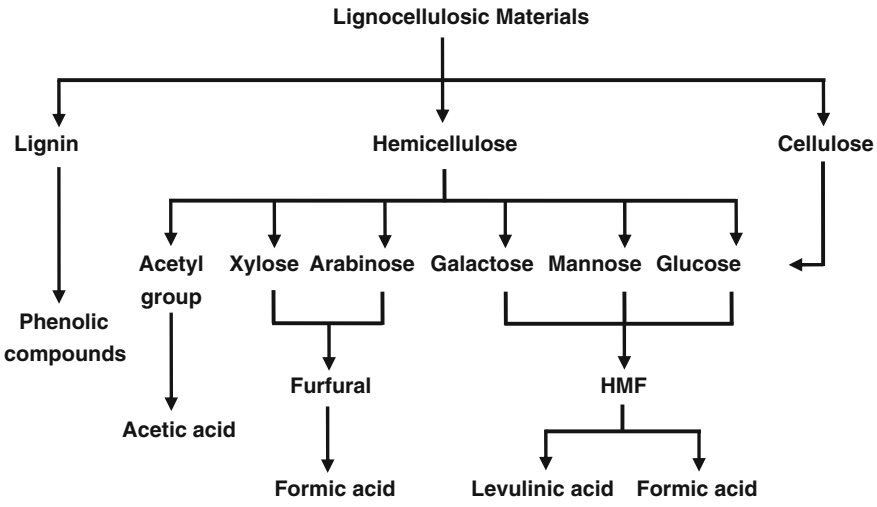

Fig. 5.4 Main inhibitory compounds formed during dilute acid hydrolysis (Talebnia 2008)

alcohol). This conversion is mediated by NADH-dependent alcohol dehydrogenase (ADH). Presence of furfural in the cultivation medium could result in reduced biomass yield, and decrease in specific growth rate and ethanol productivity. HMF is also metabolized by yeast but at a lower rate than furfural, imposing longer lag phase in growth (Talebnia 2008).

Phenolic compounds mostly originate from lignin decomposition and to a minor extent from the aromatic wood extractives (Taherzadeh and Karimi 2007a). Inhibition mechanisms of phenolic compounds on *S. cerevisiae* and other eukaryotic microorganisms have not yet been completely elucidated, largely due to the heterogeneity of the group and the lack of accurate qualitative and quantitative analyses. Phenolic compounds may partition into biological membranes, causing loss of integrity, thereby affecting their ability to serve as selective barriers and enzyme matrices (Almeida et al. 2007; Palmqvist and Hahn-Hägerdal 2000). Low molecular weight (MW) phenolic compounds are more inhibitory to *S. cerevisiae* than high MW compounds. Treatment of hydrolyzate with laccase, a lignin-oxidizing enzyme, resulted in less inhibition of fermentation (Jonsson et al. 1998).

5.3.1.3 Fermentation

Microorganisms

Microorganisms play a significant role in production of ethanol from renewable resources, and thus, selection of suitable strain is essential for the individual process. Ethanol production is much more challenging and difficult when cellulose-based materials are to be used as raw materials. Unlike the starch-based materials, pretreatment and hydrolysis of lignocellulosic materials produce a mixture of pentose and hexose sugars along with other inhibiting compounds, causing many problems in the fermentation step. Therefore, capability of consuming both pentose and hexose sugars, high tolerance against substrate, ethanol as well as inhibiting compounds, high ethanol yield, and minimum nutrient requirements are the essential features of an ideal microorganism (van Zyl et al. 2007). Although no microorganism has been yet found to meet all these requirements, development of a desirable strain is the focus of many studies. Thus far, wide varieties of microorganisms including yeasts, bacteria, and fungi have been exploited offering different advantages and disadvantages. The most frequently used microbe has been yeast, and among the yeasts, *S. cerevisiae* which can tolerate ethanol concentration as high as ca. 20 % of fermentation medium is the preferred strain (Lin and Tanaka 2006). Some species of bacteria such as *Zymomonas mobilis* and the genetically engineered *Escherichia coli* can produce ethanol at higher yields, but they are less resistant to the end product (ethanol) and other compounds present in the hydrolyzates when compared to the yeast (Huffer et al. 2012; Talebnia 2008). In contrast to many advantages offered by using yeast in ethanol production, it lacks the mechanism to take up pentose sugars as substrate. Attempts to add this ability by genetic manipulation are still at the laboratory stage (Jeffries 2006).

Fermentation Mode of Operation

A sugar such as glucose is directly metabolized by the yeast cells through the glycolysis pathway to gain energy for biosynthesis. Under anaerobic conditions, the overall reactions produce two moles of ethanol and CO_2 per mole of glucose consumed. Fermentation can be carried out with different types of industrial operation as batch, fed-batch, or continuous process. The most suitable choice depends on the kinetic properties of the microorganism as well as process economics. Batch cultivations need low investment cost and lower requirements for process control. The fed-batch operation, sometimes regarded as a combination of the batch and continuous operations, involves addition of feed at constant intervals, while effluent is removed discontinuously. When the substrate has inhibitory effects, this method is advantageous because the microorganism is exposed to low concentration of substrate (Karimi et al. 2005). Continuous operation offers ease of control and high ethanol productivity, but contamination is a serious issue to be considered (Prasad et al. 2007).

Ethanol production from biomass can be carried out by using free or immobilized cells. The natural ability of many microorganisms to adhere to different kinds of surfaces is well known. Many microbial cells grow and aggregate within or on the surfaces of natural structures. Whole-cell immobilization is defined as "the physical confinement or localization of intact cells to a certain region of space with preservation of some desired activity" and shares the same feature (Karel et al. 1985). Immobilization puts the cells in close contact together and forces them to aggregate. The resultant cell community can obtain much better protection from harsh environmental conditions. Immobilization is an efficient method for cell retention, and the cells show better tolerance in severely inhibiting medium than those from other methods such as cell filtration and recirculation (Bai et al. 2008; Brandberg et al. 2007; Westman et al. 2012). Immobilized cell system is also attractive due to other advantages including a better protection against shear force, improved cell stability, and decreased cost of recovery, recycling, and downstream processing. Furthermore, continuous processes using immobilized cells can be operated at rather high dilution rates, which lead to higher volumetric productivity and shorter residence times (Park and Chang 2000; Verbelen et al. 2006; Purwadi and Taherzadeh 2008; Talebnia and Taherzadeh 2006).

Integrated Processes for the Second-Generation Ethanol

An important factor preventing industrial utilization of lignocelluloses for bioethanol production is the lack of microorganisms able to efficiently ferment (with high yield and high rate) all sugars (both pentoses and hexoses) released during pretreatment and hydrolysis. From commercial point of view, the ideal microorganism should have broad substrate utilization, high ethanol yield and productivity, tolerance to inhibitors and to high ethanol concentrations, cellulolytic activity, and ability for sugar fermentation at high temperatures (Hahn-Hägerdal et al. 2007). No single

microorganism has been yet found to satisfy all these requirements. Those with high ethanol yield and high ethanol tolerance are usually unable to utilize C-5 sugars, and the strains capable of using both C-6 and C-5 sugars are characterized by low ethanol yields and their tendency to reassimilate the produced ethanol (Karakashev et al. 2007). Process integration has been applied to make the second-generation ethanol economically feasible by improving productivity and yield of ethanol as well as the total energy gain. Taking into account the structural complexity of raw materials for the second-generation bioethanol, the conversion process can be accomplished using different strategies, of which the most important ones include separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), simultaneous saccharification and cofermentation (SSCF), and consolidated bio-processing (CBP). Figure 5.3 represents the block diagram of cellulosic ethanol production and schematic view of possible integrated processes.

In SHF, hydrolysis and fermentation are carried out in separate vessels under their own optimal conditions; however, end product inhibition on enzymes' activity and contamination problems are associated with this process. In order to eliminate drawbacks of the SHF process, SSF that combines hydrolysis and fermentation in one vessel has been developed (Fig. 5.5). Sugars produced during hydrolysis are

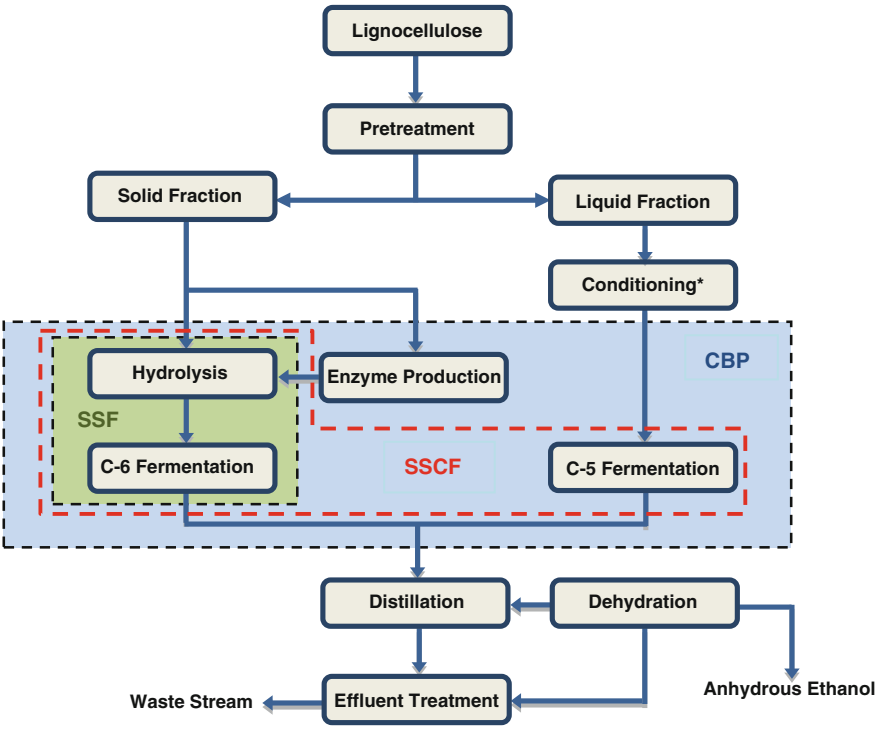

Fig. 5.5 Block diagram of the second-generation ethanol production and schematic view of suggested options for process integration, i.e., SSF, SSCF, CBP (shown in *boxes*). *Neutralization, vaporization, detoxification, etc.

immediately fermented into ethanol, and thus, problems associated with sugar accumulation and enzyme inhibition as well as contamination can be avoided (Lau et al. 2010; Galbe and Zacchi 2002). The main drawback of SSF is the different optimum temperatures of the hydrolysis and fermentation processes. Most fermenting yeasts have an optimal temperature around 30–35 °C, while hydrolyzing enzymes show optimal activities around 50 °C (Talebnia 2008). This causes the enzyme activity to be far below its potential, and as a result, more enzymes are required. To cope with this problem, a modified SSF process known as non-isothermal simultaneous saccharification and fermentation (NSSF) was suggested. In this process, saccharification and fermentation occur simultaneously but in two separate reactors, each kept under their own optimal temperature (e.g., 50 and 30 °C, respectively). The effluent from the hydrolysis reactor is recirculated through the fermentor. The cellulase activity is increased 2–3 times when the hydrolysis temperature is raised from 30 to 50 °C. The improved enzyme activity can decrease the overall enzyme requirement by 30–40 %. Higher ethanol yield and productivity of NSSF were also reported, when compared to SSF (Taherzadeh and Karimi 2007b; Wu and Lee 1998).

As mentioned above, efficient utilization of C-5 sugars is a critical issue in the second generation of ethanol production. Pentose sugars are not usually converted to ethanol in SSF process by the applied microorganisms. To address this problem, SSCF process has been developed. This process is an improvement of SSF in which both C-5 and C-6 sugars are fermented at the same time, thereby increasing the overall yield of ethanol production. Thus, SSCF is the inclusion of the pentose-fermenting microorganism in the SSF process (Fig. 5.5). This means that the hydrolysis and cofermentation (CF) of pentose and hexose sugars are integrated and carried out in one vessel. This technology is superior to SSF technology in terms of cost effectiveness, higher ethanol yield, and productivity (Lynd et al. 2005; Vohra et al. 2014; Chandel et al. 2007). The SSCF process can be carried out either by using genetically engineered microorganisms or cocultivation of two microorganisms. In the latter case, cofermenting microorganisms need to be compatible in terms of operating pH and temperature. A combination of *Candida shehatae* and *S. cerevisiae*, and *Pichia stipitis* and *Brettanomyces clausennii* was reported as suitable candidates for the SSCF process (Hickert et al. 2013; Olsson and Hahn-Hägerdal 1996). Utilization of recombinant bacterial and yeast strains including *Escherichia coli* KO11, *S. cerevisiae* 424A(LNH-ST), and *Zymomonas mobilis* AX101 in SSCF has also been reported where only the engineered yeast showed promising results (Lau et al. 2010).

The last process is CBP in which cellulase production, biomass hydrolysis, and ethanol fermentation are collectively carried out in a single vessel (Fig. 5.5) (Olson et al. 2012). This process is also known as direct microbial conversion (DMC). CBP is gaining increasing recognition as a potential breakthrough for low-cost biomass processing and seems to be the logical endpoint in the evolution of ethanol production from lignocellulosic materials. It is based on utilizing mono- or coculture of microorganisms to ferment cellulose directly to ethanol. Application of CBP requires no capital investment for purchasing or production of enzyme. The potential of this integrated process is limited by the fact that natural microorganisms exhibiting all the desired features for CBP are not readily available, although a

number of microorganisms, both bacteria and fungi, with some desirable properties for CBP have been identified (van Zyl et al. 2007; Lynd et al. 2005). At present, there is no ideal CBP microorganism or compatible combination of microorganisms able to degrade lignocellulosic biomass effectively while consuming all the sugars released from biomass to yield ethanol. Currently, CBP is not an efficient process because of poor ethanol yields and long fermentation periods (Sarkar et al. 2012). However, CBS is being investigated using two strategies: (i) engineering naturally occurring cellulolytic microorganisms to become efficient ethanol producers, and (ii) engineering non-cellulolytic organisms that exhibit high product yields and titers to express a heterologous cellulase system enabling cellulose utilization (Lynd et al. 2005). Bacteria such as *Clostridium thermocellum* and some fungi including *Neurospora crassa, Fusarium oxysporum,* and *Paecilomyces* sp. have been investigated for such activity, and intensive research is taking place to isolate new thermophilic strains with desirable properties.

5.3.2 Thermochemical Route

Three main processes in thermochemical conversion of biomass to energy are direct combustion, gasification, and pyrolysis, among which gasification is considered the most efficient process (Purohit 2009). Gasification is the conversion of biomass, or any solid fuel such as coal and oil, into an energetic gas through partial oxidation at elevated temperatures (e.g., 750–800 °C). Air, oxygen, and steam are common oxidants used in this process. The gas, called synthesis gas or syngas in short, predominantly contains H_2 and CO along with a mixture of other components including methane (CH_4), small quantities of light hydrocarbons (C_nH_m), carbon dioxide (CO_2), nitrogen (N_2), and steam (H_2O). The composition of syngas may vary depending on the feedstock inputs, and the gasification conditions, such as temperature, pressure, gasifying agent, catalyst addition, and equivalent ratio (El-Emam et al. 2012). Concentrations of these species depend on the feedstock constitution and gasification technique used. The gas mixture can be then converted into biofuels such as methanol, ethanol, and hydrogen via metal catalytic or biocatalytic methods (Pereira et al. 2012; Vohra et al. 2014). The following three reactions are the most relevant reactions for carbon conversion into gaseous components (Vessia 2005) (Table 5.2).

The biomass conversion efficiency in gasification process depends on the raw material, size and shape of the particles, gas flow, and types of reactors, among others. The classification of gasifiers is usually based on the flow direction of the gases. Common configurations include down draft (cocurrent), up draft (countercurrent), cross-draft, and fluidized bed reactors (Daniell et al. 2012). Gasification technology can be applied for energy generation in various forms from renewable resources. For instance, the process can be directed to generate heat and electricity or to produce ethanol and hydrogen. The current gasification process needs further development to address a few major challenges associated with this technology. The process is energy intensive since the biomass need to be dried before

Table 5.2 The most relevant gasification reactions and the corresponding reaction enthalpy

Reaction	ΔH_{298} (kJ mol^{-1})
$C + H_2O \rightarrow CO + H_2$	+131
$C + CO_2 \rightarrow 2CO$	+172
$C + 2H_2 \rightarrow CH_4$	−75

conversion and expensive equipment is required to free the syngas from contaminants. In addition, formation of tar during biomass gasification can adversely affect the process. Tar formation can interrupt the entire process and lead to catalyst deactivation and production of carcinogenic elements (Huang et al. 2012).

The production of higher alcohols from syngas has been an active area of research for the last few decades and has been thoroughly developed during 1930–1945 (He and Zhang 2008). A wide variety of biomass can be used for production of bioethanol via gasification technology, and this route has the advantage of utilizing the entire biomass including the lignin content, which is usually difficult to break down. However, some lignocellulosic feedstocks with high ash content, as seen in many straws, might not be preferred for gasification. The process of syngas conversion to ethanol can proceed via two different pathways which are catalytic and biocatalytic (fermentation) synthesis routes.

5.3.2.1 Catalytic Conversion Route

The catalyst with specific structure and properties is critical for ethanol production from syngas. The overall ethanol synthesis reaction is described by the following equation:

$$2CO + 4H_2 \xrightarrow{\text{catalyst}} CH_3CH_2OH + H_2O \tag{5.1}$$

Considering the several steps of this catalytic reaction, there are four specific functions that a catalyst should perform to be suitable for the process. These functions are the adsorption and dissociation properties of CO molecule and oxygen on the catalyst, hydrogenation of the adsorbed carbon to methyl species, insertion of non-dissociated CO into the methyl species to form an adsorbed acyl species, and finally hydrogenation of the acyl species to form ethanol. By far, various types of catalysts have been studied which can be roughly classified into three categories: modified Fischer–Tropsch synthesis (FTS) catalysts, groups VI–VIII metal-based catalysts, and modified methanol synthesis catalysts. In addition, catalysts with complex compositions might be used in severe conditions (He and Zhang 2008; Trippe et al. 2011). Among the studied catalysts, Rhodium (Rh) seems to be the most adaptable element in terms of its properties for catalysis, particularly for syngas conversion. Rh/SiO_2 is the baseline catalyst used for alcohol synthesis from syngas and shows high selectivity toward ethanol (He and Zhang 2008; Vohra et al. 2014).

5.3.2.2 Biological Conversion Route

The discovery of microorganisms capable of converting CO to useful products, such as butanol, ethanol, and acetic acid, provides another option for biological processing of synthesis gas. Biological conversion of synthesis gas offers several advantages over the chemical catalytic route. The process requires mild operating conditions with significantly lower temperatures and pressures compared with the chemical catalysis method which can lead to major reduction in both operating and capital costs. The presence of certain chemical species in the syngas such as sulfur may interfere with, or permanently deactivate the catalysts used. While high levels of syngas purity are required to prevent catalyst poisoning and thus, secondary equipment is necessary to remove interfering contaminants from syngas, gas fermentation has remarkable feedstock flexibility. Biological catalysts are typically more specific than their inorganic counterparts. Consequently, higher yield of desired products and lower yield of by-products including hazardous materials are expected. Therefore, process robustness, catalyst flexibility, and development potential are high in syngas fermentation. The major drawback of biological process is much lower reaction rate when compared with the catalytic process (Worden et al. 1991; Abubackar et al. 2011; Daniell et al. 2012).

A large number of bacterial strains which are able to utilize 1-carbon compounds such as CO, and CO_2 as the sole carbon and energy source, have been isolated. The anaerobic bacteria *Clostridium ljungdahlii* and *Clostridium autoethanogenum* have been widely studied and the production of ethanol and acetic acid from syngas through the acetogenic process is demonstrated. Both organisms have an optimum growth temperature of 37 °C. The strain *C. ljungdahlii* favors the production of acetate at a higher pH (5–7), but ethanol is the dominant product at pH between 4 and 4.5 (Daniell et al. 2012; Vohra et al. 2014). Other microorganisms investigated and characterized for ethanol production include *Clostridium ragsdalei, Butyribacterium methylotrophicum,* and recently isolated *Alkalibaculum bacchi.* Commercialization of syngas fermentations is currently hindered by low productivity in the bioreactor. In recent years, metabolic engineering and synthetic biology techniques are being applied to gas-fermenting organisms, in order to improve the rate and yield of process for the commercial production of increasingly energy-dense fuels and other valuable chemicals (Daniell et al. 2012).

5.4 Pilot, Demonstration, and Commercial Cellulosic Ethanol Plants

Several cellulose-based ethanol-producing plants were constructed during World War II, when wartime conditions changed the economic conditions and priorities. Many of these plants after the war could not stay viable in competition with the synthetically produced ethanol. In the following decades, interest in ethanol as a

fuel to some extent was dependent on the price of oil. However, shortage of oil supplies, the growing desire for cuts in the net emission of CO_2, and global warming together with rising demand for human food have maintained research and development on the ethanol production from celluloses as cheap and renewable raw materials. Many countries have initiated extensive programs in biofuel as a sustainable energy resource for displacement of transportation fuels (Himmel et al. 2007; Talebnia 2008). Table 5.3 summarizes some of the publicly announced pilot and demonstration plants constructed worldwide (Table 5.3). In the following section, a short description on the operational data, different technologies used, and future plans of commercialization for few of them is presented.

Iogen Corporation, based in Canada, uses an acid-catalyzed steam explosion pretreatment, followed by SHF without prior solid separation or washing. Enzymes are produced by Iogens's proprietary technology (Scott et al. 2013). Since 2004, Iogen has operated a demonstration-scale plant producing 2.5 ML/yr of ethanol from 30 t/d agricultural residues, including wheat, oat, and barley straw. The technology can also be used successfully with hardwoods. Both C-5 and C-6 sugars are fermented to ethanol with an overall yield of 340 L/t of fiber. Lignin is separated and used to generate process steam and electricity. Iogen has partnered with Shell since 2002. Iogen's first 90 ML/yr commercial plant is planned for Birch Hill, Saskatchewan, an area with sustainable supplies of straw and green residues (Johnson et al. 2009).

Bioengineering Resources Inc. (BRI) produces ethanol via fermentation of syngas. This technology is based on gasification of biomass to produce syngas followed by fermentation of produced syngas to ethanol. The company claims that this technology can handle any type of carbon-based feedstocks including MSW, biosolids, timber, forest/agricultural residues, plastics, used tires, and other waste materials. BRI uses a two-stage gasifier that raises the syngas temperature up to 1370 °C in the second stage to enable cracking of any heavy hydrocarbons to CO and H_2, thereby maximizing the ethanol yield. The hot producer gases are then cooled down to 37 °C and introduced into the bioreactor where ethanol is produced using a modified *C. ljungdahlii* strain (Vessia 2005).

DONG Energy built a bioethanol demonstration plant in 2009 at Kalundborg, Denmark, in order to prove that their second-generation technology can be profitably applied on a large scale. The plant was designed to produce 5.4 ML/yr of ethanol from 96 t/d of straw biomass. The conception principle of the plant is the Integrated Biomass Utilization System (IBUS), which is based on a symbiosis between a biorefinery and a power plant. The IBUS process is commercially exploited by Inbicon A/S, the biomass refinery division of DONG Energy. Inbicon developed a two-step pretreatment based on dilute acid which was then upgraded to a one-step autohydrolysis (AH) pretreatment, followed by two-stage SSF. The pretreatment step is based only on steam from the power plant and recycled water. The next step is the enzymatic liquefaction of the pretreated materials in a horizontal reactor designed for handling high solid concentrations. Next, the resulted slurry is sent to the fermentation tank. The product broth is stripped under vacuum, and the removed ethanol is distillated and rectified. The solids in the column

Table 5.3 Cellulosic ethanol pilot and commercial plants, operating or under construction

Company	Location	Feedstock	Capacity (feed rate)	Start date
Pilot plants				
Iogen	Ottawa, Canada	Wood chips	9.0×10^2 kg d^{-1}	1985
Iogen	Ottawa, Canada	Wheat straw	9.0×10^2 kg d^{-1}	1993
SunOpta	Norval, Canada	Various (non-woody)	4.5×10^2 kg h^{-1}	1995
Arkenol	Orange, CA	Various	9.0×10^2 kg d^{-1}	1995
NREL/DOE	Golden, CO	Corn stover, others	9.0×10^2 kg d^{-1}	2001
Pearson Technologies	Aberdeen, MS	Wood residues, rice straw	27 Mg d^{-1}	2001
NEDO	Izumi, Japan	Wood chips	3.0×10^2 l d^{-1}	2002
Dedini	Pirassununga, Brazil	Bagasse	1,600 m^3 yr^{-1}	2002
Tsukishima Kikai Co.	Ichikawa, Chiba, Japan	Wood residues	9.0×10^2 kg d^{-1}	2003
PureVision	Ft. Lupton, CO	Corn stover, bagasse	9.0×10 kg d^{-1}	2004
Universal Entech	Phoenix, AZ	Municipal garbage	1.0×10^2 l d^{-1}	2004
Sicco A/S	Odense, Denmark	Wheat straw	1.0×10^2 kg h^{-1}	2005
Abengoa Bioenergy	York, NE	Corn stover (colocated with grain ethanol plant)	2,000 m^3 yr^{-1}	2006
Demonstration plants				
BRI	Fayetteville, AR	Any carbon-based material	250–300 t d^{-1}	2003
Iogen	Ottawa, Canada	Wheat, oat and barley straw	3,000 m^3 yr^{-1}	2004
Clearfuels Technology	Kauai, HI	Bagasse and wood residues	11,400 m^3 yr^{-1}	2007
Celunol	Jennings, LA	Bagasse, rice hulls (colocated with grain ethanol plant)	5,000 m^3 yr^{-1}	2007
Mascoma	Rome, NY	Wood chips, tall grasses, corn stover and sugar cane bagasse	NA	2008
DONG Energy	Denmark	Straw biomass	4 t h^{-1} (96 t d^{-1})	2009
SEKAB	Sweden	Softwood residues (spruce, pine)	30,000 m^3 yr^{-1}	2009

Adapted from Solomon et al. (2007), Johnson et al. (2009), Larsen et al. (2008), Vessia (2005), The World's Renewable Energy Network for News, Information & Companies (2014)

bottoms are separated, and part of the liquid is recycled to the prehydrolysis reactor (Larsen et al. 2008, 2012). Lignin is recovered as biopellets to be used as solid fuel in the power plant. Another by-product is the C-5 molasses which is used for animal feed. The most relevant feature of the process is that it is entirely continuous (Prunescu et al. 2012; Scott et al. 2013).

Mascoma, a US-based company, has completed a demonstration plant in 2008 that produces 200,000 gallon (~0.8 ML) per year. They produce bioethanol via CBP, using proprietary engineered microorganisms, e.g., modified thermophilic bacteria as *Thermoanaerobacterium saccharolyticum*. Mascoma is planning to construct their first hardwood CBP facility in Kinross, Michigan, with an initial production capacity of 20 MG/yr ethanol from approximately 700 t/d of dry cellulosic materials (Scott et al. 2013; Johnson et al. 2009).

5.5 Biorefinery

Despite extensive technological advances in ethanol production from lignocellulose feedstocks over last few decades, the price of the second-generation ethanol is still high, and therefore, further development is necessary to make it economically competitive. This high price is because of some technological impediments encountered in all different steps of the process. In addition to technological progress, utilization of the process waste streams to produce power and other coproducts and chemicals can substantially improve the economy of cellulosic ethanol plants (Talebnia et al. 2010). Biorefinery is a newly developed concept which has been in focus in recent years. Several biorefinery concepts have been introduced as a solution for clean, efficient, and economically feasible utilization of lignocellulosic materials (Luo et al. 2011). The modern biorefinery parallels the petroleum refinery, and it is a processing plant that converts biomass to a wide range of products including high-value components, transportation fuels, and power through integrated processes (Ragauskas et al. 2006; Virmond et al. 2013). A closer look to the current technologies used for the first- and second-generation ethanol production can give us a clearer picture of biorefinery concept.

Dry milling process has been long used for ethanol production. In this process, grain is the feedstock and the main products are ethanol, low-value animal feed coproducts, and CO_2. Efforts in production of other high-value coproducts through dry fractionation of the corn kernel and separation of non-fermentable components have been made with little success. In that sense, this method is less versatile and has almost no flexibility in processing. The so-called wet-milling process was designed to extract the highest use and value from each component of the grains. For instance, corn wet-milling process has the capability of producing various end products such as starch, fiber, high fructose corn syrup, ethanol, corn oil, and gluten feed and meal. Therefore, this technology is much more flexible and opens numerous possibilities to connect industrial product lines with existing agricultural production units. A more advanced biorefinery is not only able to produce a variety

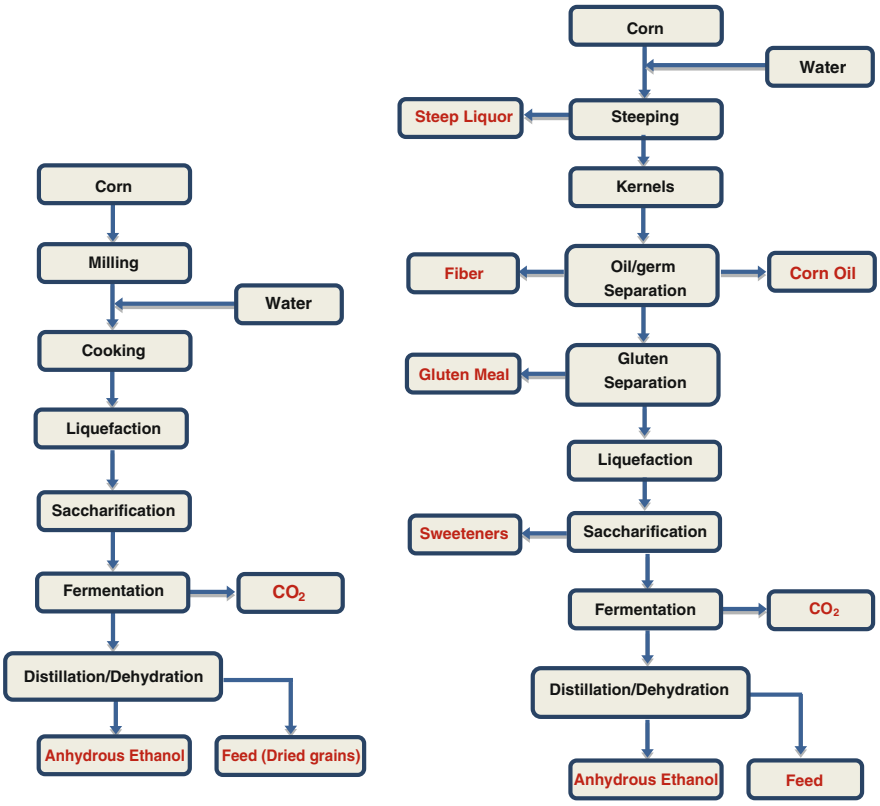

Fig. 5.6 Dry mill and wet mill processes for production of corn ethanol

of chemicals, fuels, and intermediates or end products, but can also use various types of feedstocks and processing methods to produce chemicals for the industrial market. The feedstock flexibility in this technology is a key for adaptability toward changes in demand and supply for feed, food, and industrial commodities (Kamm and Kamm 2004) (Fig. 5.6).

The production of commodity chemicals from biomass has considerable history. In the first half of the twentieth century, many industrial materials such as dyes, solvents, and synthetic fibers were made from trees and agricultural crops. Although many of these bio-based products were then replaced by oil derivatives, interest in these materials is increasing now as more advanced technology became available to make their production economically feasible. There is a wide spectrum of valuable chemicals and materials that can be produced from biomass like lignocellulose. These high-value chemicals can serve as starting materials for many chemical products via biological processes (Dodds and Gross 2007; Talebnia 2008). As described earlier, lignocellulosic materials consist of three main fractions: cellulose, hemicellulose, and lignin. Each fraction can be converted to a number of

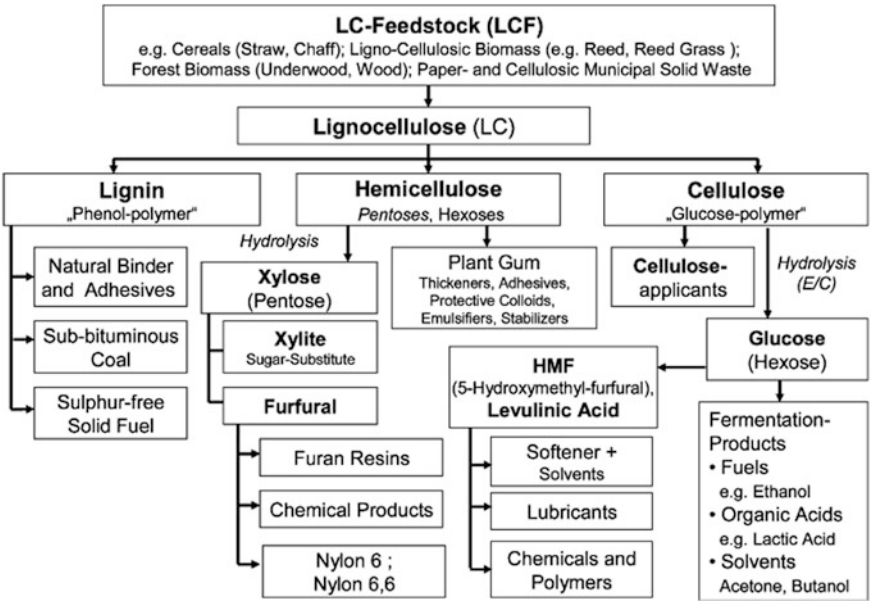

Fig. 5.7 Advanced biorefinery for lignocellulosic feedstocks (Kamm and Kamm 2004)

different chemicals or end products. Figure 5.7 summarizes the potential products of lignocellulose biorefinery.

Sugars derived after hydrolysis are suitable substrate for microbial conversion to various products such as ethanol, propanol, methane, acetone, and organic acids. Among the organic acids, lactic acid is of high interest because it can be used as start material for producing poly lactic acid (PLA) which is a biodegradable plastic with many industrial applications such as food packaging. Furfural and HMF are two other interesting sugar derivatives. As shown in Fig. 5.7, furfural can be used as the starting material in the production of Nylon 6,6 and Nylon 6 (Ragauskas et al. 2006; Kamm and Kamm 2004; Mtui 2009). Lignin as the main solid residual after hydrolysis can be burned to generate heat and power, but it can be also converted to products such as binder and adhesive. Another possibility is thermochemical conversion of the residues to syngas. The produced syngas can then be used for production of methanol, ammonia, and Fischer–Tropsch hydrocarbons (Ragauskas et al. 2006; Johnson et al. 2009; Griffin and Schultz 2012).

5.6 Summary and Future Prospects

Bioethanol can be produced from cellulosic-based feedstocks through the biological and thermochemical pathways. Both technologies need significant capital investment and consist of several sophisticated processing steps with higher operating

costs compared with the first-generation ethanol. There is currently no clear commercial or technical advantage between the biological and thermochemical pathways. The overall economics of both conversion pathways are very similar, and they are more or less the same with respect to energy efficiency and environmental merit (Foust et al. 2009). Despite extensive technological advances over the last few decades, both technologies are still under continual development and evaluation and have significant technical and environmental barriers yet to be overcome. Consequently, the price of the second-generation ethanol, around $2.65 per gallon, is still high and not economically competitive. The US Department of Energy (DOE) has determined that competitiveness with petroleum can be achieved at an ethanol production cost of US$ 1.07/US (in 2002 dollars). This compares to the production cost of Brazilian sugarcane ethanol of US$ 0.81/G (Johnson et al. 2009; Vohra et al. 2014).

The high overall cost in biological route is because of some technological impediments encountered in all different steps of the process. Improving feedstock characteristics, more efficient pretreatment methods, and hydrolytic enzymes, and improving overall process integration are among the main challenges that need further development. The current leading pretreatment methods for lignocellulosic materials are capital-intensive. Economical comparison showed that there is little differentiation between studied pretreatment methods as for instance low-cost pretreatment reactors are counterbalanced by higher cost of catalyst and/or ethanol recovery. Development of less energy intensive and more effective pretreatment methods allowing lower enzymes consumption and minimal inhibitor formation can substantially decrease the total cost of cellulosic ethanol. The next significant technical barrier is cost of enzymes which must be reduced by several folds through developing novel technologies for high solid handling and enhanced enzymes efficiency. Engineering the fermenting microorganisms to produce some or all of the cellulase enzymes in situ provides a complementary route to further reduce the enzyme cost (Talebnia et al. 2010; Vohra et al. 2014; Geddes et al. 2011). Next challenge is need for more robust biocatalysts which must be resistant to inhibitors formed during the pretreatment, be able to utilize C-5 and C-6 sugars at high yields, secrete cellulase enzymes, and remain active under conditions that are near optimal for cellulase function (pH 5, 50 °C). Here, emerging integrated technologies such as SSCF and CBP could be successfully applied since they can create new prospects for reducing operation steps as well as overall cost of process (Geddes et al. 2011; Limayem and Ricke 2012).

Although not economically competitive yet, major cost reduction has been made in biological route, and to date, it can probably provide cheaper biofuels than thermochemical route. However, there are less technical barriers in thermochemical route since much of the technology is already proven. In addition, it has the advantages of whole biomass conversion (including lignin) into syngas and significantly less freshwater consumption (Foust et al. 2009). Finally, the economy of lignocellulosic ethanol could be significantly improved by integrating multiple product lines using biorefinery concept. Therefore, new technologies for efficient fractionation of various cellulosic feedstocks, novel processing methods, and

conversion path to high-value materials and end products must be developed. Colocation with existing infrastructure and facilities, such as power plants, pulp and paper mills, and existing ethanol plants, where various synergies can be achieved, create higher operational flexibility and new opportunities for further industrial development (Johnson et al. 2009). Overall, the choice of the best technology for lignocellulose conversion to bioethanol could be made based on the overall cost, environmental impacts, and energy efficiencies.

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Chapter 6
Biogas from Lignocellulosic Materials

Maryam M. Kabir, Gergely Forgács and Ilona Sárvári Horváth

Abstract Methane production via anaerobic digestion is a steadily growing industry in Europe and all over the world. Biomethane reduces the demand for fossil fuels, since it can be used for the production of power and heat or converted to vehicle fuel. Anaerobic digestion is a renewable energy technology; however, it can also be considered as a low-cost environmental-friendly waste management process, since it reduces the emission of greenhouse gases (GHGs), while it stabilizes the wastes. Currently, mainly the organic fraction of household waste, food waste, sewage sludge, manure, and energy crops is used for biogas production; nevertheless, there are a wide range of other organic substrates which can be utilized for biogas production. Among the organic matters, lignocellulosic materials have a great potential. Great abundance worldwide and carbohydrate-rich contents make them an attractive feedstock for biofuel production. Currently, anaerobic digestion of energy crops is widespread; however, biogas production from lignocellulosic residuals and wastes is still under investigation. This chapter focuses on anaerobic digestion of lignocellulosic materials. It explains the anaerobic digestion process and the current technologies used for crops digestion. It also summarizes the biogas potential of different lignocellulosic materials and the latest research on pretreatments to improve the methane yield. Finally, this chapter compares anaerobic digestion of lignocellulosic materials with energy production from these kinds of materials through thermochemical processes.

6.1 Introduction

At present, around 80 % of the world's energy demand is provided from fossil fuels (oil, gas, and coal) (IEA BIoenergy 2013), which are limited energy sources and eventually become exhausted. Furthermore, the increasing prices of the fuels speed

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up the need for replacing fossil fuels with renewable, green alternatives. In addition to the high price of the conventional fuels and increase the energy demand, it is known that the emission of greenhouse gases (GHGs) causes severe damages in the environment, resulting in global warming and climate change. Among the GHGs, methane has a 72 times higher potential of global warming than carbon dioxide over a 20 years period (Forgács 2012; Aardenne and Fernandez 2010). Almost half of the emitted methane was generated by the agricultural sectors, mainly related to rice cultivation and enteric fermentation. Moreover, waste management sectors (e.g., wastewater treatment and landfill) generate one-third of the methane emission, while the rest of the methane is produced from combustion sectors and oil and natural gas systems (Aardenne and Fernandez 2010). The European Environmental Agency reported that a decrease of methane emission would have a significant impact on the climate change (Forgács 2012; Aardenne and Fernandez 2010). It has been shown that biogas production in a controlled environment can considerably reduce the emission of GHGs, since methane as a potent greenhouse gas can be captured (Abbasi and Abbasi 2010). In addition to that the worldwide energy demands can be largely met by production of biogas; therefore, the efforts are being made to develop and distribute technologies enabling the use of biogas as a promising substitute to fossil fuels in the production of power, heat, and gaseous vehicle fuel. (Börjesson and Mattiasson 2008; Tippayawong and Thanompongchart 2010).

Biogas is formed during microbial degradation of organic matters in oxygen-free environments, a process known as anaerobic digestion (AD). A wide variety of organic materials, i.e., food waste, municipal waste, and animal manure, have been used as feedstocks in AD. Lignocellulosic biomass, including agricultural, forestry residues, energy crops, has recently gained more attention as suitable feedstocks for biogas production due to the increased demands for bioenergy and their abundant accessibility (Montoneri et al. 2009). Lignocelluloses have been accounted for approximately 50 % of the biomass in the world. Yearly production of lignocelluloses is about 200 billion tons per year (Claassen et al. 1999; Zhang 2008). These organic residues have a high energy potential which are currently under-utilized. However, anaerobic digestion of these residues may considerably reduce the volume of waste and provide biogas as an energy source. Besides, the undigested materials can be used for production of biofertilizer and soil conditioners (Lettinga 2005).

The process flow diagram of conversion of lignocellulosic biomass to biomethane is presented in Fig. 6.1.

The methane yield during AD is affected by biodegradability and the composition of lignocellulosic biomass. However, the biodegradability of lignocellulosic biomass during AD is hindered by the recalcitrant structure attributed to the highly crystalline cellulose and lignin around carbohydrates (cellulose and hemicelluloses) (Frigon and Guiot 2010). Therefore, in most cases, the utilization of lignocellulosic biomass can only be economically feasible after pretreatment. Pretreatment processes are considered as key enabling technologies, which allow the use of these cheap and available feedstocks for design of mass- and economically efficient, second generation biofuel processes.

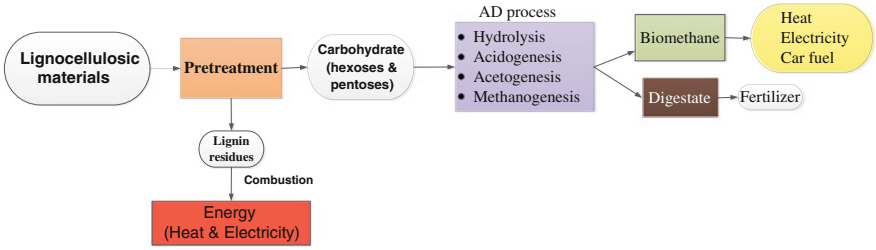

Fig. 6.1 Simplified process flow diagram of conversion of lignocellulosic materials to biomethane

6.2 Anaerobic Digestion

Biogas is a renewable energy source, which is produced by microbiological breakdown of organic matters in the absence of oxygen. One of the major benefits of anaerobic digestion is its versatility to receive wide range of organic substrates (Dolan et al. 2011). The produced gas is mainly composed of methane and carbon dioxide and some smaller amount of other gases such as hydrogen sulfide, hydrogen, nitrogen, ammonia NH_3, oxygen, and water (Ziemiński and Frąc 2012; Naik et al. 2010). The by-product of anaerobic digestion is a nutrient-rich "digestate residue" which can be utilized as a fertilizer on agricultural land (Schnürer and Jarvis 2009). This digestate residue has been proved to achieve a similar improved effect on crop production, as using the commercial fertilizers (Odlare 2005). Additional environmental benefits are the reduction of fossil energy which otherwise would be used in the production of traditional chemical fertilizers. Therefore, biogas production from organic residuals is becoming a very attractive and rapidly developing industry as it is a low-cost waste management technology and does not entail harsh conditions and a complex process design (Börjesson and Mattiasson 2008; Forgács 2012). Under optimal conditions, the energy output/input ratio can reach 28 MJ/MJ, revealing a high efficient use of the biomass (Deublein and Steinhauser 2008b).

Anaerobic digesters can be built locally, and they can be fed with a variety of substrates locally available. The largest number of digesters can be found today in developing countries, and they are small-scale household digesters. It is assumed that there are more than 30 million household digesters operating in China and 3.8 million in India, as well as 200,000 in Nepal and 60,000 in Bangladesh (Jiang et al. 2011; Rajendran et al. 2012). The biogas technology in the African countries is not developed yet; however, a few small-scale digesters are already in operation there (Amigun et al. 2008). Farm-scale digesters found in Europe and America are larger in size, compared to the household digesters in the developing countries. Approximately, 10,000 biogas plants are currently operated in Europe, producing biogas from animal manure, energy crops, sludge, and different types of wastes.

According to the prediction of the German Biogas Association, the number of the biogas plants would increase by a factor of five within the next 10 years in

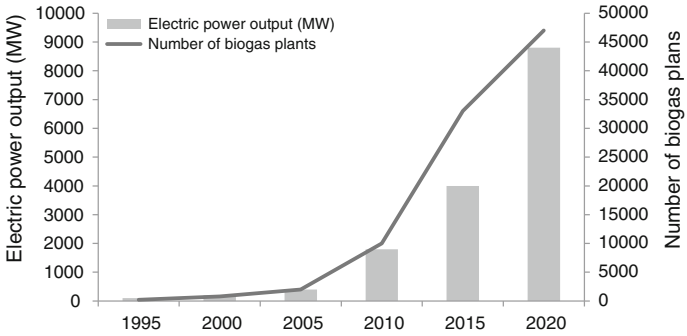

Fig. 6.2 The estimated development of biogas industry in Europe 1995–2020 (Forgács 2012)

Europe (Fig. 6.2). In China, the number of biogas plants is estimated to reach around 200 million by the year of 2020 (Deublein and Steinhauser 2008c).

6.3 Biochemistry of Anaerobic Digestion

Anaerobic digestion is regarded as a multistep biological and chemical process which is favorable not only in waste minimization but also for energy formation. As a result of anaerobic digestion process, the organic compounds are anaerobically degraded and converted to biogas by the action of different groups of microorganisms.

The main steps of the anaerobic digestion process are hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Fig. 6.3) (Ziemiński and Frąc 2012). Each individual phase is carried out by different groups of microorganisms including bacteria and archaea, which partially has syntrophic relation to each other with different needs on the environment (Deublein and Steinhauser 2008a).

6.3.1 Hydrolysis

Hydrolysis is the first step in the anaerobic digestion process. Hydrolytic bacteria (facultative anaerobes) hydrolyze the substrates using extracellular enzymes, either attached to or excreted from their cell surfaces. During this step, the polymers are broken down into soluble monomers and oligomers. The enzymes involved in this process are cellulases, hemicellulases, lipases, amylases, and proteases (Taherzadeh and Karimi 2008). Since, a variety of enzymes are in action throughout this degradation process, almost all kinds of compostable substrates can be hydrolyzed. However, waxes and lignin which are among the main components in lignocelluloses are not degraded (Fernandes et al. 2009).

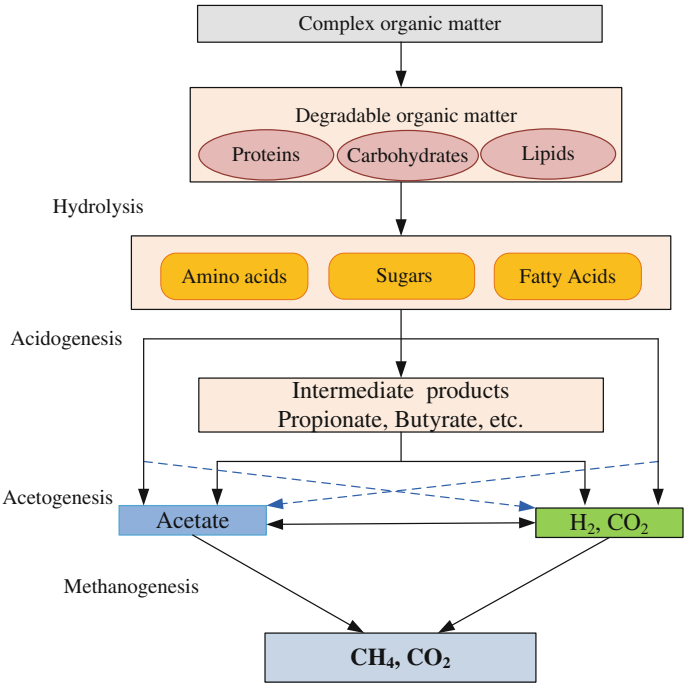

Fig. 6.3 Process flow diagram of anaerobic digestion system (Batstone et al. 2002)

The duration of the hydrolysis step is highly dependent on the characteristics of substrate. Hydrolysis can be achieved relatively fast if the suitable enzymes are produced by microorganisms and enough physical contact between the enzymes and the substrate is provided (Taherzadeh and Karimi 2008). However, substrates with recalcitrant structure, such as cellulose, require weeks to become degraded, and usually, the degradation is not completed (Deublein and Steinhauser 2008a). Hence, in biogas production from complex and rigid substrates, such as lignocelluloses, which are barely accessible to the enzymes, the hydrolysis steps are considered as the rate-limiting step (Taherzadeh and Karimi 2008) (Table 6.1).

Table 6.1 The important groups of enzymes and their functions (Schnürer and Jarvis 2009)

Enzymes	Substrate	Degradation products
Cellulases	Cellulose	Cellobiose and glucose
Hemicellulases	Hemicelluloses	Sugars such as glucose, xylose, mannose, and arabinose
Pectinases	Pectin	Sugars such as galactose and arabinose
Proteinases	Protein	Amino acids
Lipases	Fat	Fatty acids, glycerol

6.3.2 Acidogenesis

In the acid-forming phase, the products from the hydrolysis step will be further degraded by the action of both obligate and facultative anaerobes which will convert them into volatile fatty acids (VFAs) such as valeric acid, butyric acid, propionic acid, acetic acid, and formic acid, as well as hydrogen and alcohols. The partial pressure of the hydrogen regulates the expected products in this step. In general, the most favorable pathway of primary fermentative bacteria is the production of acetate via pyruvate with production of hydrogen. In a well-balanced process, with low partial pressure of hydrogen, the main products are acetate, carbon dioxide, and hydrogen. However, if the environmental conditions are not optimal, at high partial pressure of hydrogen, more intermediates such as volatile fatty acids and alcohols are formed. These products are more reduced than the products that would be produced under optimal conditions (Schnürer and Jarvis 2009; Schink 1997). Thus, these products have to be further modified before they can be converted into biogas.

Non-favorable environmental conditions are formed usually due to an overload of substrates, or the presence of toxic compounds.

6.3.3 Acetogenic Phase

Degradation products from the acidogenesis phase are undergone two different pathways.

Some of the degradation products of the acidogenesis (acetate, carbon dioxide, and hydrogen) can be directly used by methanogens to produce methane. However, VFAs containing more than two carbon atoms and alcohols containing more than one-carbon atom (Schink 1997; Bryant 1979) have to be further oxidized to acetate and H_2 in the acetogenic step by obligatory hydrogen-producing bacteria. At standard conditions, the reactions accomplished by acetogenic microorganisms are not exergonic. For the hydrogen-producing microorganisms, low partial pressure of hydrogen (lower than 10^{-5} bar) is needed for the reactions to be energetically feasible. The syntrophic association between the hydrogen-producing bacteria and the archaea in the methanogenic phase can preserve the partial pressure of hydrogen within the range suitable for the growth of the acetogenic microorganisms (Schink 1997).

6.3.4 Methanogenesis

In the methanogenesis step, obligate anaerobic archaea convert acetate or H_2 and CO_2 to CH_4 and CO_2. The methanogenic archaea can grow directly on H_2/CO_2, acetate and one-carbon compounds, such as formate and methanol (Schink 1997; Bruni 2010).

Acetoclastic microorganisms use acetate, while hydrogenotrophic microorganisms use hydrogen and carbon dioxide as substrates to produce methane. Even though acetoclastic pathway provides much lower energy for microbial growth compared to the hydrogenotrophic one (Klass 1984), approximately 70 % of methane production is performed via the acetoclastic pathway. Since the hydrogenotrophic microorganisms use hydrogen as substrate, the partial pressure of hydrogen has to be above a minimum level (higher than 10^{-6} bar) for the reaction to be exergonic.

Methanogenic archaea are more sensitive group of microorganisms compared to bacteria that are easily affected by environmental stresses in the reactor, such as changes in temperature and pH, or the presence of toxic compounds, such as heavy metals and different toxic organic substances (Chen et al. 2008; Liu and Whitman 2008). Besides, they grow slower and hence have longer generation times (2–25 days) compared to other groups of microorganisms in the reactor, which makes this step the time-limiting step for easily hydrolyzed materials (Schnürer and Jarvis 2009).

In general, as it is described above, these four groups of microorganisms involved in the anaerobic digestion process, function in sequence; in a way that the products from one group are used as feed for another group in the subsequent step (Gerardi 2003). Nevertheless, there is a closer connection between hydrolytic and acidogenic bacteria as well as between acetate- forming bacteria and methane-forming archaea. These connections divide the entire process into two main stages, with different environmental needs in each of these stages. Provided that the degradation rate is almost equal in both of these stages, the process is in balance (Weiland 2010).

6.4 Process Parameters

Accomplishment of the anaerobic digestion system relies on environmental factors, including pH, temperature, mixing rate, organic loading rate (OLR), retention time, and micro- and macronutrient availability. Therefore, to preserve a high efficiency within the process, these parameters should be effectively controlled and kept within the optimum range for the microorganisms involved in the anaerobic digestion process (Ward et al. 2008). The feedstock structure and characteristics also have a significant impact on the performance of the digestion process.

6.4.1 Organic Loading Rate and Hydraulic or Solid Retention Time

OLR is an important parameter to maintain a stable process and to measure the biological performance of anaerobic digestion systems. OLR is referred to the added solid feedstock based on volatile solids (VS) per reactor volume and time

(kg VS $m^{-3}day^{-1}$). For liquid feedstock, it is measured based on chemical oxygen demands (COD); in this case, the OLR is expressed as kg COD $m^{-3}day^{-1}$ (Vandevivere et al. 2003).

In general, the start-up period of the process needs a lower OLR, while a balanced and well-functioning process can handle a higher OLR. The biological performance of AD system is very sensitive to the composition of waste feedstock together with OLR (Zuo et al. 2013; Sharma et al. 1999). An overload into the digester normally leads to the accumulation of VFAs or other inhibitors, which may finally terminate the methane production (Bouallagui et al. 2004; Mata-Alvarez et al. 2000). However, running the digester with too low organic loading (underloaded system) is not economically feasible since the capacity of the digester is not entirely utilized.

Another important parameter that controls the rate of bioconversion of substrate to biogas is the retention time. Retention time is usually expressed as hydraulic retention time (HRT), which regarded as the estimated time that the liquid sludge is present in the anaerobic digester, or solid retention time (SRT), which refers to the time that the microorganisms/solids spend in the digester (Appels et al. 2008). HRT is calculated based on the following formula;

$$\theta = V/Q \tag{6.1}$$

where
θ is hydraulic retention time (time),
V is the volume of the digester (m^3),
Q is fluid flow rate (volume/time)

Generally, HRT is more significant if the feedstock is complex and difficult to digest, whereas SRT is important for easily degradable biomass (Speece 1983; Forgács 2012). Shorter retention time is normally favorable to increase the efficiency of the process and reduce the capital investment costs (Chandra et al. 2012). However, there must be always a balance between OLR and HRT in order to optimize digestion efficiency. Therefore, at higher OLRs, retention times should be sufficiently longer to provide enough time for the microorganisms to degrade the substrates (Demirer and Chen 2005). HRT and SRT are equal when continuously stirred tank reactors (CSTR) are employed running a continuous or semi-continuous process. Nevertheless, in processes, when a part of the residues are recirculated back to the digester tank, SRT gets longer than HRT. In digestion of industrial sewage sludge, where the feedstock has a low total solid content, returning the thickened digestate sludge residue including the biomass, would allow longer retention time for the microorganisms to degrade the organic matter (Dererie et al. 2011). SRT can be also prolonged in proportion to HRT using high-rate processes, such as fluidized bed reactors and anaerobic expanded bed reactors where the microorganisms are attached to a certain carrier material, or UASB reactor in which the microorganisms are forming granules remaining in the system. New technologies for cell immobilization by using specific capsules made of a membrane which is permeable to nutrients and

metabolites retaining biomass (Cheng and Timilsina 2011; Youngsukkasem et al. 2012; Chaudhary 2008) are also in development to increase the efficiency of the process. An additional benefit with a longer SRT is to enable the viable biomass adapted to possible inhibitors, such as ammonia, sulfides, and other substances that might otherwise be toxic at high concentrations (Dererie et al. 2011).

6.4.2 Temperature

Temperature is also one of the most vital factors affecting the activity of anaerobic digestion microorganisms. Temperature fluctuations might be favorable to a certain group and unfavorable to other groups. Among the microorganism in AD system, methane-forming archaea are the most strongly affected by changes in temperature. For instance, an increase of 10 °C in temperature can terminate the methanogenic activity within 12 h; however, it increases the rate of production for acid-forming bacteria. Therefore, the system might suffer from accumulation of VFAs which cannot be utilized by the methane-formers, affecting the overall balance of the digestion process (Gerardi 2003) (Table 6.2).

The anaerobic digestion process can be operated at three different temperature ranges; the psychrophilic range, where the growth optima is around 10 °C, the mesophilic range with an optima at around 37 °C, and the thermophilic range with an optimum at above 50 °C (Mesbah and Wiegel 2008; Kashyap et al. 2003; Coelho et al. 2011). Psychrophilic temperatures can be used for small-scale digesters without heating. However, biogas production at psychrophil temperature is much slower compared to at higher that temperature conditions (Collins et al. 2006; Bohn et al. 2007; Hesselgren et al. 2005). The large-scale anaerobic digesters in Europe are mostly run at mesophilic or thermophilic conditions (Table 6.3).

Table 6.2 Temperature intervals for methane producers (based on Schnürer and Jarvis 2009; Gerardi 2003)

Temperature range	Temperature
Psychrophile	4–25
Mesophile	25–40
Thermophile	50–60
Hyperthermophile	>65

Table 6.3 Comparison of mesophilic and thermophilic digesters (adapted from Gerardi 2003)

Feature	Mesophilic digester	Thermophilic digester
Loading rate	Lower	Higher
Destruction of pathogens	Lower	Higher
Sensitivity to toxicants	Lower	Higher
Operational cost	Lower	Higher
Temperature control	Less difficult	More difficult

A greater diversity of methanogenic microorganisms are found in the mesophilic group (Sekiguchi et al. 1998; Sung and Santha 2003). At mesophilic conditions, the stability and growth conditions of the methanogens in the digester are more likely provided. Due to a greater diversity of the microorganisms at this range of temperature, the process is more robust and has more resistance to different process disturbances (e.g., accumulation of ammonia) which may occur (Zhao and Kugel 1996; Levén et al. 2007).

In general, under thermophilic conditions, methanogens have higher metabolic rates and higher specific growth rates (Lier 1995). Due to this fact, the digesters operated at thermophilic temperature may be constructed in smaller dimensions (which has lower capital costs), while maintaining high levels of biogas production (Duran and Speece 1997). However, in thermophilic range, a smaller group of methanogenic organisms are active. One of the drawbacks is therefore the high sensitivity of thermophilic methanogens to changes in process conditions, since even a small change of the operating parameters can negatively influence their activity (Hwu and Lettinga 1997; Duran and Speece 1997; Lier 1995). For example, a change in temperature with more than 1–2 °C causes a significant reduction in the amount of produced biogas (Chae et al. 2008) due to the fact that a sudden temperature alteration leads to a simultaneous rise in the concentration of all VFAs, particularly in acetic acid and propionic acids (Ahn and Forster 2002; Dohanyos et al. 1985).

Moreover, a range of substrates that can be processed in anaerobic mesophilic condition is higher than those at thermophilic conditions, mainly due to the chemical composition and the stronger influence of some inhibitors in the process (Braun et al. 1981). Several studies showed that anaerobic digestion of wastes with a high concentration of ammonia was less stable and more easily inhibited at thermophilic temperatures than at mesophilic temperatures (Parkin and Miller 1983).

6.4.3 pH and Alkalinity

pH is a vital factor in the anaerobic digestion system. The different microorganisms involved in the biogas process have widely varying requirements on pH for their best growth (Mittal 1996). Most of the microorganisms prefer a neutral pH range, i.e., between 7.0 and 8.5 (Kanokwan 2006). However, there are organisms which are active at both lower and higher pH values. Acid-forming microorganisms can survive in relatively acidic environments (pH 5.0). However, in favor of all the organisms in the digester, neutral pH needs to be maintained (Ferry 1993). The pH out of neutral range results in imbalances in the system by negatively affecting the microorganisms, especially the methanogens (Schnürer and Jarvis 2009; Dague 1968). Since the pH in the process directly depends on the production rates of intermediates, such as volatile fatty acids, during the digestion, in order to keep the pH in optimum range, the system needs to be fed at an optimal OLR. Generally, to

have a stable process, the concentration of volatile fatty acids, particularly acetic acid, should be below 2,000 mg/L (Jain and Mattiasson 1998).

Buffering capacity or alkalinity is referred to the equilibrium between carbon dioxide and bicarbonate, with ammonia as the major cation, which cause a significant resistance to pH changes. In order to preserve optimum pH in the digester, it is vital to have a high stable alkalinity. In AD process, the buffering capacity is mainly provided by few acid-base pairs including, carbon dioxide–bicarbonate, ammonium–ammonia, and dihydrogen phosphate–hydrogen phosphate. The major buffer, produced in anaerobic digesters, is bicarbonate (HCO_3^-), with a pKa of 6.3, and the main acids are VFAs, with an aggregate pKa around 4.8 (Kanokwan 2006). For the process stability, the recommended VFA: alkanity ratio should be maintained less than 0.3 (Ross et al. 1992). The higher the bicarbonate concentration in the digester medium, the greater the alkalinity and resistance to changes in pH (Alvarez et al. 2006). However, a sudden change in pH can occur, for instance, if the system is overloaded and the feed rate is significantly increased. Since the methanogens grow slower than the fermentative bacteria, VFA accumulations will result in a pH drop. In addition, feeding the digester with materials with low buffering capacity, such as lignocelluloses, can also lead to low pH in the digester (Banks and Humphreys 1998). Volatile fatty acid concentrations, specially propionic and acetic acid and butyric acids, are important intermediates to monitor the anaerobic digestion process (Björnsson et al. 1997). In order to maintain the pH in favor of fermentative bacteria and methanogenic archaea, the phase separation (two-staged and multi-staged digesters) is introduced, where the first phase can be optimized at optimal conditions for the growth of hydrolytic and acidogenic microorganisms, while the second phase can operate at conditions optimal for the acetate and methane formation (Ince 1998).

6.4.4 Nutritional Requirements

Nutrients are vital for synthesis and growth, enzymes, cofactors involved in biochemical and metabolic pathways of anaerobic digestion microorganisms. Methanogens have a wide range of mineral nutrient needs for robust metabolism (Blanchard 1992; Rowell and Young 1997). Nutrients are categorized into two types, the macronutrients and the micronutrients and to have a well-balanced system both macro- and micronutrients ought to be present in the digester in right ratios and concentrations. It is reported that in an ideal AD system, the nutrients should be found in excess in the digester as even small shortage may inhibit the overall process (Mara and Horan 2003).

Therefore, in case of feedstock nutrient deficiencies, supplementary nutrients must be added to stimulate the digestion process. However, it should be noted that the inhibition can also occur from the substrate fed to the reactor such as presence of heavy metals and other chemical compound, i.e., limonene in fruit such as citrus

waste, and toxic impurities from batteries and electronic waste mixed with organic fraction of municipal solid waste (OFMSW) (Nayono 2010).

Fundamental macronutrients such as carbon (C), nitrogen (N), phosphorus (P), and sulfur (S) are necessary for growth and multiplication of microorganism. The nitrogen content of a substrate also has a key role in this process since it results in neutral pH stability by liberating ammonium ions (Speece 1983; Gunnerson et al. 1986).

6.4.5 C/N Ratio

There is a vital connection between the utilization of carbon and nitrogen source within the biogas production process. Nitrogen is necessary for the growth of the microorganisms. In one hand, nitrogen deficiency results in insufficient consumption of the carbon source which prohibits the growth of the microorganisms which would accordingly decrease the biogas production (Resch et al. 2011). On the other hand, the degradation of the proteins and other nitrogenous materials would give rise to the high concentration of the nitrogen in the form of ammonium ion (NH_4^+) or ammonia (NH_3) in the system (Chandra et al. 2012; Hobson et al. 1981). Changes in temperature and pH are the main factors to control the chemical equilibrium between the ammonium and the ammonia. As the temperature or the pH increases, this equilibrium would shift more toward NH_3 resulting in ammonia inhibition (Chen et al. 2008). The free ammonia is a main source of inhibition as it can diffuse into the cell and cause proton imbalance in the AD systems (Chen et al. 2008). Therefore, the C/N ratio is considered as a vital parameter for the anaerobic digestion systems, which can be adjusted by feeding the digester with a proper substrate mixture (Chandra et al. 2012; Hobson et al. 1981).

6.4.6 Trace Elements

Among micronutrients, the elements which are known to be the most crucial ones are iron (Fe), nickel (Ni), cobalt (Co), molybdenum (Mo), and wolfram (W) (Zandvoort et al. 2006).

Micronutrients play an important role to form the active sites for several key enzymes; thus, several functions of anaerobic microorganism are dependent on the presence of sufficient micronutrients (Oleszkiewicz and Sharma 1990). The optimum micronutrient requirements in the digester have to be optimized based on the inherent micronutrient concentrations of the substrate, inocula, and the general process conditions within the digester (Jagadabhi 2011).

6.5 Lignocelluloses as Substrates for Anaerobic Digestion

Biogas today is mainly produced from the following: sewage sludge, the organic fraction of municipal solid waste (OFMSW), agricultural residues, energy crops, and waste from the food industry (Angelidaki et al. 2003). However, the current used feedstocks for anaerobic digestion are limited; therefore to reserve the growing needs to feed the digesters, the introduction of new substrates is highly demanded.

The abundant availability of lignocellulosic biomass worldwide with their high carbohydrate content makes them an attractive feedstock for biofuel production. Available lignocellulosic materials can be divided into two different groups: cultivated feedstocks, known as energy crops, and lignocellulosic residuals. Energy crops are mainly composed of cellulose, hemicellulose, and a smaller amount of lignin (Kabir et al. 2013). In addition to these compounds, they contain non-structural carbohydrates such as glucose, fructose, sucrose and fructans, extractives, and pectins (Kabir et al. 2014), which make them an ideal source for biomethane production. The utilization of the energy crops such as corn silage is already extensively common especially in Germany, where approximately 90 % of the digesters utilize crops as main or co-substrate (Weiland 2003; Braun et al. 2008).

Lignocellulosic residuals have a higher amount of lignin content, which is a major drawback regarding their application as a feedstock for anaerobic digestion. Currently, the utilization of lignocellulosic residues as feedstock for biomethane production is not widespread, due to the relatively low methane yield (Seppälä et al. 2007; Lehtomäki 2006). Generally, most lignocellulosic residuals such as straw and woody biomass are not degradable due to their native structure and composition (Hendriks and Zeeman 2009).

In organic wastes, VS are measured as total solids minus the ash content, as achieved by complete combustion of the wastes. The VS are referred to two groups, i.e., the biodegradable volatile solids (BVS) fraction and the refractory volatile solids (RVS). Only the BVS of the VS are potential for bioconversion during the anaerobic digestion. Therefore, knowing the BVS fraction of VS in individual fraction of any kind of heterogeneous waste streams allows a better estimation of the biodegradability, the organic loading, the C/N ratio, and lastly the biogas production (Golush 2008; Monnet 2003). The RVS in organic wastes are mainly lignin which is associated with cellulose and hemicelluloses in plant materials. Lignin is a complex polymer which is difficult to degrade and usually needs a long period of time for complete degradation (Golush 2008; Kayhanian 1995).

Thus, lignocellulosic waste, characterized by high VS and low RVS fraction, is more suitable for biogas production (Monnet 2003). For that reason, the inert fraction of the lignocellulosic waste is better to be removed prior to digestion, since in this case it will not increase the digester volume and slow down the digestion process. For example, in balanced condition in case of waste streams high in sewage and manure, the microorganisms thrive and hydrolyze the organic fraction rapidly while, for the more resistant waste materials, such as native lignocelluloses; i.e., forest residues and straw, the digestion is limited.

6.5.1 Specific Surface Area of Lignocelluloses

Specific surface area has been identified as a particularly important factor affecting enzymatic deconstruction rate and yield (Meng et al. 2013; Mansfield et al. 1999). To improve the biochemical reaction during the digestion process, the accessible surface area of the substrate needs to be increased (Deublein and Steinhauser 2008a). Therefore, in the case of lignocellulosic biomass, the main challenge is to enhance the susceptibility to biodegradation of the material (Bruni et al. 2010; Bruni 2010). The porosity of the lignocelluloses per gram of the substrate is found to be 600–800 m^2; however, the size of each pore is only about 5 nm due to the firm connection between the main three constituents; i.e., cellulose, hemicelluloses, and lignin (Bruni 2010).

Research in connection to biomass pore size and enzymatic hydrolysis propose that small pores with diameters smaller than the cellulase enzymes diameters can hinder, and conversely, large pores enhance enzymatic hydrolysis (Tanaka et al. 1988). When pores are small, only small cellulase components can slowly penetrate inside the pores and may become trapped there, causing a decrease in synergistic interactions, and eventually lowering the rate of cellulose deconstruction. This explains why enzymes with dimensions between 5 and 18 nm depending on the shape need long reaction times (Grethlein and Converse 1991; Schacht et al. 2008). However, if the pores are large, the enzyme accessibility to the substrate will increase and synergistic catalytic actions will take place, and subsequently, the enzymatic hydrolysis yield and rate will increase (Foston and Ragauskas 2010; Meng et al. 2013).

6.5.2 Microbial Degradation of Cellulose and Hemicellulose

Hydrolysis of cellulose necessitates the concerted action of three enzymes, including, endoglucanases, exoglucanases, and β-glucosidase. The function of endoglucanases is to randomly break intermonomer bonds, while exoglucanases are responsible for removing mono- and dimers from the end of the glucose chain; and finally, β-glucosidase hydrolyzes the glucose dimmers (Malherbe and Cloete 2002; Tomme et al. 1995). The rate-limiting factor in the hydrolysis step is due to the ability of endoglucanases to reach amorphous regions within the crystalline matrix of cellulose to create new chain ends, there exo-cellobiohydrolases can attack.

Similar types of enzymes are needed for the hydrolysis of hemicelluloses; however, other enzymes rather than cellulase are required for its complete degradation because of its greater complexity compared to cellulose.

Aerobic fungi and bacteria normally have non-complexed cellulase systems, which lead to the excretion of the cellulose hydrolyzing enzymes into the culture medium. The most reviewed is the fungi, *Trichoderma reesei*, which has been used industrially for production and extraction of cellulases (Wilson 2008). Nevertheless, anaerobic bacteria such as *Clostridium spp* and fungi including, genera

Neocallimastix, Piromonas, and *Sphaeromonas* comprise complexed cellulase systems, where the cellulose hydrolyzing enzymes are enclosed in membrane-bound enzyme complexes (called cellulosomes). The unique components that distinguish the cellulosome from free enzyme systems are the cohesion-containing scafoldin(s) and the dockerin-containing enzymes (hemicellulases, cellulases, and pectinases). Moreover, free non-cellulosomal enzymes usually contain a cellulose-binding domain, called carbohydrate-binding module (CBM), that is attached to the substrate (Fig. 6.4) (Shoham et al. 1999). In rumen and large intestine of herbivorous mammals, cellulose and hemicelluloses are anaerobically degraded by complex cellulase systems. These microorganisms produce short-chain fatty acids that are absorbed and used as energy sources by the mammals (Flint 2008). In anaerobic digester, the same microorganisms that perform cellulolytic and hemicellulolytic activities are present. The only difference between the rumen and anaerobic digester is that the short-chain fatty acids are further converted by methanogens into methane and carbon dioxide.

Generally, aerobic microorganisms utilize far more energy per degraded sugar than anaerobic microorganisms (38 mol ATP versus 2–4 mol ATP per mole of glucose) (Malherbe and Cloete 2002). Cellulose hydrolysis efficiency in anaerobic fungi and bacteria is higher than that in aerobic systems. The reason for that is the presence of cellulosome systems, which allow better coordination between the different cellulose hydrolyzing enzymes. Their close connection will limit the loss of degradation intermediates due to dynamic environmental conditions. Another reason is that the anaerobic microorganisms are more limited in the amount of produced enzymes; so they have a need for a more energy-efficient system (Bayer et al. 2008; Himmel 2009; Doi and Kosugi 2004).

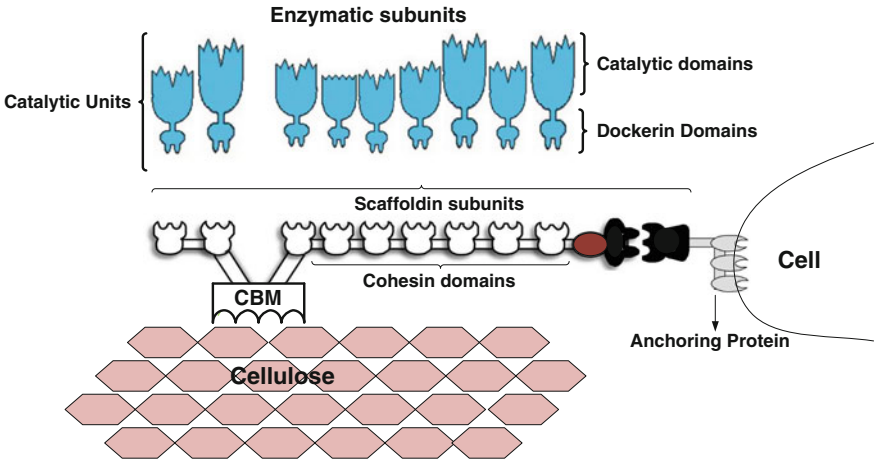

Fig. 6.4 Cellulosome complex structure, adapted from Shoham et al. (1999)

6.5.3 Microbial Degradation of Lignin

Lignin is the most recalcitrant constituent of the plant cell wall. The content of lignin and the biodegradability of the substrate are inversely proportional. The effect of lignin on the biodegradability of cellulose and hemicelluloses is considered to be largely a physical restriction, since the presence of lignin molecules will decrease the available surface area for enzymatic penetration and activity (Haug 1993). Lignin degradation is principally an aerobic process, and in anaerobic conditions, lignin is preserved for a very long period of time (Van Soest 1994). Lignin degradation by white-rot fungi is an oxidative process, and the key enzymes are phenol oxidases. Conditions which favor the lignin decomposition by white-rot fungi are adequate nitrogen level, moisture, temperature, all appear to be important in encouraging lignin decomposition, as does the composition of the lignocellulosic substrate itself (Kuhad et al. 1997; Leonowicz et al. 1999).

Laccase has broad substrate specificity and oxidizes lignin and phenols substructures with forming oxygen radicals. The other enzymes that contribute to the lignin degradation are H_2O_2-producing enzymes and oxidoreductases, which can act either intra- or extracellularly. Fungal and bacterial feruloyl and *p*-coumaroyl esterases are rather novel enzymes, and they are able to liberate feruloyl and *p*-coumaroyl which play a key role in biodegradation of recalcitrant cell wall in grasses (Kuhad et al. 1997). The above-mentioned enzymes can act synergistically with xylanases to disrupt the hemicellulose-lignin link, without mineralization of the lignin (Borneman et al. 1990).

6.6 Anaerobic Digestion of Energy Crops

Energy crops are plants which are dedicated for bioenergy production. Ideal crops for biogas production have the following characteristics: (1) high yield (maximum production of dry matter per hectare), (2) high methane yield (3) low energy input to produce, (4) low cost, (5) low content of contaminants and (6) low nutrient requirements (Koçar and Civaş 2013). Even though successful digestion of energy crops was demonstrated from 1930s, the practical application did not start due to economic reasons. In the 1990s, increasing oil prices and supportive European and National legal frameworks of eco-tariffs facilitated the spread of energy crop digestion (Braun et al. 2008). Moreover, crops digestion facilitates the growing activity in the agricultural sector due to the increasing demand for biomass.

6.6.1 Crops Used in Anaerobic Digestion

Various plant species and plant residues have been investigated for their biogas potential (Table 6.4) (Lehtomäki 2006; Amon et al. 2007). Many of them including

Table 6.4 Biomass and methane yield of various energy crops (Braun et al. 2008; Braun 2007)

Crop	Methane yield (m^3 CH_4/kg VS)	Crop yield (t TS/ha)	Calculated energy potential (GJ/ha)
Maize (whole crop)	205–405	9–30	59–435
Potatoes	276–400	10.7–50	95–644
Grass	298–467	10–15	96–226
Wheat (grain)	384–426	3.6–11.75	45–161
Oats (grain)	283–492	4.1–12.4	33–146
Triticale	337–555	3.3–11.9	36–213
Sorghum	295–372	8–25	76–300
Barley	353–658	3.6–4.1	41–87
Red clover	300–350	5–19	48–214
Alfalfa	340–500	7.5–16.5	82–266
Hemp	355–409	8–16	92–211
Flax	212	5.5–12.5	38–85
Nettle	120–420	5.6–10	22–135
Miscanthus	179–218	8–25	46–176
Sunflower	154–400	6–8	30–103
Oilseed rape	240–340	2.5–7.8	19–85
Jerusalem artichoke	300–370	9–16	87–191
Peas	390	3.7–4.7	47–59
Rhubarb	320–490	2–4	21–63
Turnip	314	5–7.5	51–76
Kale	6–45	240–334	46–484
Sugar beet	236–381	9.2–18.4	70–226

hemp, flax, potatoes, beets, kale, grass, and rape showed relatively high biodegradability and methane yield (Braun et al. 2008). Most of the successfully tested crops showed similar methane yields per VS. However, different crops have different biomass yield per hectare (Braun et al. 2008). Therefore, information regarding the overall energy methane yield per hectare of cultivated land is a more useful parameter from agricultural and economical point of view.

It is worth to mention that cultivation of energy crops has a certain energy requirement. This energy requirement includes the cultivation of the plant, harvesting and processing. Furthermore, significant energy is needed for the production and application of pesticides, herbicides, and fertilizers (Dalla Marta et al. 2011). Currently, maize and grass are the most common energy crops. Maize has high yield per hectare, while grass has relatively low energy requirement. Additionally, grass because of its perennial, it is associated with improved soil quality as well (Amon et al. 2007; Weiland 2006; Murphy and Power 2009). Both maize and grass are characterized by high net (energy yield/energy requirement) energy yield per hectare (Table 6.5).

Table 6.5 Energy calculation of net energy yield and energy output/input ratio for maize and grass, recalculated from Braun et al. (2008)

	Maize	Grass
Energy yield (GJ/ha)	247	161
Energy demand of cultivation (GJ/ha)	17	17
Energy requirement of digestion (GJ/ha)	33	24
Total energy requirement (GJ/ha)	50	41
Net energy yield (GJ/ha)	197	120
Energy output/input ratio	5.0	3.9

6.6.1.1 Stages of Crops Utilization in Anaerobic Digestion Process

Biogas production from crops can be divided into four district stages: (1) harvest, preprocessing and storage, (2) anaerobic digestion, (3) treatment and usage of biogas, and (4) treatment and usage of digestate. As Table 6.4 shows, various annual and perennial plants can be used as crops for AD. It is worth to mention that AD of crops as mono- substrate is not common, in practice crops are co-digested with liquid manure or other liquid substrates to obtain homogenous mixture in the digester and/or providing a more balanced C/N ratio in the system (Giuliano et al. 2013). Germany and Austria are the market leaders regarding to crop digestion with ca 7,800 and 290 digesters, respectively, utilizing mainly crops as feedstock. Other countries including Sweden, Finland, and France use crops as co-substrate (IEA Bioenergy 2014).

6.6.1.2 Harvest, Preprocessing and Storage

Crops can be used in the digestion process straight after harvest. However, for year-round availability of the feedstock, crops are usually stored in silage clamps. The time of the harvest is significantly influence the biomass composition, thus the biodegradability. Late harvest typically leads to higher lignin content, which results in lower methane yield in the subsequent AD process. Therefore, early harvest is recommended to maximize the methane yield. Ensiled biomass has a dry solid content between 20 and 40 %. During the ensiling, a rapid lactic acid and acetic acid fermentation take place, causing a sharp pH decrease to between 4 and 4.5 within a few days (Herrmann et al. 2011). Due to the low pH, the butyric acid fermentation is hindered. Furthermore, the acetic acid formation improves the aerobic stability of the silage and protects it from the growth of specific species of yeasts that are responsible for heating upon exposure to oxygen (Driehuis et al. 1999). Under these conditions, the ensiled crops can be stored for months (Weiland 2003). The harvesting and ensiling processes result in energy losses between 8 and 20 % which are mainly the results of the undesirable aerobic degradation process (Weiland 2010).

6.6.1.3 Anaerobic Digestion Process of Crops

Numerous technical solutions exist for anaerobic digestion of crops. These solutions can be divided into two groups, based on the solid content. Wet digesters operate with solid content less than 15 %; therefore, dilution of the feedstock with other liquid substrates or with process water is usually necessary (Redman 2008). According to Weiland (2010), a majority of the crop digestion plants uses wet processes. The most common reactor configuration applies single-stage digestion using a vertical continuously stirred tank reactor (CSTR) as digester; however, many plants have two-stage processes (Fig. 6.5) (Cirne et al. 2007; Parawira et al. 2008). In these two-stage systems, the second digester is often combined with a membrane type gas holder. Typically, the loading rate of wet crop digestion system is between 1.2 and 4.3 kg VS m^{-3} day^{-1} and the retention time varies between 50 and 150 days, although digesters with retention time longer than 200 days are also exist (Braun et al. 2008).

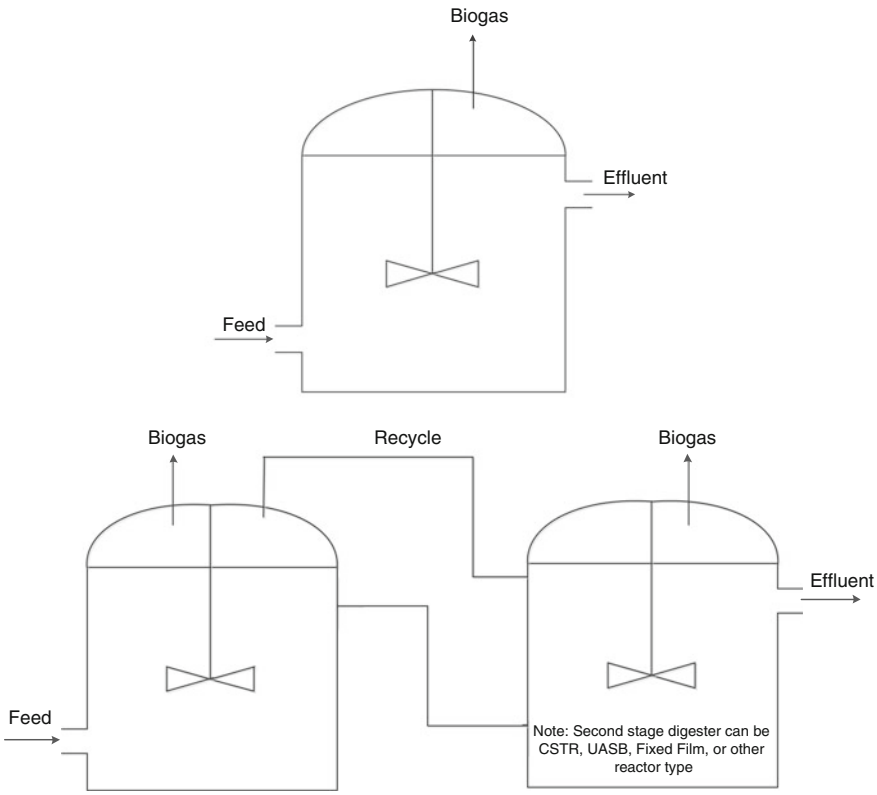

Fig. 6.5 Schematic figure of one-stage CSTR and two-stage wet anaerobic digesters

The other type of digestion is called dry digestion. The solid content in dry digestion systems is between 20 and 40 % (Karthikeyan and Visvanathan 2013). Minority of the crop digestion plants utilizes the crops through dry digestion. For dry digestion of crops, both batch and continuous processes are applied. Batch operations are mainly vertical reactors with or without mixing. During batch process, the feedstock is placed in the reactor followed by the addition of microbes from the inoculum (percolate and or digestate). The gas production starts, reaches the maximum rate then decreases, and finally stops. After the biogas production is stopped or nearly stopped, half/major part of the feedstock is removed and the remainder part acts as inoculum for the next batch. Figure 6.6 shows the schematic set-up of a simple dry batch digestion system

However, gas engine and turbines, there the produced gas is utilized, require relatively stable gas quality and quantity, and the production rate and composition of the gas vary during the batch operation. Therefore, numerous batch digesters coupled in series are used and fed sequentially to be able to produce gas with a stable quality and quantity.

For continuous dry digestion, the vertical and horizontal reactor designs are equally common (Fig. 6.7). The horizontal design has the advantage over the vertical design that the retention time of the feedstock is more controlled, but the construction and operation costs are higher than those for the vertical design, because vertical design always contains mixing devices (Karthikeyan and Visvanathan 2013). During continuous dry digestion, the feedstock is mixed with the digestate to ensure the inoculation, but in many cases, the process water is also recycled.

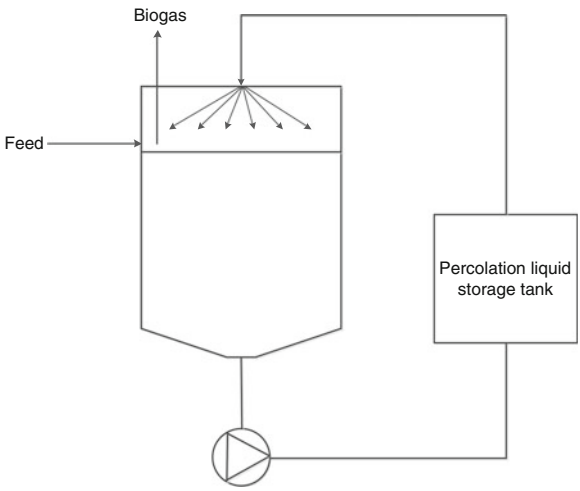

Fig. 6.6 Schematic figure of a dry batch digestion system

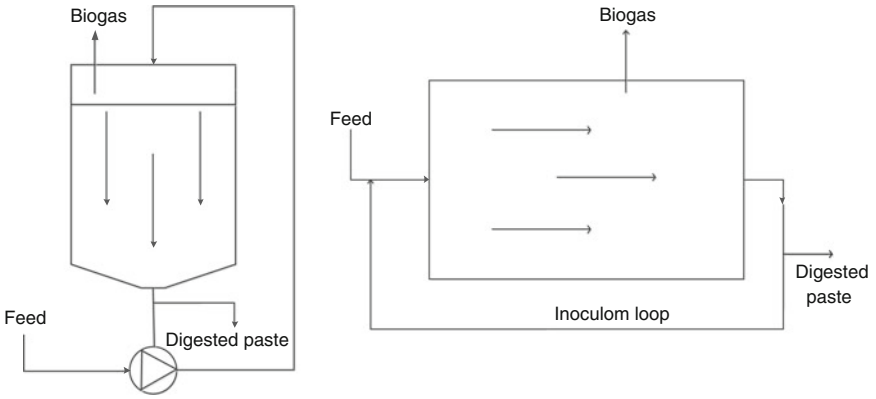

Fig. 6.7 Schematic figure of dry continuous digestion systems

6.6.1.4 Treatment and Usage of Biogas

Biogas collected from digestion can be used directly in a gas boiler for generating heat or burned in a combined heat and power plant (CHP) to produce heat and electricity. Currently, biogas utilization in CHP units dominates in Europe. The produced electricity is usually distributed through the public electricity net. The heat is used to provide energy for the process, and the remaining part can be sold for central and district heating. However, since during the summer, heat is not required in rural areas, an other possibility to utilize the biogas is to upgrade it to biomethane (Ryckebosch et al. 2011). Biomethane can be used as vehicle fuel or injected to the national gas grid. Several existing upgrading techniques are available, including water scrubbing, pressure swing adsorption, chemical absorption as well as cryogenic and membrane separation (De Hullu et al. 2008).

6.6.1.5 Treatment and Usage of the Digestate Residue

The digestate residue is the secondary product of anaerobic digestion. Approximately 80 % of the volume of the feedstock fed to the digester ends up as digestate residue. The solid content of digestate depends on the process, but generally varies between 5 and 30 %. Regardless of the applied process, the digestate residue contains almost the same quantities of macronutrients (nitrogen, potassium, phosphorous), micronutrients (Fe, Ni, Co, etc.), and trace elements as the original feedstock. Therefore, digestate can be used as natural fertilizer that recycles the organic matter and nutrients to the soil. In most cases, digestate can be directly applied to agricultural lands.

6.7 Anaerobic Digestion of Lignocellulosic Residues and Waste

Lignocellulosic residues and waste can be divided into four main groups: agricultural residues (straw), fruit and vegetable waste, forestry residues (woody biomass), and paper waste. These wastes are generated in a huge amount; however, the utilization is not always resolved. Anaerobic digestion is a possible solution, but the methane yield of these kinds of wastes is low and their degradation requires a very long process. Research was focused therefore during the last decades on suitable pretreatment methods that can increase the degradation rate of lignocelluloses, leading to increased methane yields (Yang and Wyman 2008; Taherzadeh and Karimi 2008).

6.7.1 Pretreatment Affecting Anaerobic Digestion of Lignocelluloses

Several pretreatment technologies are available in the literature on lignocellulosic materials. An ideal pretreatment would aim to complete or partial decomposition of the feedstock into fermentable sugars, thus increasing the rate of hydrolysis. The final goal of the pretreatment is to eliminate the resistance of lignin and decrease the crystalline structure of cellulose, and subsequently make the substrate more accessible for the microorganisms in the anaerobic digestion system.

So far numbers of promising pretreatment methods (discussed in detailed in Chap. 3) have been suggested for enhancing the biogas production from lignocellulosic biomass, such as physical, physicochemical, chemical, and biological pretreatments (Taherzadeh and Karimi 2008; Yang and Wyman 2008; Hendriks and Zeeman 2009; Chandra et al. 2007). Milling, among the physical pretreatments, was proven to be effective for increasing the specific surface area, reducing the degree of polymerization (DP), and also causes the shearing, thus improving the hydrolysis yield by 5–25 %. This improvement depends on type of biomass, duration and type of milling (Zeng et al. 2007; Jin and Chen 2006). Additionally, it is repeatedly shown that the smaller particle size of the lignocelluloses results in higher yield in biofuel production (Jin and Chen 2006; Monavari et al. 2009; Teghammar et al. 2012; Lennartsson et al. 2011). That is why the physical pretreatment is often carried out in combination with other pretreatment methods.

According to Hendrik and Zeeman (2009), the pretreatment methods such as steam, lime, liquid-hot-water and ammonia-based steam explosion, thermal hydrolysis, wet oxidation, and ultrasound and radiation are offering potential for improving biogas yield from lignocelluloses (Hashimoto 1986; Fox and Noike 2004). However, methods that result in a very high methane yield, such as steam explosion, wet explosion, and ammonia fiber explosion (AFEX), are energy-intensive pretreatments. Hence, the energy cost of applying these pretreatments is high and the net energy gain of these techniques is required to be clearly evaluated.

Furthermore, using these methods there is also a risk for production of inhibitory products such as furfural, HMF, and soluble phenolic compounds. Although the methane-producing bacteria are capable of adapting to a very low concentration of such compounds, the methane production rate would decrease at the beginning of the digestion (Fox et al. 2003).

Apart from the pretreatment methods, the results of anaerobic digestion are influenced by many different other factors, including inoculum, substrate to inoculum ratio, OLR, and process conditions (Angelidaki et al. 2009). Therefore, distinction between the methane productions of the same substrates, described in the next part of this chapter, do not necessarily show differences between the effects of pretreatments.

6.7.2 The Inhibition Effect of Pretreatment on the Digestion Process

As it was discussed previously, due to the recalcitrant structure and the high lignin content of the lignocellulosic biomass, the rate of anaerobic digestion of these materials is relatively slow. Therefore, pretreatments are performed to increase the rate of degradation and to improve the methane yield. However, in some cases the chemical agent used for pretreatment can act as a potential inhibitor for the microbial community of the anaerobic digestion. It was found that after pretreatment with the organic solvent *N*-methylmorpholine-*N*-oxide, the remaining solvent affected the digestion process negatively even though it was present in as low concentrations as 1 % (Kabir et al. 2013a). Besides, pretreatment might lead to the production of inhibitory products, such as furans in dilute acid and steam explosion pretreatments, furfural from alkaline pretreatments (Ahring et al. 1996; Taherzadeh and Karimi 2008). The problem of inhibitory by-products might be solved after a long hydraulic retention time since the microorganisms may adapt or degrade these by-products after a while, although, the kinetic of the process might be affected.

6.7.3 Anaerobic Digestion of Woody Biomass

Methane yield of woody biomass have been found to be not economically feasible without pretreatment. There are many factors influencing anaerobic digestion of wood, such as low moisture content, high lignin content, cellulose crystallinity, and degree of association between lignin and carbohydrates. Additionally, certain plants produce resin acid extracts for protection from microbial attack and biological damages, which might be inhibitory to the microorganisms carrying out the anaerobic digestion. Therefore, several pretreatment methods have been investigated aiming to improve the biogas production from this group of biomass (Tong et al. 1990; Cowling 1975; Chandler and Jewell 1980; Jerger et al. 1982; Kenney et al. 1990).

Generally, there is an inverse linear relationship between VS reduction and lignin content in anaerobic degradation of woody biomass (Chandler and Jewell 1980). Biodegradability of several woody species was investigated for biogas production by using biomethane potential (BMP) assay. Some of the results found in the literature presenting the methane potential from woody biomass before and after pretreatment are discussed in this section and summarized in Table 6.6.

Salehian et al. (2013) investigated the effect of alkaline pretreatment on pine (softwood) using 8 % NaOH. The pretreatment was performed in different conditions: at two temperatures (0 and 100 °C) in different duration times (10, 30, and 60 min). The results of anaerobic digestion in batch mode showed that while 0.065 m^3/kg VS CH_4 was produced from the untreated pine, methane yield of 0.178 m^3 CH_4/kg VS could be achieved after the most successful pretreatment (8 % NaOH, 10 min and 100 °C). This corresponds to 181 % improvement comparing to that of the untreated assay. Further analyses of pretreated assays with scanning electron microscopic (SEM) and Fourier transform infrared (FTIR) spectroscopy revealed that alkaline conditions at higher temperature resulted in the disintegration of the biomass structure, while the pretreatment at low temperature led to decrease in cellulose crystallinity. Mirahmadi et al. (2010) have also examined the effect of alkaline pretreatment using 7 % w/w NaOH on two different wood species, milled spruce (softwood) and birch (hardwood), at different temperatures ranging between −15 and 100 °C. Batch anaerobic digestion assay was then carried out at thermophilic conditions (55 °C) for 30 days. Treatment of birch at 100 °C led to a methane yield of 0.46 m^3/kg VS, compared to 0.25 m^3/kg VS obtained from untreated birch. The best result for spruce was achieved with NaOH pretreatment at 5 °C, resulting in a 74 % improvement in the methane production compared to that from untreated spruce. Furthermore, it was concluded that there was roughly no destruction of lignin during the pretreatments neither for softwood nor for hardwood. However, applying alkaline treatment to improve the methane production was more successful for hardwood than that for softwood.

The pretreatment of forest residues (mixture of spruce, pine, bark, etc.) using N-methylmorpholine-N-oxide (NMMO) was carried out in another study. The pretreatment with NMMO could effectively decrease the cellulose crystallinity of the wood without leading to a loss in carbohydrates. The best methane yield of the forest residues was achieved using 85 % NMMO for 15 h at 120 °C which corresponds to 85 % of the expected theoretical yield, assuming that only the carbohydrate fraction present in forest residues is utilized for methane production (Kabir et al. 2013a). Similarly, Teghammar et al. (2012) studied the effect of NMMO-pretreatment on spruce (softwood) for biogas production. Pretreatments were carried out at 130 °C for 1–15 h followed by anaerobic batch digestions for six weeks. The NMMO-pretreatment significantly improved the methane yields counting up to improvements between 400 and 1,200 %. The anaerobic digestion of untreated spruce chips (10 mm) and milled (<1 mm) spruce resulted in methane yields of 0.011, 0.066, Nm^3/kg raw material, respectively. Hence, only milling resulted in sixfold improvement in the methane yield. Moreover, increasing the pretreatment time for NMMO treatment led to better results. After the pretreatments

Table 6.6 Methane potential of different woody biomass

Substrate/wood	Pretreatment condition	Digestion condition	Methane yield	Methane yield m^3/kg raw material	References
Untreated wood chips (Eucalyptus globules)	Untreated	Mesophilic	0.014 m^3/kg TS	NA	Nakamura and Mtui (2003)
	Extraction using hot water 125 °C in 20 min		0.124 m^3/kg TS	NA	Nakamura and Mtui (2003)
	Extraction using 1 % NaOH, 125 °C in 20 min		0.134 m^3/kg TS	NA	(Nakamura and Mtui 2003)
	Steam explosion at 25 atm and 3 min		0.194 m^3/kg TS	NA	Nakamura and Mtui (2003)
Japanese cedar chips	Untreated	Mesophilic	0	NA	Take et al. (2006)
	Steam Explosion 4.5 MPa and 258 °C, 5 min		0.180 m^3/kg TS	NA	Take et al. (2006)
	Fungal treatment (*Cyathus stercoreus AW 03*-72)		0.043 m^3/kg TS	NA	Take et al. (2006)
Japanese cedar wood chips	Untreated	Mesophilic	NA	0.002	Amirta et al. (2006)
	Fungal pretreatment *C. subvermispora ATCC 90467* + wheat bran media for 8 weeks		NA	0.0835	Amirta et al. (2006)
	Fungal pretreatment C. subvermispora CBS 347.63 for 8 weeks		NA	0.0265	Amirta et al. (2006)
Japanese beech (Fagus crenata)	Untreated	Thermophilic	0	0	Yoshida et al. (2010)
	Supercritical water treatment at 380 °C and pressure 30 MPa		NA	0.105	Yoshida et al. (2010)
	Supercritical water treatment at 380 °C and pressure 100 MPa		NA	0.068	Yoshida et al. (2010)

(continued)

Table 6.6 (continued)

Substrate/wood	Pretreatment condition	Digestion condition	Methane yield	Methane yield m^3/kg raw material	References
Spruce chips (*Picea abies*)	Untreated (10 mm chips)	Thermophilic	17 ± 69 ml/g CH	0.011	Teghammar et al. (2010)
	6 % spruce in the NMMO solution (85 %), at 130 °C and 1 atm 15 h		202 ± 88 ml/g CH	0.125	Teghammar et al. (2010)
Milled spruce	Untreated (less than 1 mm)		106 ± 80 ml/g CH	0.066	Teghammar et al. (2010)
	6 % spruce in the NMMO solution (85 %), at 130 °C and 1 atm 15 h		395 ± 88 ml/g CH	0.245	Teghammar et al. (2010)
Spruce	Untreated	Thermophilic	0.030 m^3/kg VS	NA	Mirahmadi et al. (2010)
	7 % (w/w) NaOH, at 5 °C, 2 h		0.050 m^3/kg VS	NA	Mirahmadi et al. (2010)
Birch	Untreated	Thermophilic	0.250 m^3/kg VS	NA	Mirahmadi et al. (2010)
	7 % (w/w) NaOH, at 100 °C, 2 h		0.469 m^3/kg VS	NA	Mirahmadi et al. (2010)

for 15 h, methane productions of 0.125 and 0.245 Nm^3 CH_4/kg raw materials were obtained from spruce chips and milled spruce, respectively.

Nakamura and Mtui (2003) applied steam explosion, pretreatment on wood chips (Eucalyptus globules) at pressure of 25 atm and steaming time of 3 min. The obtained methane yield after the steam explosion treatment was 0.194 m^3 CH_4/kg TS, while only 0.014 m^3/kg TS methane was produced from the untreated material. The improvement of the methane yield was due to the high decrease in Klason lignin. Moreover, the pretreatment led to conversion of 80 % of the holocellulose into methane. Similarly, a considerable improvement was observed in a study performed by Take et al. (2006) who applied steam explosion treatment on wood (Japanese cedar chips), prior to biogas production. The pretreatment was performed at 4.51 MPa (258 °C) for 5 min. The pretreated wood yielded to 0.180 m^3/kg TS methane, while the methane yield for untreated wood samples was almost zero.

Biological pretreatment of Japanese cedarwood was carried out by Amirta et al. (2006) prior to anaerobic digestion. Pretreatment was performed using two different strains of white-rot fungi, i.e., *Ceriporiopsis subvermispora*, CBS 347.63 and ATCC 90467. The wood chips were subjected to cultivation of these two strains with and without the addition of wheat bran during 4–8 weeks. The methane production obtained during the subsequent anaerobic digestion of treated Japanese cedar wood enhanced with increased cultivation time of the fungi on the material. The longest pretreatment time, i.e., 8 weeks with *C. subvermispora* ATCC 90467 in the presence of wheat bran led to the highest methane yield of 0.083 m^3/kg raw material, which corresponds to 35 % of the theoretical yield based on the holocellulose content in the decayed wood.

6.7.4 Anaerobic Digestion of Straw

The results of various studies on anaerobic digestion of straw showed that the gas production varied depending on what kind of cereals were being used in anaerobic digestion system. Besides, investigations on straw also reveal that the physical pretreatment such as milling is one of the significant factors for improving the anaerobic digestion yield. Some of the results found in the literature from straw are discussed in this section and summarized in Table 6.7.

Alkaline pretreatment using 96 % lime ($Ca(OH)_2$) containing 3 % $CaCO_3$ was applied on milled oat straw with particle size of 5–15 mm at 55 °C for 24 h. The treated samples were then subjected to anaerobic batch digestion for 35 days resulting in a methane yield of 0.287 m^3/kg VS. Other pretreatment methods applied on the same substrate, such as steam explosion and steam explosion with addition of acid, resulted in lower methane yields of 0.197 and 0.201 m^3/kg VS, respectively, comparing to that obtained after the lime pretreatment (Dererie et al. 2011).

Table 6.7 Methane potential of different kind of straw

Substrate/ Straw	Pretreatment condition	Digestion condition	Methane yield	Methane yield m^3/kg raw material	References
Wheat Straw	Untreated	Mesophilic	0.189 m^3/kg VS	NA	KTBL (2005; Bauer et al. 2009)
Wheat Straw	Milled (0.5–1.0 mm)		0.275 m^3/kg VS	0.235	Bauer et al. (2009)
	Steam explosion 180 °C, 15		0.331 m^3/kg VS	0.283	Bauer et al. (2009)
Oat Straw	Untreated	Mesophilic	No data	No data	Dererie et al. (2011)
	Lime pretreatment		0.287 m^3/kg VS	0.252	Dererie et al. (2011)
	Steam explosion		0.197 m^3/kg VS	0.173	Dererie et al. (2011)
	Acid + steam explosion		0.201 m^3/kg VS	0.1845	Dererie et al. (2011)
Rice Straw	Untreated (3–5 mm particle size)	psychrophilic	0.240 m^3/kg VS	0.214	Lei et al. (2010)
	Phosphate supplementation 155 mg-P/l		0.250 m^3/kg VS	0.223	Lei et al. (2010)
Straw	Untreated	Mesophilic HRT, 28 days	0.165 m^3/kg VS	0.140	Hjorth et al. (2011)
	Extruded straw		0.281 (+70 %) m3/kg VS	0.239	Hjorth et al. (2011)
	Untreated	Mesophilic HRT, 90 days	0.320 m^3/kg VS	0.272	Hjorth et al. (2011)
	Extruded straw		0.355 (+11 %) m^3/kg VS	0.301	Hjorth et al. (2011)
Corn Straw*	Untreated	Mesophilic	0.1537 m^3/kg VS	0.1185	Zhong et al. (2011)
	NaOH 8 % Wt		0.472 m^3/kg VS	0.364	Zhong et al. (2011)
	Ammonia 5 % Wt		0.316 m^3/kg VS	0.2435	Zhong et al. (2011)
	Urea 4 % Wt		0.178 m^3/kg VS	0.137	Zhong et al. (2011)

* The yield for corn straw, represents the total biogas production.

In another study, wheat straw was subjected to steam explosion pretreatment at 180 °C and 15 min [48]. Methane production from the pretreated assay was 0.331 Nm^3/kg VS, while the untreated wheat straw yielded 0.275 Nm^3/kg VS methane. This corresponds to 20 % increase in methane production comparing to that of the untreated substrate. From this study, it was concluded that the longer residence time and higher temperature did not considerably increased the methane yield. The optimum temperature for steam explosion pretreatment was suggested to be between 160 and 200 °C (Panagiotou and Olsson 2007).

Another study investigated biogas production from rice straw after different pretreatments, i.e., mechanical, thermal, and chemical using ammonia in high-rate anaerobic digestion system. The results of this study reveal that the combination of milling to 10 mm particle size, and thermal treatment at 110 °C with addition of 2 % ammonia was the most successful method, which led to about 25 % improvement in biogas production comparing to that of the untreated assay. Biogas obtained from untreated and pretreated assays were 0.38 and 0.47 m^3/kg VS, respectively (Zhang and Zhang 1999).

Zhong et al. (2011) investigated the effect of three different alkaline pretreatment including 8 % NaOH, 5 % ammonia, and 4 % urea on corn straw prior to anaerobic digestion. The pretreatments were carried out at an ambient temperature of (15 ± 2 °C) for 20 days. All the pretreatments caused significant degradation of lignin, hemicellulose, and also cellulose. However, the treatment with 8 % NaOH resulted in the highest methane yield of 0.472 m^3/kg VS, which corresponds to 207 % increase compared to that of the untreated assay.

Teghammar et al. (2012) studied the effect of an organic solvent, i.e., N-methylmorpholine-N-oxide on triticale straw and rice straw aiming to enhance the methane production. The pretreatments were carried out at 130 °C for 1–15 h prior to batch anaerobic digestion assays running at thermophilic conditions for 6 weeks. The digestion of untreated rice straw and triticale straw resulted in methane yields of 0.022 and 0.030 Nm^3/kg raw material, respectively. The NMMO-pretreatment significantly improved the yield of anaerobic digestion leading to methane productions of 0.157 and 0.203 Nm^3 CH_4/kg for the pretreated rice straw and triticale straw, respectively.

6.7.5 Anaerobic Digestion of Paper Waste

Derived from literature the BMP from paper is highly dependent on the type of the paper, i.e., pulp and paper sludge, paper tube residual, the pretreatment method applied and the inoculum used. Generally, the methane yield from untreated paper is found to be between 0.1 and 0.2 m^3/kg VS.

Some of the results found in the literature regarding anaerobic digestion of different fractions of paper wastes are presented in this section and summarized in Table 6.8.

Table 6.8 Methane potential of paper wastes

Substrate/ paper	Pretreatment	Digestion condition	Methane yield	Methane yield ml/g raw material	References
Newsprint	Untreated	Mesophilic	–	No data	Fox et al. (2003)
	Alkaline supercritical water treatment, 190 °C pH = 11		No data	327 ml/g. day	
Newsprint	Untreated	Mesophilic	80 ml/g COD	No data	Clarkson and Xiao (2000)
	NaOH 10 %, 1 day at 25 °C		120 ml/g COD	No data	
Office paper	Untreated	Mesophilic	360 ml/g VS	No data	Xiao and Clarkson (1997)
Paper tube residual	Untreated	Thermophilic	222 Nml/g VS	188	Teghammar et al. (2010)
	Non-exclusive pretreatment 100 ml suspension of 50 g/l dry milled paper + 2 % NaOH at 190 °C for 30 min		269 Nml/g VS	228	Teghammar et al. (2010)
	Steam explosion 3 liter suspension of 50 g/l dry milled + both 2 % NaOH and 2 % H_2O_2 at 220 °C for 10 min		493 Nml/g VS	419	
	Steam explosion 3 liter suspension of 50 g/l dry milled + 2 % NaOH at 220 °C for 30 min		405 Nml/g VS	344	
	Steam explosion 3 liter suspension of 50 g/l dry milled + 2 % NaOH at 220 °C for 10 min		403 Nml/g VS	342.5	
Pulp and paper sludge	Untreated	Mesophilic	190 ml/g VS	36.5	Lin et al. (2009)
	0.6 % NaOH at 37 °C for 6 h		320 ml/g VS	61.5	

Xiao and Clarkson (1997) applied acetic acid and nitric acid reagent targeting the lignin fraction of newsprint waste prior to anaerobic digestion. The results of their investigation showed that even though the pretreatment was carried out using high concentration of acetic acid (80 %) at elevated temperature (boiling water bath), it could not successfully dissolve lignin. On the other hand, 80 % lignin removal from newsprint residues was observed when using 35 % acetic acid together with the addition of 2 % nitric acid. These treatment conditions increased the methane production from 0.100 m^3/kg VS (obtained from untreated) to 0.270 m^3/kg VS as it was observed during the subsequent anaerobic digestion tests.

In another study, newsprints were subjected to alkaline pretreatment using 10 % NaOH which significantly improved the biodegradability of the substrate. The NaOH pretreatment was also performed with increased concentrations of 15 and 20 %; however, no significant differences in terms of methane production were observed. Newsprint undergone alkaline pretreatment with 10 % NaOH resulted in 0.120 m^3/kg COD methane production, while 0.08 m^3/kg COD methane was obtained from the untreated assay (Clarkson and Xiao 2000). Similarly, the alkaline pretreatment of pulp and paper sludge using NaOH (8 g NaOH /100 g TS sludge), resulted in 184 % increase in methane yield (0.32 m^3CH_4/kg VS pretreated sludge) compared to that from the untreated paper sludge (Lin et al. 2009).

Paper tube residuals were used as a substrate for biogas production in a study by Teghammar et al. (2010). Steam explosion treatment was applied with the addition of sodium hydroxide and/or hydrogen peroxide to improve the biogas production. The untreated assay resulted in 0.238 Nm^3/kg VS methane. While, using steam explosion at 220 °C for 10 min and with addition of both 2 % NaOH and 2 % H_2O_2, the methane production was enhanced by 107 %, i.e., 0.493 Nm^3/kg VS methane was obtained.

Wet oxidation was also investigated to enhance methane production from newspaper waste. Pretreatments were carried out at 170, 190, and 210 °C, with a retention time of 1 h. The highest lignin removal was achieved at 190 °C in which about 65 % was isolated. Furthermore, the batch anaerobic digestion tests showed that the highest methane yield could be achieved after pretreatment at 190 °C, which converted 59 % of the initial total COD to methane (Fox and Noike 2004).

6.8 Co-digestion

Simultaneous digestion of homogenous mixture of two or more substrates is called co-digestion. Recently, co-digestion has taken much attention since it is one of the interesting ways of improving the yield of anaerobic digestion. The co-digestion causes improvement in yield of anaerobic digestion due to its positive synergisms established in the digestion medium and supplying the missing nutrients and sometimes by addition of suitable moisture contents required in the digester (Mata-Alvarez et al. 2000).

As mentioned earlier in this chapter, C/N ratio of the feedstock has an important role for a well-balanced digestion system. According to the literature, the optimum level of the C/N ratio is between 20 and 30 (Sreekrishnan et al. 2004; Liu and Whitman 2008). But this is only an approximate suggestion, since in the case of lignocellulosic biomass the nitrogen can be also bound in lignin structure (Deublein and Steinhauser 2008a). The C/N ratio of the lignocellulosic substrates is too high therefore; mixing it with high nitrogen content substrates can be beneficial to acquire optimal nutritional conditions. For instance, the co-digestion of manure and plant materials provides a better nutritional balance in AD system, which reduces the risk for inhibition. The manure fraction supply a wide range of nutrients, and the addition of plant materials with high carbon content would balance the C/N ratio of the feedstock (Lehtomäki et al. 2007).

The viability of co-digestion of two or more organic waste streams (e.g., organic fraction of municipal solid waste (OFMSW), sewage sludge or biosolids, animal waste, and agricultural solid waste) has been investigated at both laboratory-scale (Rivard et al. 1990; Zhang et al. 2013; Pagés-Díaz et al. 2014) and full-scale level (Cecchi et al. 1988).

Lehtomäki et al. (2007) investigated anaerobic co-digestion of grass silage, sugar beet, and oat straw together with manure in semi-continuously fed CSTRs. The results showed that co-digestion of manure with 40 % VS loading coming from the crop feedstock was advantageous to improve the yield of methane production. The methane yield obtained from manure was 0.155 dm^3 CH_4/kg VS, while co-digestion of manure with grass, sugar beet tops, and straw resulted in 268, 229, and 213 dm^3 CH_4/kg VS, respectively.

In another study, different mixture ratios of straw and manure were subjected for anaerobic co-digestion. The digestions were performed in bath reactors for 28 days at mesophilic conditions (35 °C). The results of this investigation revealed that co-digestion of manure and straw with a mixing ratio of 1:1 (VS) led to significant decrease in methane yield (0.182 m^3 CH_4/kg VS) production compared to the methane production of only manure, which was 0.234 m^3/kg VS (Demirbas 2006).

Müller and Trösch (1986) examined the effects of biological treatment on a mixture of straw and manure on anaerobic digestion. Batch digestion assays were carried out with loading of 40 g/L solids in mesophilic digesters. The results showed that pre-treated straw/manure mixture using *Pleurotus florida* pretreatment in 60 and 90 days showed higher methane yields, i.e., 0.318 and 0.343 dm^3/g raw material, respectively, while the biogas yield from untreated straw/manure was 0.293 dm^3/g raw material.

6.9 Anaerobic Digestion Versus Thermochemical Biofuel Production

Thermochemical processes are the other alternatives to biochemical methods for converting lignocellulosic materials into energy (Verma et al. 2012; McKendry 2002) Thermochemical conversion technologies have certain advantages and

drawbacks over biochemical conversion technologies. This section briefly describes the main thermochemical processes including combustion, pyrolysis, and gasification and compares to the anaerobic digestion process.

6.9.1 Thermochemical Conversions

6.9.1.1 Combustion

During the combustion of lignocellulosic biomass, heat is produced by chemical reaction, where carbon, hydrogen, oxygen, combustible sulfur, and nitrogen contained in biomass react with air or oxygen (Demirbas 2004). Currently, combustion is the most common technology converting biomass to usable heat energy is through straightforward combustion, and it accounts for around 90 % of all energy attained from biomass (Bhaskar et al. 2011). Combustion of lignocellulosic material consists of five main steps: drying, pyrolysis, gasification, char combustion, and gas-phase oxidation (Nussbaumer 2003). During the drying, the biomass first loses its moisture at temperatures up to 100 °C. Followed by pyrolysis and gasification steps, where the solid biomass chemically converted into fuel gases, volatile liquids, and a carbon-rich solid residue called char (Bhaskar et al. 2011). After all volatiles are removed, char combustion stage starts producing the fuel gases including hydrogen and carbon monoxide. Finally, in the gas-phase oxidation, the produced gases burn with oxygen from the air producing water vapor and carbon dioxide. It is worth to mention that only burning of the fuel gases generates heat, and solids and liquids do not burn themselves, but consume heat and energy during the beginning of the process. Currently, combustion is widely used on various scales to convert biomass into bioenergy; however, its efficiencies are the lowest among thermochemical processes (McKendry 2002; Demirbaş 2001).

6.9.1.2 Gasification

Gasification is an environmental-friendly way to produce energy from lignocellulose. The gasification conversion is taken place at temperatures of 500–1,300 °C in an oxygen-deprived environment (Goyal et al. 2008). The result of the gasification process is an energy-rich combustible gas mixture called producer gas which mainly consists of H_2, CO, and CH_4; however, it also contains impurities such as nitrogen, CO_2, sulfur, alkali compounds, and tars (Damartzis and Zabaniotou 2011). Tar is a complex mixture of hydrocarbons, which can condensate and form tar aerosols and polymers causing problems in the process equipment as well as it damages engines and turbines (Meng et al. 2011; Chiang et al. 2013). Temperature has a significant role in the destruction and reforming of tar and it influences the gas yield (Kumar et al. 2009; Narvaez et al. 1996; González et al. 2008; Gupta and Cichonski 2007). Among all thermochemical processes, gasification is one of the

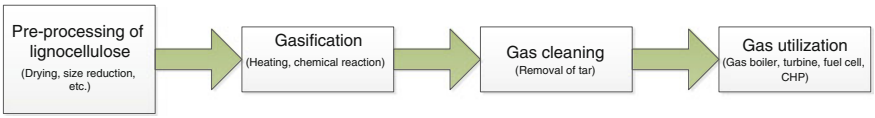

Fig. 6.8 Schematic figure of gasification of lignocellulosic biomass

promising, since the conversion efficiency is relatively high. Lignocellulosic biomass has an especially low sulfur content which is a major advantage when SO_2 emission is taken into account (Basu 2013). The main steps involved in the gasification process of lignocellulosic biomass are shown in Fig. 6.8.

6.9.1.3 Pyrolysis

Pyrolysis is the third basic thermochemical process for converting biomass to a more useful fuel. In respect of combustion and gasification during pyrolysis, biomass is heated in the absence of oxygen or with such a limited oxygen supply that gasification does not occur to an appreciable extent. The results of the pyrolysis are hydrocarbon-rich gas mixture, an oil-like liquid, and a carbon-rich solid residue. Usually, pyrolysis is optimized prior to maximize the liquid fuel yield. Fuel type, temperature, pressure, and heating rate affect the quality and quantity of the formed products. In the case of pyrolysis of lignocellulosic biomass, a considerable amount of carbon monoxide and carbon dioxide is formed due to the high oxygen content of the fuel. Fast pyrolysis conducted at temperatures between 400 and 550 °C and lasted for 0.5–3 s with small biomass particle size (up to 2 mm) results in high liquid production (Meier and Faix 1999). Pyrolysis at lower temperatures (250–350 °C) with long residence time (few minutes to hours) and larger particle size favors the production of solid char (Kersten and Garcia-Perez 2013; Verma et al. 2012). Among lignocellulosic materials forest residue, sawdust and straw are the most common feedstocks (Mohan et al. 2006). Pyrolysis produces energy fuels with high fuel-to-feed ratios, making it the most efficient process for biomass conversion; however, because of some problems related to the conversion process and poor thermal stability and corrosively of the products, pyrolysis technology is currently still at pilot stage (Verma et al. 2012).

6.9.2 Comparison of Anaerobic Digestion and Thermochemical Conversion Processes

As it mentioned in the previous sections, the main products of AD process are biogas and digestate. Typically, the biogas used for generation of heat and electricity or it is upgraded to biomethane and used as a biofuel in the public transportation sector or

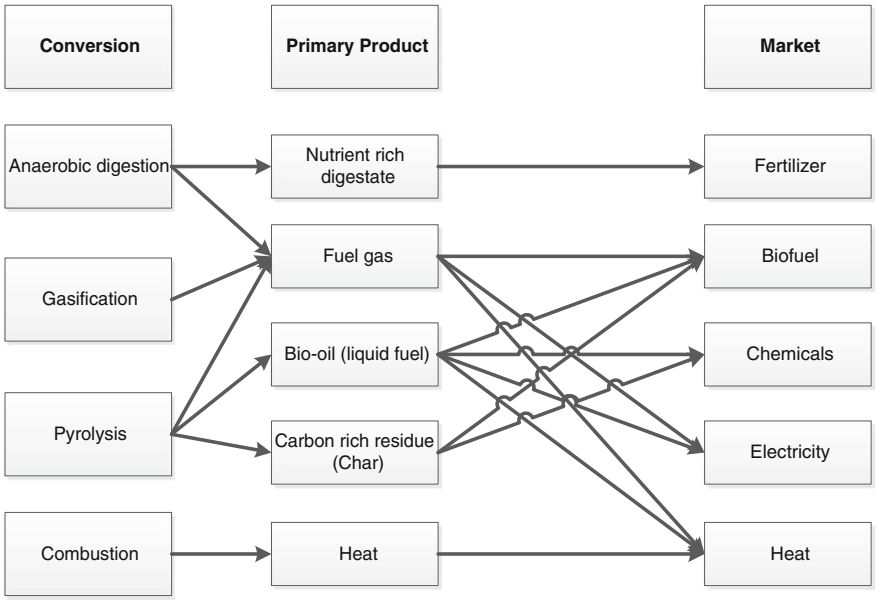

Fig. 6.9 Main conversion processes applied on lignocellulosic biomass together with their primary products and market

injected to the national gas grid, while the digestate is applied on farms as fertilizer (Kabir et al. 2013b; Forgács et al. 2014). Thermochemical conversion has multiple products including gases, liquids, and solids, which can be converted to a variety of fuels (H_2, Fischer-Tropsch (FT) diesels, and synthetic gasoline) and chemicals (methanol, urea). Figure 6.9 summarizes the main conversion processes applied on lignocellulosic materials including their primary products and market.

Anaerobic digestion of lignocellulosic biomass is a completely sustainable waste management technology which beside the production of biogas can considerably reduces the GHGs emission. It also allows almost complete nutrient and water recovery through the application of the digestate as fertilizer. However, the process efficiency greatly depends on the type of the lignocellulose, and in many cases, pretreatment is needed to improve the productivity. In contrast, thermochemical conversion can be effectively applied on any types of lignocellosic biomass, and generally it has a higher productivity due to the nature of the chemical reaction and the fact that it completely utilizes the lignocellulose except its inorganic fraction (ash). The major drawbacks are the high cost associated with cleaning of the product gas from the unwanted chemicals such as tar and alkali compounds and the inefficiency of the process due to the application of elevated temperature. Table 6.9 compares anaerobic digestion process with combustion, gasification, and pyrolysis.

Table 6.9 Comparison of AD process with combustion, gasification, and pyrolysis

	Anaerobic digestion	Combustion	Gasification	Pyrolysis
Technology status	Commercial	Commercial	Commercial	Demonstration
Preprocessing step	Not essential, but can help	Not essential	Necessary	Necessary
Temperature (°C)	Low 35–55	Very high 700–1,400	Very high 500–1,300	High 380–550
Sustainable	Yes carbon neutral	No fertilizer loss	No fertilizer loss	No fertilizer loss
Environmental impact	Positive GHGs mitigation	Negative toxic ash	Neutral pollutants locked in slag	Negative toxic ash
Energy recovery	Depends on the type of lignocellulose	High energy loss	High energy loss	High energy loss
Water recovery (%)	100	0	0	0
Nutrient recovery	All nutrients recovery possible	Some P and K N loss	Some P and K N loss	Some P and K N loss

6.10 Concluding Notes

Anaerobic digestion is an effective biological process for treating a broad range of biodegradable feedstocks for biogas production. However, the efficiency of the entire process is greatly dependent on the type of feedstock. For instance, digestion of manure is easier than digestion of other lignocellulosic biomass, such as wood and straw. Lignocelluloses are the building blocks of all plants with high carbohydrate content. Their worldwide availability makes them an attractive feedstock for biogas production. However, the arrangements of the components of lignocelluloses, i.e., cellulose, hemicellulose, and lignin, have a profound effect on lignocellulose tertiary structure. These complex associations create physical and chemical hindrances to lignocellulose biodegradation in natural and man-made environments.

Therefore, to achieve a stable and cost-efficient methane production from lignocelluloses, the following developments can be pursued:

1. Adjustment of the carbon/nitrogen ratio with co-digestion with other nitrogen-rich substrate
2. Addition of macronutrients and other trace metals
3. Integration of effective pretreatments on the feedstock prior to anaerobic digestion

Even though there have been so many studies available on investigations of the biodegradability of lignocelluloses for biogas production, more detailed research is needed in the future to emphasize on the following:

- Development of new and cost-effective pretreatments that are suitable for AD processes
- Collection of techno-economic data for AD systems that adopt biomass pretreatment processes
- Combination of AD with other biofuel processes such as bioethanol, biohydrogen, or biobutanol to obtain a more energy-efficient biorefinery process.

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Chapter 7
Biohydrogen from Lignocellulosic Wastes

Hamid Zilouei and Mohsen Taherdanak

Abstract Hydrogen has a high potential of being renewable and environmentally friendly alternative for the future energy carriers. Dark fermentative biohydrogen production has been received considerable attention because of the potential utilization of a wide variety of carbohydrate-rich wastes, lower operational costs, higher efficiency, simpler control requirements, and considerable role in waste reduction. Different types of biomasses, e.g., lignocellulosic wastes, have been used as feedstocks for this purpose. Biohydrogen production using dark fermentation can be performed by either pure cultures or anaerobic microbial consortia. The higher efficiency of hydrogen generation through control and reducing by-products is the main advantages of using pure cultures. However, mixed anaerobic consortia are usually preferred because of potential expression of a wide range of hydrolytic activities to enhance substrate utilization especially for complex lignocellulosic compounds, no need to medium sterilization, simpler control and operation, and more robust to changes in the environmental conditions such as pH and temperature. Therefore, anaerobic cultures from different sources have been tested as inocula for hydrogen fermentation from lignocellulosic wastes. However, manipulation and modification of the microbial community of anaerobic cultures toward reducing or even inhibiting the reactions of hydrogen consumption and by-products formation is the primary and necessary step. Moreover, it has been concluded from the literatures that there is no consistent procedure for microbial pretreatment and it should be checked based on the sources of inoculum and types of the substrate. Dark fermentative hydrogen production is influenced by several different factors, including type and source of inoculum, environmental parameters (e.g., temperature, pH, and partial pressure of hydrogen), and metal ions. This chapter reviewed and discussed different basic and applied aspects of biohydrogen production.

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7.1 Hydrogen: The Cleanest Energy Carrier

Hydrogen, an environmentally safe renewable energy, can be regarded as an excellent candidate for future energy carriers (Elbeshbishy 2011; Kapdan and Kargi 2006). It has the highest energy content per unit weight of any known fuel (142 kJ/g), which is approximately 2.7 times greater than that for hydrocarbon fuels. It should be noted that hydrogen produces only energy and water when it is combusted as a fuel or converted to electricity. CO_2 emissions from fuels depend primarily on their carbon content as well as their hydrogen/carbon ratio (Lam and lee 2013). Therefore, the utilization of hydrogen to provide energy can play a significant role in the reduction of greenhouse gas emissions. Moreover, new regulations for desulfurization of transportation fuels and growth in the consumption of transportation fuels have stimulated researchers to produce hydrogen as an alternative fuel. Hydrogen is considered as a clean, environmentally friendly, and attractive energy carrier by its potentially higher efficiency of conversion, low generation of pollutants, high energy density, and its sustainability (Sinha and Pandey 2011; Mohan and Pandey 2013).

It has been reported that 50 million tons of hydrogen is traded annually worldwide with a growth rate of 10 % per year. The demand for hydrogen is expected to grow exponentially by its entrance into the transportation sector. About 40 million tons of hydrogen per year is expected to be required to fuel about 100 million fuel cell-powered cars after full market utilization (Clinton and Scott 2004).

Despite its attraction in terms of energy content, environmental benefits, and utilization technology, hydrogen constitutes only about 3 % of the world's total energy consumption. Based on the national hydrogen program of the USA, the contribution of hydrogen to total energy market will be 8–10 % by 2025. It has been reported by the US department of energy that hydrogen power and transport systems will be available in all regions of this country by the year 2040 (Levin and Azbar 2012; Kapdan and Kargi 2006).

7.2 Biological Hydrogen Production

The industrial and global production of hydrogen is for the most part (approximately 96 %) from fossil sources through the conventional steam reforming of natural gas or methane, partial oxidation of fossil fuels, and as a by-product of some industrial processes. These are all energy intensive processes requiring high pressures and temperatures (>850 °C) (Kapdan and Kargi 2006). Hydrogen production from fossil fuels is usually associated with the production of high levels of greenhouse gases such as CO_2 and CH_4. For example, methane steam reforming and coal gasification yield 0.25 and 0.83 mol CO_2 per each mole of the produced H_2, respectively (Nath and Das 2011). On the other hand, biological processes are carried out at ambient temperatures and atmospheric pressures, thereby being less energy intensive than chemical and electrochemical ones. Therefore, based on the sustainable development and waste

minimization purposes, biohydrogen production from renewable resources seems to be a more promising energy carrier and it has received considerable attention in recent years (Azwar et al. 2014; Cheng et al. 2012).

Biological hydrogen production processes are usually classified into light-independent fermentation and photosynthetic processes (Fig. 7.1). The photobiological processes are classified into either a photosynthetic or carbon-source-dependent fermentation process (Zaborsky 1997). *Cyanobacteria* and microalgae produce hydrogen in the presence of light and water utilizing CO_2 as a carbon source (Eqs. 7.1–7.3):

$$2H_2O + \text{light energy} \rightarrow 4H^+ + O_2 \tag{7.1}$$

$$2H^+ + 2Fd_{red} + 4ATP \rightarrow H_2 + 2Fd_{ox} + 4ADP + 4P \tag{7.2}$$

$$2H^+ + 2e^- \rightarrow H_2 \tag{7.3}$$

This process requires only water and sunshine, making it attractive from the viewpoint of environmental protection. However, these systems have several drawbacks including low rates of hydrogen production by organisms, the need for a carrier gas to collect the evolved hydrogen gas from the culture, and separation of the produced hydrogen from oxygen and nitrogen (Mohan and Pandey 2013; Yue et al. 2014). Photosynthetic bacteria produce hydrogen through photofermentation by consuming a wide variety of inorganic and organic substrates in the presence of light (Eq. 7.4):

$$\text{Glucose} + 6H_2O \rightarrow 24H^+ + 6CO_2 + 24e^- \rightarrow 12H_2 + 6CO_2 \tag{7.4}$$

There are various kinds of photosynthetic bacteria and many different types of organic substrates, e.g., fatty acids, sugars, starch, and cellulose. As photosynthetic bacteria completely decompose the organic substances, photosynthetic processes of hydrogen production are environmentally advantageous due to their organic waste treatment.

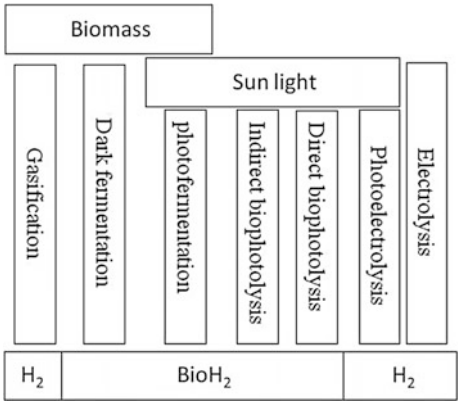

Fig. 7.1 Different methods for biological production of hydrogen

In dark fermentation process, anaerobic microorganisms produce hydrogen from organic substrates (Eqs. 7.5–7.6), together with volatile fatty acids (VFA) and CO_2:

$$\text{Glucose} + 2H_2O \rightarrow 2\text{Acetae} + 2CO_2 + 4H_2 \tag{7.5}$$

$$\text{Glucose} \rightarrow \text{Butyrae} + 2CO_2 + 2H_2 \tag{7.6}$$

Although fermentative bacteria degrade organic substrates as the initial step, this decomposition is incomplete. So the remaining organic substance (acetic acid) is considered as a by-product of anaerobic fermentation. This process could be suitable as the initial step of wastewater treatment along with hydrogen production (Wang and Wan 2009a; Gupta et al. 2014). While direct and indirect photolysis systems produce pure H_2, dark fermentation processes yield a mixed biogas primarily containing H_2 and carbon dioxide (CO_2), but they may also have minor amounts of methane (CH_4), CO, and/or hydrogen sulfide (H_2S) (Levin et al. 2004).

7.3 Dark Fermentation of Hydrogen Production

The efficiency of photosynthetic hydrogen production is low, and it cannot be operated in the absence of light. This makes it necessary to utilize high cost surface reactors, while fermentative hydrogen production can produce hydrogen all day long without light using different kinds of substrates such as organic wastes. Dark fermentation is a promising alternative to light-dependent processes, particularly when waste biomass is used as a feedstock for the generation of H_2. Since fermentation does not need a constant light supply, it can be run continuously using inexpensive and commercially used processes. Dark biohydrogen fermentation has higher hydrogen production efficiency and stability, simpler control requirements, lower operating costs, and more feasible for industrial production. In addition, it is of great value to produce hydrogen from various organic and carbohydrate-rich wastes by fermentative hydrogen production, because it plays the dual role of waste reduction and sustainable low-cost biohydrogen energy production. Therefore, dark fermentative hydrogen production appears to be the most favorable process which has received considerable attention in recent years (Hallenbeck 2012; Kuan-Yeow and Duu-Jong 2013).

7.4 Microbiology of Dark Fermentative Hydrogen Production

Dark fermentative production of hydrogen is often expressed with anaerobic digestion of organic compounds to biogas (Tan et al. 2011). Anaerobic digestion is characterized by diverse ecology and complex synergy of microorganisms so that the interrelated microbial conversions of hydrolysis, acidogenesis, acetogenesis,

and methanogenesis can be performed. The final production of carbon dioxide and methane through the intermediates of hydrogen and volatile fatty acids from complex carbohydrates is a summary of mass flow through anaerobic digestion (Fig. 7.2) (Chandra et al. 2012; Mohan et al. 2013). More details of this process are presented in Chap. 6.

Complex compounds such as cellulose, proteins, and fats are broken down into their fragments using hydrolytic exoenzymes of facultative and obligate anaerobic bacteria. The hydrolysis of carbohydrates takes place within a few hours, while that of proteins and lipids may take a few days. The degradation of lignocellulose and lignin is slow and incomplete too (Sinha and Pandey 2014). The products of hydrolysis phase are fermented by different facultative and obligate acidogenic bacteria in the second phase, generating a mixture of low molecular weight organic acids (e.g., butyric acid, propionic acid, acetate, and acetic acid), with hydrogen production as a key intermediate (Fig. 7.2) (Mohan and Pandey 2013; Motte et al. 2014). The products of acidogenic phase serve as a substrate in the third phase *via* the reversible interconversion of H_2 and CO_2 to produce acetate by acetogens and homoacetogens. During this phase, organic acids and alcohols are converted into acetate. Finally, acetate is used as a substrate by the conversion of acetoclastic methanogens to CH_4 and CO_2 through methanogenesis. Acetogenic bacteria grow in a syntrophic relationship with H_2 consuming methanogens to maintain a low H_2 partial pressure in order to allow acidogenesis to become thermodynamically favorable. The accumulation of hydrogen and increasing H_2 partial pressure inhibits the activity of acetate-forming bacteria (Mohan and Pandey 2013). The methane formation by methanogenesis takes place under strict anaerobic conditions in an

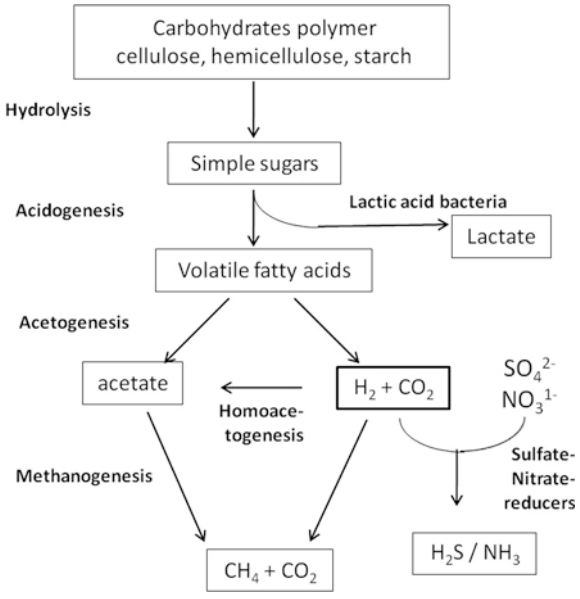

Fig. 7.2 Microbial pathways in the ecosystem of anaerobic dark fermentation of carbohydrates

exergonic reaction. In anaerobic digestion, different by-products (hydrogen, volatile fatty acids (VFAs), and alcohols) are used as substrates for methanogenesis to convert into CH_4 as shown below (Chandra et al. 2012; Saady 2013):

$$\text{Acetoclastic methanogenesis} \qquad \text{acetate} \rightarrow CH_4 + CO_2 \tag{7.7}$$

$$\text{Hydrogenotrophic} \qquad H_2 + CO_2 \rightarrow CH_4 \tag{7.8}$$

$$\text{Methylotrophic methanogenesis} \qquad \text{methanol} \rightarrow CH_4 + H_2O \tag{7.9}$$

7.4.1 Hydrogen-Producing Bacteria

It has been mentioned in the previous section that hydrogen is an internal by-product of anaerobic digestion of organic matter to biogas. It means that some microorganisms produce H_2, while some others consume it. Therefore, it is necessary to understand and manipulate these groups.

A wide variety of bacteria are capable of producing hydrogen, including obligate anaerobes (clostridia), facultative anaerobes (*Escherichia coli* and *Enterobacter*), and aerobic microorganisms (*Alcaligenes* and *Bacillus*) (Show et al. 2012; Balachandar et al. 2013). The isolated and identified mesophilic H_2 producers mainly belong to facultative *Enterobactericeae* and strictly anaerobic *Clostridiaceae,* whereas most thermophiles relate to genus *Thermoanaerobacterium* (Lee et al. 2011). Strictly anaerobic bacteria are the most common class of bacteria for biohydrogen production. However, since facultative anaerobes can grow more easily and simply than the obligate ones, pure cultures of facultative anaerobes of genus *Enterobacteriaceae* have been used too (Chong et al. 2009; Show et al. 2012).

- **Obligate anaerobic hydrogen producers**
  Obligate anaerobic bacteria have shown a higher rate of hydrogen production compared to facultative anaerobes. Hydrogen is generated during the exponential phase of growth, while their metabolism is shifted to solvent production in the stationary phase. *Clostridium* sp. is a typical hydrogen producer capable of utilizing a wide range of carbohydrate-based feedstocks and a wide range of fermentation by-products such as acetate, butyrate, and organic solvents (Chong et al. 2009). *Clostridium butyricum*, *Clostridium acetobutyricum*, *Clostridium beijerinckii*, *C. thermolacticum*, *C. saccharoperbutylacetonicum*, *C. tyrobutyricum*, *C. thermocellum,* and *C. paraputrificum* are examples of anaerobic spore-forming hydrogen producers. Usually, a mixture of products is obtained using *Clostridia,* in which the yield of hydrogen production is proportional to butyrate/acetate ratio. For example, the yield of produced hydrogen using different species of *Clostridium* has been reported to be between 1.5 and 2.8 mol per each mol glucose (Zaborsky 1997; Pandey et al. 2013).

- **Facultative anaerobic hydrogen producers**
  Enterobacter sp. is the most common facultative anaerobe which is able to generate hydrogen. Since facultative anaerobes can grow more easily than the obligate ones, attempts have been made to work with the pure cultures of genus *Enterobacteriaceae*. As a result, some bacterial strains of genus *Enterobacteriaceae* have been isolated and used for hydrogen production, including *Enterobacter aerogenes* strain E. 82005, *E. aerogenes* strain HO 39, *E. aerogenes* HU-101 strain AY2, and *E. cloacae* IIT-BT 08 (Lee et al. 2011; Chong et al. 2009). Members of *Enterobacteriaceae* have been shown as a favorable hydrogen producer because of their several advantages over the obligate anaerobes. The most important one is that they are not sensitive to the presence of trace oxygen, and therefore, they are easier to handle. Moreover, they can sustain higher concentrations of hydrogen as higher H_2 partial pressure (Pandey et al. 2013).
- **Co-culture of anaerobic hydrogen producers**
  Cultivation of anaerobic bacteria is difficult as their growth and activity are inhibited in the presence of trace amounts of oxygen. *Clostridia* can be regarded as a kind of strict anaerobic hydrogen producer extremely sensitive to oxygen, and hydrogen production is completely stopped in the presence of trace oxygen. In other words, the rate of hydrogen production by obligate anaerobes (*Clostridium* sp.) is about 2 mol H_2 per each mol of glucose, while it is 1 mol H_2 per each mol of glucose by facultative anaerobes (*Enterobacter* sp.). Therefore, in a co-culture of these bacteria, dissolved oxygen will be first consumed by facultative anaerobic *E. aerogenes,* leading to some anaerobic condition favorable for obligate anaerobic *C. butyricum* and accelerating the start-up of the process. Therefore, a hydrogen yield of up to 2 mol H_2 per each mol of glucose can be obtained without the addition of any reducing agent (Chong et al. 2009; Lee et al. 2011).
- **Thermophilic anaerobic hydrogen producers**
  Hydrogen production at high temperatures, using thermophilic or hyperthermophilic bacteria, has received growing attention due to some useful properties of these microorganisms. These H_2 producing bacteria are less sensitive to contaminations by undesirable compounds, prevent contamination by hydrogen-consuming bacteria, and better resistance against high hydrogen partial pressures, which can be regarded as an inhibitor for fermentative H_2 production (Ntaikou et al. 2010; Chong et al. 2009). However, hyperthermophilic bacteria exhibit much lower production rates of hydrogen, compared to mesophiles, due to their slow-growing characteristics (Lee et al. 2011).
  Different thermophiles have been isolated and identified as hydrogen producers, including *Caldicellulosiruptor saccharolyticus*, *Thermotoga* sp., *T. maritime*, *T. neapolitana, T. elfii*, and *Thermoanaerobacterium* sp. such as *T. thermosaccharolyticum* PSU-2. *Thermoanaerobacter thermohydrosulfuricus*, isolated from hot spring environments, has shown the potential of producing hydrogen and ethanol from biomass and organic wastes. *Caldoanaerobacter subterraneus*, as a

hydrogen-producing thermophile, was found to be dominant in an extreme thermophilic microflora enriched with cow manure (Pandey et al. 2013; Chong et al. 2009).

7.4.2 Hydrogen-Consuming Bacteria

In a mixed anaerobic fermentation culture, H_2 is consumed by some groups of bacteria as it is produced by some other groups in the consortia, and therefore, the yield of hydrogen production is reduced. The main groups of hydrogen-consuming bacteria have been identified as methanogenic archaea, sulfate-, nitrate- and iron-reducing bacteria, lactic acid bacteria, and homoacetogenic bacteria which produce propionate, lactate, butyrate, valerate, and alcohols by consuming H_2 as an electron donor (Lee et al. 2011; Guo et al. 2010; Saady 2013).

- **Homoacetogenic H_2-consuming bacteria**
  Homoacetogenic bacteria are strict anaerobic and fast-growing microorganisms which catalyze the formation of acetate by consuming H_2 and CO_2. They can convert different substrates to acetate as a major end product. Therefore, it means that the measured hydrogen production may be lower than the expected value calculated from the accumulation of acetate. Homoacetogenic bacteria belonging to *Acetobacterium*, *Butyribacterium*, *Clostridium*, *Eubacterium*, *Peptostreptococcus*, and *Sporomusa* have been widely reported (Guo et al. 2010; Saady 2013). Homoacetogens have the ability to switch between various substrates, to grow autotrophically using H_2 and CO_2, to use organic compounds heterotrophically (e.g., sugars, alcohols, and methanol) as energy and carbon sources, or even to utilize them simultaneously, producing acetate, lactose, succinate, and ethanol. These properties enable them to persist in any anaerobic environments and compete for H_2 consumption. Since some homoacetogenic bacteria are spore forming and belong to the same genus of *Clostridium*, heat treatment of the mixed culture is not suitable to remove them from the anaerobic community to prepare inoculum. Therefore, the use of some operating parameters such as CO_2 removal from the headspace is likely to improve biohydrogen production yield (Guo et al. 2010; Saady 2013).
- **Methanogenic H_2-consuming bacteria**
  Methanogens, as mentioned in the previous sections, are the main hydrogen-consuming microorganisms in the mixed culture dark fermentation (Eq. 7.10). However, methanogenic bacteria do not form spore at high temperatures and cannot survive in such conditions. Therefore, fortunately, for the purpose of hydrogen production, the treatment of inoculum by heating the medium for a short time (for example, 100 °C for a few minutes) may enrich spore-forming hydrogen-producing bacteria by inhibiting methanogenesis. Moreover, since most methanogens can only grow at a narrow pH range from 6 to 8, pH treatment is also a useful way to inhibit them in the mixed anaerobic

fermentation culture. In the continuous mode, application of short HRT leads to washing these microorganisms out from the reactor because of their low growth rate (Guo et al. 2010).

$$\text{Methanogenic: } 4H_2 + CO_2 \rightarrow CH_4 + 2H_2O \quad \Delta G = -135.0\ \text{kJ} \tag{7.10}$$

$$\text{Homoacetogenic: } 4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O \quad \Delta G'^0 = -104.0\,\text{kJ} \tag{7.11}$$

$$\text{Sulfate reducing: } 4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4H_2O \quad \Delta G'^0 = -152.2\,\text{kJ} \tag{7.12}$$

- **Sulfate-reducing of H_2-consuming bacteria**
  Sulfate-reducing bacteria (SRB) consume H_2 as an electron donor in the presence of sulfate or nitrate as an electron acceptor in a thermodynamically efficient reaction, even at the very low hydrogen concentration of only 0.02 ppm.
  Based on a thermodynamic viewpoint, sulfate-reducing bacteria are advantageous over homoacetogenic and methanogenic bacteria (Eqs. 7.10–7.12). These microorganisms can grow on a variety of fermentative by-products as the electron donor. In the absence of sulfate, some groups such as *Desulfococcus*, *Desulfosarcina,* and *Desulfobotulus* have been reported to be very competitive for hydrogen in which they use substrate level phosphorylation for growth through interspecies H_2 transfer (Guo et al. 2010; Saady 2013). In order to inhibit the activity of SRB, application of pH values lower than 6 has been reported as a significant kind of treatment.
- **Lactic acid bacteria as H_2 consumers**
  Lactic acid bacteria (LAB) have been recognized by their potential in inhibiting H_2 production through dark fermentation. Different species of LAB which are common in dark fermentation mixed cultures, including *Lactobacillus paracasei*, *Lactobacillus ferintoshensis,* and *Enterococcus durans,* inhibit H_2 production via the secretion of bacteriocins. Increasing lactic acid concentration from 2.3 to 4.4 g/L in the medium has caused approximately 30 % reduction in the yield of H_2 production. It has been reported that lactic acid bacteria can be dominant under mesophilic culture and also high organic loading. Therefore, temperatures higher than 50 °C and low organic loading may inhibit their presence in the mixed cultures (Saady 2013; Guo et al. 2010).
  Based on the above sections, in order to produce and increase the yield of H_2 as a final product through using the mixed dark fermentative culture, H_2-consuming microorganisms and reactions should be eliminated or inhibited. To reach this purpose, different strategies for the manipulation of microbial diversity and operational parameters (medium composition, pH, temperature, and organic loading) have been proposed. Elimination of sulfate, nitrate, and iron from the fermentation medium can inhibit the use of H_2 as an electron donor. Removal of the headspace CO_2 can increase the yield of H_2 through the

elimination of the reactions of homoacetogens and hydrogenotrophic methanogens (Eqs. 7.11–7.12). However, it should be mentioned that the manipulation of the microbial diversity and culture medium may affect the microbial ecology of anaerobic digestion. The inhibition of acetogens causes the accumulation of VFAs, while the inhibition of methanogens leads to the accumulation of acetate (Azwar et al. 2014).

7.4.3 Hydrogen Production Improvement Through Microbial Diversity Modification

Dark fermentative hydrogen production can be carried out using either pure cultures of selected hydrogen producers or mixed anaerobic cultures. The main advantages of using pure cultures are the substrate selectivity, metabolism manipulation, higher yields of hydrogen through reducing the by-products, and the repeatability of the process. By using sorghum extract as the feedstock for fermentative hydrogen production, yields of hydrogen with the mixed culture and the pure culture of *Ruminococcus albus* were reported to be 0.86 and 2.6 mol H_2 for each mol of glucose, respectively (Lee et al. 2011). Table 7.1 represents the capabilities of hydrogen production using different pure cultures of H_2 producers. According to Table 7.1, species of genus *Clostridium*, which are obligate anaerobes, gram-positive, endospore former, and rod-shaped, have been widely used as the pure culture inoculum (Wang and Wan 2009a). However, using the pure culture for H_2 production is very sensitive to contamination with hydrogen-consuming bacteria, and therefore, their utilization should be in the presence of aseptic conditions as they significantly increase the energy requirements and the overall cost of the process (Ntaikou et al. 2010; Show et al. 2012).

Mixed anaerobic consortia are usually preferred when a complex material, such as sewage sludge, solid waste, or lignocellulosic compounds, is going to be used as the feedstock for hydrogen production. One of the most important advantages of using mixed cultures is that they usually express a wide range of hydrolytic activities and enhance substrate utilization. Therefore, feedstocks containing a variety of biodegradable organic compounds as well as complex polymeric substrates can be used efficiently for H_2 production. No need for medium sterilization, especially in large-scale processes, is another advantage of using the mixed culture, which improve the control and operation and reduce the overall cost of the process. Furthermore, higher volumetric hydrogen production rates can be obtained in the continuous mode. Self-immobilized granules are usually obtained when the reactors are seeded with the mixed culture, giving high volumetric H_2 production rates as a result of biofilm performance (Ntaikou et al. 2010; Pandey et al. 2013). Moreover, mixed cultures are potentially more robust to the changes in environmental conditions such as pH and temperature (Pandey et al. 2013; Show et al. 2012).

Table 7.1 Anaerobic fermentative hydrogen production using pure microorganisms

Inoculum	Substrate	Yield (mol H_2/mol hexose)	References
Escherichia coli NCIMB 11943	Starch hydrolysate	1.8	Lee et al. (2011)
Escherichia coli BL-21 (recombinant)	Glucose	3.2	Das (2009)
Enterobacter aerogenes HU-101 AY2	Glucose	1.2	Lee et al. (2011)
Enterobacter aerogenes	Starch	1.1	Kapdan and Kargi (2006)
Enterobacter cloacae IIT-BT08	Starch-based distillery effluent	7.4[b]	Mishra and Das (2014)
Enterobacter cloacae IIT-BT08	Xylose	0.95[c]	Ren et al. (2009)
Enterobacter cloacae IIT-BT08	Potato starch	0.20	Lee et al. (2011)
Rhodopseudomonas palustris P4	Glucose	2.76	Lee et al. (2011)
Bacillus coagulans IIT-BT 08	Glucose	2.3	Das (2009)
Citrobacter sp. Y19	Glucose	2.49	Lee et al. (2011)
Clostridium sp. YM1	Glucose	1.7	Abdeshahian et al. (2014)
Clostridium butyricum	Glucose	2.2	Beckers et al. (2013)
C. amygdalinum C9	Xylan	0.04	Lee et al. (2011)
C. amygdalinum C9	Xylose	2.2	Lee et al. (2011)
C. amygdalinum C9	Starch	390[d]	Lee et al. (2011)
C. acetobutylicum M121	Glucose	2.29	Chong et al. (2009)
C. paraputrificum M-21	Glucose	1.1	Lee et al. (2011)
C. tyrobutyricum FYa102	Glucose	1.47	Chong et al. (2009)
C. thermolaiticum DSM2910	Lactose	1.5	Lee et al. (2011)
C. bifermentans	Wastewater sludge	2.1[b]	Lee et al. (2011)
C. beijerinckii L9	Glucose	2.81	Chong et al. (2009)
Clostridium sp. DMHC-10	Distillery wastewater	3.35	Lee et al. (2011)
Klebsiella sp. TR17	Crude glycerol	44.27[e]	Chookaew et al. (2014)
Halanaerobium saccharolyticum	Glycerol	0.58	Lee et al. (2011)

(continued)

Table 7.1 (continued)

Inoculum	Substrate	Yield (mol H_2/mol hexose)	References
Halanaerobium senegalensis	Glycerol	1.21	Lee et al. (2011)
Thermococcus kodakaraensis KOD1	Starch	3.33	(Lee et al. 2011)
Thermotoga maritime	Glucose	1.67	Cheng et al. (2011)
Thermotoga neapolitana	Glucose	1.84	Cheng et al. (2011)
Thermotoga neapolitana	Hydrolyzed potato steam peels	3.3	Lee et al. (2011)
Thermotoga elfii	Glucose	3.3	Ren et al. (2009)
Thermoanaerobacterium thermosaccharolyticum PSU-2	Sucrose	2.53[f]	Chong et al. (2009)
Caldicellulosiruptor saccharolyticus	Sucrose	3.33	Lee et al. (2011)
Caldicellulosiruptor saccharolyticus	Xylose	2.24[c]	Ren et al. (2009)
Caldicellulosiruptor saccharolyticus	Hydrolyzed potato steam peels	3.4	Lee et al. (2011)
Klebsiella oxytoca HP1	Sucrose	1.5[f]	Kapdan and Kargi (2006)
Enterobacter sp. CN1	Xylose	2.0[c]	Lee et al. (2011)
Ethanoligenens harbinense Yuan-3	Glucose	2.81	Ren et al. (2009)

[a] continuous
[b] mmol H_2/g COD
[c] mol H_2/mol Xylose
[d] ml H_2/g starch
[e] mmol H_2/mol glycerol
[f] mol H_2/mol sucrose

The mixed anaerobic fermentative inocula have been derived from a variety of different natural environments such as soil, sewage sludge, compost, animal manure, cow dung composts, cattle or dairy residue composts, and sludges from anaerobic digesters of different organic wastes and wastewaters (Pandey et al. 2013; Reith et al. 2003; Ntaikou et al. 2010; Guo et al. 2010). However, it has been shown that the origin of the inoculum can affect the overall performance of hydrogen production process. Moreover, acclimation and adaptation of the seed source is important to improve the anaerobic dark fermentation of hydrogen production (Manish and Banerjee 2008). However, the main limitation in using these types of consortia is the presence of hydrogen-consuming bacteria, such as methanogens, homoacetogens, and lactic acid bacteria, within their community, reducing the total yield of hydrogen production. Therefore, it is necessary to inhibit the activity of hydrogen-consuming bacteria or remove them from the culture (Ntaikou et al. 2010; Pandey et al. 2013).

In order to increase the hydrogen yield, the anaerobic mixed culture should be pretreated to suppress as much hydrogen-consuming bacterial activity as possible while preserving and enriching the activity of the desired hydrogen-producing bacteria (Chang et al. 2011). In fact, pretreatment is a way through which mixed cultures are treated under harsh conditions in which hydrogen-producing bacteria would have a better chance than some hydrogen-consuming bacteria to survive (Wang and Wan 2009a). Different pretreatment methods have been applied to enhance H_2 production including thermal pretreatment (heat shock), alkaline pretreatment, acidification, aeration, ultrasonic pretreatment, freezing and thawing, and chemical pretreatment (e.g., chloroform, sodium 2-bromoethanesulfonate, and iodopropane) (Mohan et al. 2008). Various pretreatment methods of microbial diversity have been applied to enhance hydrogen production yield of mixed anaerobic cultures as summarized in Table 7.2.

Heat-shock pretreatment is the most common method used by researchers. Heat-shock pretreatment eliminates non-spore-forming methanogenic bacteria from the mixed culture, while hydrogen-producing bacteria can form protective spores under such an extreme environment and survive these conditions. Temperature range of 75–121 °C and exposure time of 15–120 min have been used in the literature as thermal pretreatment of anaerobic mixed fermentative culture (Ren et al. 2008; Ntaikou et al. 2010). For this reason, using higher temperature for fermentative hydrogen production by the mixed culture usually avoids contamination by hydrogen-consuming bacteria (Guo et al. 2010). Heat-shock pretreatment is an efficient method that has also been used in combination with other methods to be mentioned in the next sections.

Acidic treatment at pH values of 2–4 has been successfully applied by different researchers for improving hydrogen-producing consortia. It has been shown that by increasing acid concentration, non-spore-former methanogens are more severely affected and H_2-producing *Clostridium* sp. can be selectively enriched. Therefore, for example, it has been found that inoculum of sludge pretreated with HCl at pH values of 2 and 3 results in hydrogen production volumes of 3.2 and 2.8 times higher, respectively, compared to that obtained without acid pretreatment.

Table 7.2 Different microbial pretreatments used to modify the anaerobic fermentative H_2 production community based on H_2 production

Inoculum source	Pretreatment methods studied	Substrate	Optimal pretreatment method	Yield	References
Digested sludge	Acid, base, heat shock, aeration, and chloroform	Glucose	Heat shock	1.8 mol H_2/mol glucose	Sinha and Pandey (2011)
Methanogenic granules	Acid, heat shock, and chloroform	Glucose	Chloroform	1.2 mol H_2/mol glucose	Sinha and Pandey (2011)
Digested sludge	Heat shock, aeration, acid, base, iodopropane, and 2-bromoethanesulfonic acid	Sucrose	Base	6.12 mol H_2/mol sucrose	Sinha and Pandey (2011)
Anaerobic sludge	Sodium 2-bromoethanesulfonate, acid, heat shock, and their four combinations	Dairy wastewater	Sodium 2-bromoethanesulfonate	0.0317 mmol H_2/g COD	Sinha and Pandey (2011)
Digested sludge	Heat shock, acid, base, aeration, chloroform	Glucose	Heat shock	221.50 ml H_2/g glucose	Dong et al. (2010)
Digested sludge	Acid, base, ionizing irradiation, and heat shock	Glucose	Ionizing irradiation	2.15	Yin et al. (2014)
Anaerobic sludge	Heat shock, methanogenic inhibitor, freezing and thawing, acid	Glucose	Acid	128.49 ml H_2/g glucose	Dong et al. (2010)
Excess sludge	Heat shock, acid, base, and aeration	Glucose	Aeration	149.67 ml H_2/g glucose	Song et al. (2012)
Cow dung compost	Infrared dryer, aeration, and soaking	Sucrose	Infrared dryer	146.50 ml H_2/g sucrose	Song et al. (2012)
Cow dung compost	Microwave radiation	Corn stalk	Microwave radiation	131.7 ml H_2/g corn stalk	Song et al. (2012)
Cattle manure	Forced-air pumping for 24 h at ambient temperature	Corn stalk	Aeration	129.2 ml H_2/g corn stalk	Song et al. (2012)
Cattle manure	Freezing and thawing, acid, heat shock, and sodium 2-bromoethanesulfonate	Glucose	Acid	1.0 mol H_2/mol glucose	Sinha and Pandey (2011)
Mixed culture		Glucose	Microwave radiation and acid	2.07 mol H_2/mol glucose	Faloye et al. (2014)
Mixed culture		Distillery wastewater	Ultrasonication	10.95 mmol/g COD	Gadhe et al. (2014)

Moreover, methane will not be produced from sludge pretreated at pH values less than 3 (Manish and Banerjee 2008). Combined heat-shock and acid-shock treatment of the microbial seed has also been used for the manipulation of hydrogen-producing microbial community. Alkaline pretreatment of sewage sludge at the pH of 11 has also been used to increase the yield of hydrogen through the enrichment of alkalophilic H_2 producers (Wu and Juan 2013). No consistent results have been obtained by different researchers, due to the source of seeds, types of organic constituents, and pretreatment exposure time and strength (Ren et al. 2008).

Aeration pretreatment has been reported as a method of enriching hydrogen-producing bacteria through the removal of H_2-consuming bacteria. However, H_2 consumers such as homoacetogens, sulfate-reducing bacteria, and lactic acid bacteria have been shown to tolerate the exposure to oxygen. Different aeration conditions, from complete aeration for 30–60 min to incomplete aeration for 5–7 days, have been used. Different aeration times and intensities have resulted in different hydrogen production yields (Wu and Juan 2013).

Combinations of different pretreatment methods have been applied and compared in different studies on different mixed bacterial seeds. Usually, unfortunately, no similar pattern has been obtained for different individual or combined pretreatment methods, depending on the sources of the seeds. Several different pretreatment methods have been applied on the sludge of municipal wastewater treatment plant, and the hydrogen yield efficiency has been obtained to be in the order of repeated aeration > heat shock > control > alkaline > acidic pretreatment (Ren et al. 2008). In another study, the efficiency of different pretreatment methods on anaerobic mixed fermentative H_2 production using dairy wastewater as the substrate was obtained to be in the following order: C > PH > PC > PHC > HC > control > H > P (C: chemical treatment, P: pH treatment, H: Heat treatment) (Mohan et al. 2008).

In the continuous mode, the hydraulic retention time (HRT) influences the hydrogen yield via impacts on the microbial community (Show et al. 2012). In fact, the HRT is able to reduce the diversity of microbial community through eliminating the propionate producers without any impact on the existence of dominant hydrogen-producing bacteria, thereby causing the increase in hydrogen yield. Higher dilution rates, in continuous fermentations, have been used to wash out the slow growing methanogens and therefore to enrich the hydrogen-producing bacteria (Reith et al. 2003).

7.5 Metabolism of Dark Fermentative Hydrogen Production

The majority of microbial hydrogen production is generated through the anaerobic metabolism of pyruvate formed during the catabolism of various organic substrates. Figure 7.3 represents a schematic illustration of hydrogen production mechanism

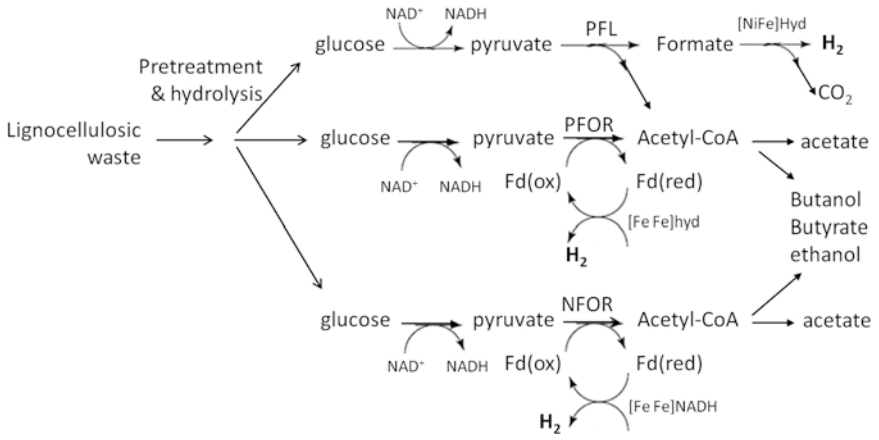

Fig. 7.3 Metabolic pathways leading to hydrogen production in anaerobic dark fermentation

during dark fermentation of the organic substrate. Pyruvate, as a central molecule of microbial fermentation, has a diverse fate under anaerobic fermentation based on the operating conditions. Pyruvate enters the acidogenic pathway and generates volatile fatty acids (VFA), mainly acetic acid, propionic acid, butyric acid, and malic acid, in association with the H_2 generation (Pandey et al. 2013). Three different biochemical reactions have been reported to involve in dark fermentation process, from pyruvate to molecular hydrogen.

Facultative anaerobes, typically *Enterobacteriaceae*, convert pyruvate to acetyl-CoA and formate (Eq. 7.13) in the presence of pyruvate formate-lyase (PFL) and under anaerobic condition. Then, H_2 and CO_2 are generated through break downing formate (Eq. 7.14) by a special class of [Ni–Fe] hydrogenase which is a part of formate hydrogen lyase (FHL) complex. Increasing the activity of this enzyme complex can be a means of increasing hydrogen production. This enzyme is induced upon media acidification, as it mainly serves to reduce acidity by removing formic acid. Therefore, the concentration of formate should be high enough to activate the FHL transcription (Wu and Juan 2013).

In obligate anaerobes, typically *Clostridium sp*., pyruvate is oxidized into acetyl-CoA and CO_2 (Eq. 7.15) through the reduction of ferredoxin (Fd) by pyruvate–-ferredoxin oxidoreductase (PFOR) (Reith et al. 2003). Then, the reduced ferredoxin (Fd(red)) is reoxidized, regenerating oxidized ferredoxin (Fd(ox)) by [Fe–Fe] hydrogenase (HydA), together with the release of electrons as the molecular hydrogen (Eq. 7.16). A variety of reduced products such as butyrate, butanol, ethanol, and acetone can be produced depending on the species and environmental conditions (Mohan et al. 2013).

The third type of biochemical reaction of H_2 production, typical in many thermophilic bacteria and several *Clostridium* species, is catalyzed by using two major enzymes, NADH–ferredoxin oxidoreductase (NFOR) and HydA. The oxidized ferredoxin (Fd(ox)) is reduced by NADH (Eq. 7.17), which is generated during

glycolysis and pyruvate formation. Then, molecular hydrogen is generated through the transfer of the electrons in reduced ferredoxin (Fd(red)) to protons using enzyme HydA (Eq. 7.18). However, under standard conditions, the reduction of hydrogenase by NADH is an energetically unfavorable reaction, proceeding at very low partial pressures of hydrogen. Usually, the NADH is used to drive the more energetically favorable formation of butyrate or butanol. Thus, a lower actual biohydrogen yield has been observed (Wu and Juan 2013).

$$\text{Pyruvate} + \text{CoA} \overset{\text{PFL}}{\rightarrow} \text{Acetyl} - \text{CoA} + \text{Formate} \tag{7.13}$$

$$\text{Formate} \overset{\text{FHL}}{\rightarrow} \text{H}_2 + \text{CO}_2 \tag{7.14}$$

$$\text{Pyruvate} + \text{CoA} + 2\text{Fd(ox)} \overset{\text{PFOR}}{\rightarrow} \text{Acetyl} - \text{CoA} + \text{CO}_2 + 2\text{Fd(red)} \tag{7.15}$$

$$2\text{H}^+ + 2\text{Fd(red)} \overset{\text{HydA}}{\rightarrow} \text{H}_2 + 2\text{Fd(ox)} \tag{7.16}$$

$$2\text{NADH} + 4\text{Fd(ox)} \overset{\text{NFOR}}{\rightarrow} 2\text{NAD}^+ + 4\text{Fd(red)} \tag{7.17}$$

$$4\text{H}^+ + 4\text{Fd(red)} \overset{\text{HydA}}{\rightarrow} 2\text{H}_2 + 2\text{Fd(ox)} \tag{7.18}$$

[Fe–Fe] hydrogenase and [Ni–Fe] hydrogenase are two important enzymes involved in microbial H_2 production. Both enzymes contain the complex metal clusters at their active site with diverse subunits. They catalyze the reduction of protons to H_2 by oxidizing a strong reductant, including natural electron carrier proteins known as ferredoxin. The nitrogenase enzyme contains two component protein systems, Mo–Fe protein and Fe protein, which are involved in the H_2 production process. Nitrogenases use Mg-ATP and electron to reduce a variety of substrates during H_2 generation. Dehydrogenase is another important enzyme involved in the interconversion of metabolites and the transfer of proton between metabolic intermediates through redox reactions using several mediators (NAD, FAD). Both dehydrogenase and hydrogenase functions are important in maintaining H^+ equilibrium in the cell and reducing them to H_2 (Mohan and Pandey 2013).

7.6 The Yield of Hydrogen Production

Carbohydrate-based feedstocks are the preferred substrate for fermentative hydrogen production and estimation of potential yields is mostly and commonly based on hexose conversions (Manish and Banerjee 2008). As previously mentioned, H_2 is produced as an intermediate by-product of acetate and butyrate formation (Fig. 7.4) (Levin et al. 2004; Saady 2013). Theoretically, when H_2-consuming microorganisms are completely inhibited, different yields of H_2 are obtained depending on the

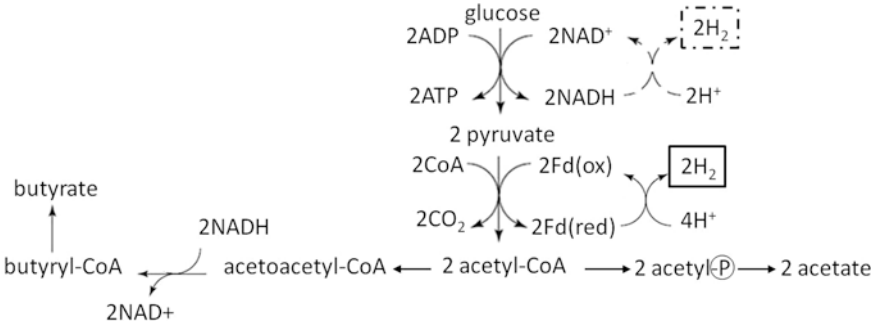

Fig. 7.4 Acetate and butyrate pathways for H_2 production

fermentation pathway and end products. A theoretical maximum yield of 4 mol H_2 per mole of glucose is expected if all substrate is converted to acetic acid (Eq. 7.19). If all substrate is converted to butyric acid, the maximum yield of 2 mol H_2 per more of glucose is expected (Eq. 7.20). Acetate formation is usually preferred because it regenerates the reducing equivalents, allowing the microorganism to synthesize energy currency of ATP; however, at high partial pressure of H_2, butyrate is produced to avoid the accumulation of inhibitory reducing equivalents (Saady 2013). Therefore, the yield of hydrogen from glucose can be determined by the ratio of butyrate/acetate produced during fermentation (Pandey et al. 2013; Lee et al. 2011). The highest theoretical yield of H_2 is associated with acetate as the fermentation end product (Levin et al. 2004). However, it should be noted that the accumulation of acetate in the medium is not an indicator of higher biohydrogen production yield since in some microbial species, acetate is produced from the conversion of hydrogen and carbon dioxide (Eq. 7.21) (Guo et al. 2010).

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 4H_2 + 2CO_2 \tag{7.19}$$

$$C_6H_{12}O_6 + 2H_2O \rightarrow CH_3CH_2CH_2COOH + 2H_2 + 2CO_2 \tag{7.20}$$

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \tag{7.21}$$

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2CH_3CH_2COOH + 2H_2O \tag{7.22}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2 \tag{7.23}$$

$$C_6H_{12}O_6 \rightarrow 2CH_3CHOHCOOH + 2CO_2 \tag{7.24}$$

The yield of hydrogen during dark fermentation is actually affected by the partial pressure of the product. At high H_2 partial pressures, a metabolic shift to the production of more reduced products, e.g., lactate or alanine, occurs, thereby decreasing the yield of H_2 (Fig. 7.4) (Reith et al. 2003). Therefore, high yields of H_2 are associated with a mixture of acetate and butyrate fermentation products, and low yields of H_2 are related to propionate and reduced end products such as alcohols

and lactic acid (Guo et al. 2010; Levin et al. 2004). No hydrogen is produced when ethanol, lactic acid, or propionic acid are the sole metabolic end product (Pandey et al. 2013). Propionate is a metabolite of a hydrogen-consuming pathway (Eq. 7.22), while ethanol (Eq. 7.23) and lactic acid (Eq. 7.24) are known to be involved in a zero-hydrogen-balance pathway (Azbar and Levin 2011).

In order to improve the H_2 production yield in dark fermentation, the production of H_2 from NADH/NADPH, directly or indirectly *via* Fd, has been suggested. However, this route is significantly affected by several factors, such as H^+ concentration, NADH/NAD ratio, H_2 partial pressure, and temperature. Because NADH/NADPH- or Fd-dependent hydrogenases are reversible, the forward reaction leading to H_2 formation is inhibited by H_2 accumulation. Compared to CH_4 fermentation, limited substrate availability is another problem encountered with dark H_2 fermentation. For example, lignocellulose and starch are not used readily for H_2 production by most H_2-producing bacteria. Moreover, some facultative anaerobes may carry out anaerobic respiration instead of fermentation using nitrate or fumarate as terminal electron acceptors. Therefore, in order to produce hydrogen, media should be empty of these electron acceptors (Pandey et al. 2013).

The complete oxidation of glucose to H_2 and CO_2 yields a stoichiometry of 12 mol H_2 per mole of glucose, gaining no metabolic energy by the cell. The maximum yield in dark fermentation is 4 mol H_2 for each mol of glucose, that is, only 33 % of the stoichiometric maximum. This low H_2 yield is linked to some microbial metabolism which requires sufficient energy to support microbial growth (Reith et al. 2003).

7.7 Feedstocks

Any organic substrate rich in carbohydrates, fats, or proteins could be theoretically considered as the possible substrate for biohydrogen production. However, from a thermodynamic point of view, the conversion of carbohydrates to hydrogen is preferred. Therefore, wastes and biomasses rich in sugars and/or complex carbohydrates are the most suitable feedstocks for dark fermentative hydrogen generation, while proteins and amino acids are less suitable, and lipids may be inappropriate. This has been confirmed by a study showing approximately 20 times higher hydrogen production potential of carbohydrate-rich waste (rice and potato) compared to fat-rich waste (fat meat and chicken skin) and protein-rich waste (egg and lean meat).

The major criteria that should be considered for the selection of suitable substrates for fermentative hydrogen production are carbohydrate content, availability, cost, and biodegradability. Although simple sugars such as glucose, sucrose, and lactose are readily biodegradable, and thus, preferred as model substrates for hydrogen production, pure carbohydrate sources are expensive raw materials for real scale hydrogen production. Different types of biomasses or wastes have been used as feedstocks for dark fermentative hydrogen production as reviewed below.

7.7.1 Energy Crops

Energy crops refer to certain plants cultivated solely for the further utilization of their biomass as the feedstock for energy production. It can be directly exploited for its energy content via combustion or transformed biologically to biofuels. In order to have sustainable and suitable feedstocks for hydrogen production via dark fermentation, energy crops should have high sugar and/or carbohydrates content, low lignin content, low production cost, and high biomass yield. Energy crops used for fermentative hydrogen production are divided into sugar-based crops (e.g., sweet sorghum, sugar cane, and sugar beet), starch-based crops (e.g., corn and wheat), lignocellulose-based crops (e.g., switch grass and fodder grass), and woody (e.g., miscanthus and poplar). A review of different types of energy crops was used for dark fermentative hydrogen production as summarized in Table 7.3. Although energy crops are quite sufficient for hydrogen generation, the food versus fuel conflict has led to an adverse response against the use of energy crops as the feedstocks for biofuels generation. Therefore, attention has been given to the use of alternative feedstocks not competitive to crops such as wastewaters, solid wastes, and lignocellulosic residues.

7.7.2 Wastewaters

Different types of wastewaters have been considered as suitable feedstocks for hydrogen production via dark fermentation. Food processing wastewaters, such as olive mill wastewater, rice winery, noodle, sugar and molasses manufacturing, olive pulp, and cheese whey, have been successfully tested for hydrogen production, and quite high yields of hydrogen have been achieved without any pretreatment. However, in most cases, the dilution of the raw wastewater has been performed in order to reduce the organic loading, which can be otherwise inhibitory for the goal of H_2 production. Moreover, it should be noted that such wastewaters can have a quite complex chemical composition, including different organic and inorganic substances with varying concentrations that may limit the reproducibility of the proposed process. Some examples of different wastewaters have been used for H_2 production as listed in Table 7.3.

Cheese and dairy wastewaters (rich in lactose up to 5 %) have been applied as the substrate for fermentative hydrogen production, yielding 2.7 mol H_2 for each mol lactose (Chang et al. 2011).

Palm oil mill effluent (POME) with high COD in the range of 70–100 kg COD/L is generated from the processing of fresh fruit bunch. These types of wastewaters with high COD and BOD content are the sufficient feedstock for fermentative hydrogen production. Possibility of hydrogen production from raw POME could be compared with the results of hydrogen production from carbohydrate-rich wastewaters (Chang et al. 2011).

Table 7.3 Different feedstocks used for fermentative H_2 production

Feedstock	Microorganism	Max. H_2 yield (mol H_2/mol hexose)	References
Glucose	*Clostridium butyricum*	2.2 mol H_2/molhexose	Beckers et al. (2013)
Glucose	Mixed culture	2.07 mol H_2/molhexose	Faloye et al. (2014)
Starch	*Clostridium* CGS2	2.0 mol H_2/molhexose	Pandey et al. (2013)
Starch	*E. aerogenes*	1.1 mol H_2/molhexose	Pandey et al. (2013)
Starch	*Thermoanaerobacterium*	92 ml/g starch	Pandey et al. (2013)
Starch	Mixed culture	0.6 mol/mol starch	Pandey et al. (2013)
Crude glycerol	*Klebsiella* sp. TR17	44.27 mmol H_2/mol Gly	Chookaew et al. (2014)
Cellulose	*Clostridium* sp.	2.2 mmol/g cellulose	Pandey et al. (2013)
Cellulose	Mixed culture	2.4 mol H_2/mol hexose	Pandey et al. (2013)
Sweet sorghum extract	*Ruminococcus albus*	2.61 mol H_2/molhexose	Ntaikou et al. (2010)
Wheat starch	Mixed cultures	1.26 mol H_2/molhexose	Ntaikou et al. (2010)
Sugar beet juice	Mixed cultures	1.9 mol H_2/molhexose	Ntaikou et al. (2010)
Corn starch	Mixed cultures	0.51 mol H_2/molhexose	Ntaikou et al. (2010)
Domestic sewage	Mixed cultures	6.0 mmol H_2/gCOD	Pandey et al. (2013)
Rice spent wash	Mixed cultures	464 ml H_2/g sugar	Pandey et al. (2013)
Rice winery WW	Mixed cultures	2.1 mol H_2/molhexose	Pandey et al. (2013)
Dairy waste	Mixed culture	1.1 mmol H_2/m^3/min	Pandey et al. (2013)
Cheese processing WW	Mixed culture	3.2 mmol H_2/gCOD	Pandey et al. (2013)
Cheese whey	*C. saccharoperbutylacetonicum*	2.7 mol H_2/mollactose	Wang and Wan (2009a)
Olive pulp water	Mixed culture	2.8 mol H_2/molhexose	Pandey et al. (2013)
POME	Mixed culture	0.4 l H_2/gCOD	Pandey et al. (2013)
Potato processing WW	Mixed culture	5.7 mmol H_2/gCOD	Pandey et al. (2013)
Food waste	Mixed culture	57 ml H_2/gVS	Pandey et al. (2013)
Olive mill wastewater	Mixed culture	0.5 mmol H_2/gCOD	Wang and Wan (2009b)
Brewery wastewater	Mixed culture	6.1 mmol H_2/gCOD	Wang and Wan (2009a)

Crude glycerol from the biodiesel production industry is another interesting example. The increase in the biodiesel production from vegetable oils and fats has nowadays led to the generation of large quantities that need to be disposed. However, the crude glycerol waste has been diluted to lower the organic loading and the salt concentrations, both of which seem to be inhibitory above a certain limit. Moreover, addition of nutrients, such as yeast extract and peptone, could significantly enhance the hydrogen-producing capacity of the microorganism from a particular waste; this means that the waste is poor in nitrogen source, which is necessary for microbial growth. The maximum hydrogen yield observed was 0.77 mol H_2 per mol glycerol, with ethanol being the dominant by-product.

7.7.3 Solid Waste

Complex solid wastes, such as food processing and kitchen wastes, municipal solid wastes, and waste-activated sludge, have been tested as the feedstocks for biological hydrogen production. Food wastes from the industry and household contain high levels of carbohydrates and proteins. The organic constituent, especially carbohydrate in food wastes, could be a potential substrate for anaerobic hydrogen production. Waste-activated sludge from wastewater treatment plants contains high levels of organic matter which can be a potential feedstock for hydrogen production (Wang and Wan 2009a). The organic fraction of municipal solid waste can also be promising as a potential feedstock for fermentative hydrogen production, since it can represent up to 70 % of the total MSW produced. It consists of paper (up to 40 %), garden residues, food wastes, and wood.

However, in order to use these types of feedstocks for fermentative hydrogen production, two steps are generally followed before the main process to prevent the additional costs in the overall process. The first step is the initial separation of the suitable substrates from the solid wastes (carbohydrates prefer proteins and fats). The second one is the appropriate pretreatment of feedstocks, such as physical, chemical, or biological pretreatment, in order to increase the availability and degradability of the substrate for fermentative hydrogen-producing microorganisms (Wang and Wan 2009a).

The biotransformation of solid wastes and wastewaters toward hydrogen can be considered quite attractive from both environmental (pollution control, renewable energy) and economical (resources recovery, low total cost of waste management) standpoints. Criteria according to which a solid waste or wastewater would be characterized as the efficient feedstock for hydrogen generation are a high concentration of degradable organic compounds, the high proportion of readily fermentable compounds such as sugars and carbohydrates, and the low concentration of inhibitory compounds to microbial activity.

7.7.4 Lignocellulosic Residues

Different types of lignocellulosic residues have been used as a potential renewable feedstock for fermentative hydrogen production, including agricultural residues such as sugarcane and sweet sorghum bagasse, corn stalks and stover, rice straw, wheat straw, and forestry residues. Food and Agriculture Organization reported the plentiful sources of lignocelluloses such as annual worldwide production of around 2,900 million tons of cereal crops, 1,600 million tons of pulse crops, and annual generation of 350–450 million tons of agricultural wastes in the USA. Hydrogen generated from such feedstocks has been characterized as 'second-generation hydrogen' since its production is not competitive to food chain (FAO November 2007; Limayem and Ricke 2012).

Although agricultural and forestry residues are abundant and almost zero cost feedstocks, they do not contain easily fermentable free sugars. They include complex carbohydrate polymers, i.e., cellulose and hemicellulose, which are tightly bonded to lignin and covered by it. Detailed description of lignocellulosic structure and composition is presented in the previous chapters. Cellulose and hemicellulose are renewable substrates for fermentative hydrogen production, while lignin is not degraded under anaerobic conditions. Lignin strongly inhibits the availability and utilization of cellulose and hemicellulose. Therefore, the bottleneck is the proper pretreatment process used to remove lignin, open up the crystalline structure of cellulose, and degrade cellulose to simple sugars. Hydrogen production from lignocellulosic materials could be a potential process with the help of pretreatment on materials and the use of cellulolytic microorganisms.

Delignification of lignocellulosic feedstocks is an important step in dark hydrogen fermentation. The most commonly applied methods include physicochemical treatment (steam explosion, acidification), enzymatic treatment, biological treatment, and the combination of mechanical (i.e., extrusion or milling) and chemical pretreatment (Table 7.4). Development of cost-effective pretreatment methods with a low energy demand is necessary to produce cheap feedstocks for dark hydrogen fermentation (Reith et al. 2003). No matter which method is employed, critical factors that should be considered to make this process economically viable are optimizing sugar recovery and minimizing process cost and environmental impact.

For the efficient hydrolysis of cellulosic materials, the bacterial cell should be adhered to the cellulose. During the hydrogen production phase, *Clostridium cellulolyticum* has been found to be in close contact with cellulose and bacterial cell has been seen to be released at the end of growth, thereby indicating the exhaustion of accessible cellulose (Chang et al. 2011). Even in the case cellulolytic microorganisms are used for fermentative hydrogen production, residues have to be subjected to some kind of pretreatment to achieve delignification and facilitate the subsequent liberation and uptake of sugars. In Table 7.4, different types of lignocellulosic residues used as feedstocks for hydrogen production are presented, along with the achieved hydrogen yields and rates.

Table 7.4 Lignocellulosic feedstocks together with pretreatment methods used for anaerobic dark fermentative H_2 production

Feedstock	Pretreatment	Microorganism	Yield*	References
Wood fibers	Mechanical	*Clostridium thermocellum*	1.47 mol/mol Hexose	Ntaikou et al. (2010)
Sugarcane bagasse hydrolysate	Acid thermal hydrolysis	*Clostridium butyricum*	1.73 mol/mol total sugar	Ntaikou et al. (2010)
Sweet sorghum residues	Mechanical	*Rumicococcusalbus*	2.59 mol/mol Hexose	Ntaikou et al. (2010)
Wheat straw	Mechanical	*Caldicellulosiruptor saccharolyticus*	3.8 mol/mol Hexose	Reith et al. (2003)
Maize leaves	Mechanical	*Caldicellulosiruptor saccharolyticus*	3.6 mol/mol Hexose	Ntaikou et al. (2010)
Corn stover	Acid thermal hydrolysis H_2SO_4 0.25–4 (v/v), 121 °C, 30–180 min	*Thermoanaerobacterium thermosaccharolyticum*	2.24 mol/mol Hexose	Ntaikou et al. (2010)
Corn stalk wastes	_	Cow dung compost	149.69 ml/g TVS	Wang and Wan (2009a)
Corn stalk	0.5 % NaOH	_	57 ml/g VS	Guo et al. (2010)
Corn stalk	0.2 % HCl boiled 30 min	_	150 ml/g VS	Guo et al. (2010)
Corn stalk	Lime (ambient temperature for 69 h)	–	155.4 ml/g TVS	Cao et al. (2012)
Corn stalk	Steam explosion	–	12 L/Kg TS	Liu et al. (2014)
Delignified wood fiber	_	*C. thermocellum* 27405	1.6 mol/mol Hexose	Chong et al. (2009)
Corn stover	_	*Thermoanaerobacterium thermosaccharolyticum* W16	2.24 mol/mol Hexose	Cheng et al. (2011)
Corn stover	_	*Clostridium butyricum* AS1.209	68 ml H_2/g straw	Cheng et al. (2011)
Corn stover	_	Activated sludge	1.53 mol/mol Hexose	Cheng et al. (2011)
Bagasse	Alkali–thermal, 0.2–4 g/L of NaOH, 100 °C, 2 h	Mixed cultures	13.39 mmol/g TVS	Ntaikou et al. (2010)
Sweet sorghum	_	Mixed cultures	15.1–127.3 ml H_2/gTVS	Cheng et al. (2011)
Wheat straw	_	Mixed cultures	61.8 ml H_2/g TVS	Cheng et al. (2011)
Wheat straw[1]	_	Activated sludge	212.0 ml H_2/g Sugar	Cheng et al. (2011)
Corn stover[2]	Steam explosion (90–220 °C, 3–5 min)	Mixed cultures	3 mol H_2/mol glucose	Ntaikou et al. (2010)

* All experiments have been performed in batch mode except for 1 in UASB and 2 in continuous mode

Production of biohydrogen from lignocellulosic feedstocks could be broken down into three main steps: pretreatment, hydrolysis, and fermentation. Pretreatment is required to modify the structural and chemical composition as well as microscopic and macroscopic size of feedstocks, thereby achieving the microbial hydrolysis of lignocelluloses more rapidly, with greater yields. The following criteria for a proper pretreatment lead to improvement in the hydrolysis of lignocellulosic material and fermentative hydrogen production (Menon and Rao 2012; Taherdanak and Zilouei 2014; Taherzadeh and Karimi 2008):

- Removal of lignin and release of hemicelluloses
- Increase of the surface area and porosity
- Production of opened structure cellulosic fiber for enzymatic attack
- Prevention of the formation possible inhibitors for hydrolytic fermenting microorganisms
- Prevention of the destruction of hemicelluloses and cellulose
- Reduction of the crystallinity of cellulose
- Partial depolymerization of hemicelluloses
- Production of fewer residues
- Low energy consumption
- Consumption of little or no chemicals and use of cheap chemicals.

The feasibility of biohydrogen production from wheat straw was investigated in a mixed substrate with cow dung compost. The maximum hydrogen yield of 68.1 ml H_2/g TVS was obtained as the raw wheat straw was pretreated with HCl and microwave heating, which was comparable to some reported values for carbohydrates-rich wastes. This value was about 136-fold as compared with that of fermenting untreated wheat straw, thereby indicating that an appropriate pretreatment is necessary for microbial hydrogen fermentation of complex agricultural wastes.

7.8 The Effect of Different Factors on Fermentative Hydrogen Production

Several different factors have been demonstrated to be effective in fermentative dark hydrogen production. These include feedstock composition and structure, type of inoculum, environmental parameters (temperature, pH, and partial pressure of hydrogen), type of reactors, metal ions, nitrogen, and phosphates. On the other hand, the yield of hydrogen production via dark fermentation is influenced by the integration of the above parameters (Sinha and Pandey 2011). Some of the most effective parameters on dark fermentative hydrogen production process are presented below.

7.8.1 Inoculum

Selection of inoculum [pure H_2-producer culture (Table 7.1) versus mixed anaerobic fermentative consortium (Table 7.2)] is the first and most important step which can influence the total yield and costs and even type of the biohydrogen production process. Each of these selections has its advantages and drawbacks as mentioned in the previous sections. Mixed consortia of H_2 producer have been obtained from different natural habitats by following different pretreatment methods to enrich H_2 producers and inhibit H_2 consumers, as previously mentioned (Table 7.2). Therefore, they respond differently to operational parameters. This will be reviewed briefly below.

7.8.2 Temperature

Process temperature is one of the most effective parameters in dark hydrogen fermentation as it influences the substrate conversion and product formation through its effect on chemical and enzymatic reaction rates, stability of enzymes, microbial community compositions, metabolic pathways, and rate and yield as well as the lag time of hydrogen production (Dong et al. 2010). Although microbial hydrogen can be produced over a wide temperature range of 15–85 °C, most studies have been performed under mesophilic temperature (Kuan-Yeow and Duu-Jong 2013). Thermal activity of hydrogenase enzymes, which may occur at higher temperatures, can lead to a higher yield of hydrogen production. According to the standard enthalpy of the conversion of one mole of glucose to acetate (theoretical yield of 4 mol hydrogen per mole of glucose) (Eq. 7.25) and the changes in the Gibb's free energy, the reaction is endothermic and can occur spontaneously (Pandey et al. 2013).

$$\begin{gathered} C_6H_{12}O_6 + 4H_2O \rightarrow 2CH_3COO^- + 2HCO_3^- + 4H^+ + 4H_2 \\ \Delta G^\circ = -176 \frac{KJ}{mol}, \qquad \Delta H^\circ = +90.69\,KJ/mol \end{gathered} \tag{7.25}$$

Since the reaction is endothermic, equilibrium kinetic constants can be expected to be enhanced by increasing the temperature. Therefore, increasing the temperature of glucose fermentation (at the constant concentration of substrate) will increase the hydrogen concentration. Several different studies have investigated the effect of temperature on dark fermentation process. As listed in Table 7.5, most of these studies have been conducted at mesophilic temperature and batch reactors mode (Sinha and Pandey 2011).

Table 7.5 Effect of temperature on anaerobic dark fermentative H_2 production

Inoculum	Substrate	Temperature (°C)		Yield of hydrogen	References
		Range studied	Optimum		
Citrobacter CDN1	Glucose	27–40	30	2.1 mol/mol glucose	Sinha and Pandey (2011)
Ethanoligenes harbinense YUAN-3		20–40	37	1.34 mol/mol glucose	Sinha and Pandey (2011)
Anaerobic sludge	Glucose	25–55	40	275 ml/g glucose	Sinha and Pandey (2011)
Mixed cultures	Glucose	30–45	37.8	–	Wang and Wan (2011)
Anaerobic mixed culture	Sucrose	25–45	35	371.7 ml	Zhang and Shen (2006)
Thermoanaerobacterium Thermosaccharolyticum PSU-2	Sucrose	40–80	60	2.53 mol/mol hexose	Wang and Wan (2009a)
Municipal sewage sludge	Sucrose	30–55	40	3.88 mol/mol sucrose	Sinha and Pandey (2011)
Municipal sewage sludge	Starch	37–55	55	1.44 mmol/g starch	Wang and Wan (2009a)
Cow dung	Cow dung	37–75	55	743 ml/Kg cow dung	Sinha and Pandey (2011)
Anaerobic digester sludge	Organic waste	37–55	55	360 ml/g VS	Wang and Wan (2009a)
Seed sludge	Distillers grains	40–60	50	0.4 mmol/g COD	Chuang et al. (2012)

7.8.3 PH

The pH of medium is another major environmental factor affecting the fermentative hydrogen production through its potential influence on the diversity of microbial community, metabolic pathways, relative VFA production, and hydrogenase activity (Ntaikou et al. 2010). It strongly influences the relative amounts of fatty acids. The values of pH between 4.0 and 5.0 favor propionate production (H_2-consuming reaction), while pH values of 6.0–7.0 promote acetate and butyrate formation (H_2-producing reaction). Moreover, pH influences the growth rates of different groups, and therefore, the pH range of optimal growth is different for each group present in anaerobic fermentative hydrogen production. For example, the optimum pH for acidogens is 5.9, while for acetogens and hydrogenotrophic and acetoclastic methanogens, it is 7.0. Low values of pH have been considered to enrich H_2 producers via the inhibition of hydrogenotrophic methanogens and sulfate-reducing bacteria (Show et al. 2012). As a general rule, the optimum pH range to obtain maximum hydrogen yield has been suggested to be between 5.0 and 6.0 for both pure and mixed cultures (Wang and Wan 2009a; Kuan-Yeow and Duu-Jong 2013). In other words, the yield of hydrogen production is related to the pathways of pyruvate metabolism formed through the glycolysis of glucose using facultative anaerobes (Ntaikou et al. 2010; Wu and Juan 2013). All enzymes are active only in a certain range of pH, and their maximum activity is at the optimum pH. Studies have demonstrated that in an appropriate range of pH, increasing the pH value can improve the ability of bacterial hydrogen production. However, higher pH levels from this appropriate range could decrease their activity (Wang and Wan 2009a). Several different studies addressing the effect of the initial pH on dark fermentation process in batch reactors are listed in Table 7.6.

7.8.4 Metal Ions

Metal ions could participate in cellular transport processes and act as enzyme cofactors. Therefore, trace metal ions could be effective in any fermentative process (Pandey et al. 2013). Metal ions of Fe, Na, Mg, and Zn are needed by bacterial enzymes cofactors, dehydrogenases, and cellular transport processes. Thus, they can affect the metabolism in hydrogen-producing bacteria. However, at higher concentrations, metal ions could inhibit the activity of hydrogen production, leading to less hydrogen yield. Several studies investigating the effect of metal ions on dark fermentative hydrogen production are presented in Table 7.7.

- **The effect of iron**
  Hydrogenases, key enzymes in dark fermentative H_2 production, contain a central bimetallic Fe–Fe surrounded by Fe–S protein clusters (Pandey et al. 2013). Studies have demonstrated that iron–sulfur influences the protein functions primarily as an electron carrier involved in the conversion of pyruvate to

Table 7.6 Effect of pH on anaerobic dark fermentative H_2 production

Inoculum	Substrates	Reactor type	pH		Yield of H_2	References
			Range studied	Optimal		
Anaerobic digester sludge	Rice slurry	Batch	4.0–7.0	4.5	346 ml/g starch	Sinha and Pandey (2011)
Anaerobic digester sludge	Sucrose	–	3.0–12.0	9.0	126.9 ml/g sucrose	Wang and Wan (2009a)
Anaerobic microflora	Glucose	Batch	5–6	5.61	2.54 mol/mol glucose	Mullai et al. (2013)
Anaerobic sludge	Sucrose	Batch	4.7–6.3	5.5	3.7 mol/mol sucrose	Sinha and Pandey (2011)
Anaerobic sludge	Sucrose	Batch	4.5–6.5	5.5	252 ml/g sucrose	Sinha and Pandey (2011)
Compost	Sucrose	–	4.5–6.5	4.5	214 ml/g COD	Wang and Wan (2009a)
Cow dung compost	Corn stalk wastes	–	4.0–9.0	7.0	149.69 ml/T VS	Wang and Wan (2009a)
E. cloacae IIT-BT 08	Sucrose	Batch	4.5–7.5	6.0	29.63 mmol/g DW h	Sinha and Pandey (2011)
Mixed cultures	Glucose	Batch	6–9	7.1	–	Wang and Wan (2011)
Mixed cultures	Sucrose	–	4.0–10.0	8.48	3.21 mol/mol sucrose	Han et al. (2011)
Mixed cultures	Sucrose	Contin.	3.4–6.3	4.2	1.61 mol/mol glucose	Sinha and Pandey (2011)
Anaerobic sludge	Glucose	Contin.	4.0–7.0	5.5	2.1 mol/mol glucose	Sinha and Pandey (2011)
Mixed cultures	Sucrose	Contin.	6.1–9.5	7.0	1.61 mol/mol glucose	Sinha and Pandey (2011)
Seed sludge	Distillers grains	Batch	6–8	6.0	0.4 mmol/g COD	Chuang et al. (2012)

Table 7.7 Effect of metal ions on anaerobic dark fermentative H_2 production

Inoculum	Substrate	Reactor type	Metal ion	Concen. (mg/L)		Yield of H_2	References
				Studied	Optimum		
Cracked cereals	Starch	Batch	Fe^{2+}	1.2–100	10	140 ml/g starch	Wang and Wan (2009a)
Anaerobic sludge	Starch	Batch	Fe^{2+}	0–1,473.7	55.3	296.2 ml/g starch	Wang and Wan (2009a)
Grass compost	Food wastes	Batch	Fe^{2+}	0–250	132	77 ml/g TVS	Wang and Wan (2009a)
Anaerobic sludge	Palm oil effluent	Batch	Fe^{2+}	2–400	257	6.33 L/L substrate	Sinha and Pandey (2011)
Digested sludge	Glucose	Batch	Fe^{2+}	0–1,500	350	311.2 ml/g glucose	Sinha and Pandey (2011)
Anaerobic mixed cultures	Sucrose	Batch	Fe^{2+}	0–12,800	200	371.7 ml	Zhang and Shen (2006)
Anaerobic sludge	Sucrose	Batch	Fe^{2+}	0–1,763.8	352.8	131.9 ml/g sucrose	Wang and Wan (2009a)
Cracked cereals	Sucrose	Batch	Fe^{2+}	0–1,842.1	589.5	2.73 mol/mol sucrose	Wang and Wan (2009a)
Anaerobic sludge	Glucose	Batch	Cu^{2+}	0–400	400	1.74 mol/mol glucose	Wang and Wan (2009a)
Anaerobic sludge	Glucose	Batch	Zn^{2+}	0–500	250	1.73 mol/mol glucose	Sinha and Pandey (2011)
Hydrogen-producing bacterial B49	Glucose	Batch	Mg^{2+}	1.2–23.6	23.6	1.73 mol/mol glucose	Sinha and Pandey (2011)
Anaerobic microflora	Glucose	Batch	Ni0	10–30	5.67	2.54 mol/mol glucose	Mullai et al. (2013)
Digested sludge	Glucose	Batch	Ni^{2+}	0–50	0.1	296.1 ml/g glucose	Wang and Wan (2009a)
Digested sludge	Sucrose	Cont.	Ca^{2+}	0–300	150	3.6 mol/mol sucrose	Sinha and Pandey (2011)
Municipal sewage sludge	Sucrose	Cont.	Ca^{2+}	0–27.2	27.2	2.19 mol/mol sucrose	Sinha and Pandey (2011)

acetyl-CoA, CO, and H_2. Iron could also be effective on metabolic alteration (Wang and Wan 2009a).

- **The effect of nickel**
  Two major types of hydrogenase in dark fermentative H_2 production are [Ni–Fe] hydrogenases and [Fe–Fe] hydrogenases. The [Ni–Fe] hydrogenases are abundant among bacteria in comparison with [Fe–Fe] hydrogenases (Mullai et al. 2013). Hydrogen catalysis by [Ni–Fe] hydrogenases leads to electron transfer from the redox partner of the [Ni–Fe] hydrogenase (e.g., NADH) to the active site, and simultaneously, protons are transferred there; therefore, hydrogen is produced by the reduction of protons by the electrons at the active site. Although nickel as a fundamental component of [Ni–Fe] hydrogenases can improve the yield of dark fermentation process, its higher concentration could have an inhibition effect on bacterial activity, thereby contributing to fermentative H_2 production.
- **The effect of magnesium**
  Magnesium ion, an important element within microorganisms, is one of the constituents of cell walls and membranes. It can also act as the cofactor of glycolytic enzymes including hexokinase, phosphofructokinases, glyceraldehyde-3-phosphate dehydrogenases, and enolases. Therefore, appropriate concentrations of magnesium ions would favor the glycolysis. However, at higher concentrations of magnesium ions, the increased concentration of glycolytic metabolites (e.g., fructose 1, 6-bisphosphate, 3-phosphoglycerate, and phosphoenol pyruvate) inhibits glycolysis via feedback regulation. Through dark fermentation process, hydrogen is produced by electron transfer from reduced ferredoxin to a proton in the presence of hydrogenase. The reduced ferredoxin could be generated either by the oxidation of NADH (catalyzed by NFOR system) or the oxidation of pyruvate (catalyzed by PFOR system). Inhibition of glycolysis, which is caused by the increased concentration of magnesium ion, can cease the production of pyruvate and NADH. Therefore, further oxidation of pyruvate and NADH may reduce the yield of the produced hydrogen (Wang and Wan 2009a).
- **The effect of other heavy metals**
  Studies have shown that the heavy metals, including Cr, Cu, and Zn, could affect the metabolic pathways. For example, Cr, Cu, and Zn affect acidogenesis and methanogenesis phases of anaerobic digestion. Since hydrogenesis process is similar to acidogenesis in biochemical characteristics, heavy metals might play an important role in dark fermentation process. However, no significant effect of heavy metals on the efficiency of dark hydrogen process has been reported (Sinha and Pandey 2011).

7.8.5 Nitrogen and Phosphate

Nitrogen and phosphate are essential supplements, especially for carbohydrate-based feedstocks, that can increase hydrogen production yield through anaerobic

Table 7.8 Effect of Nitrogen source and concentration on anaerobic dark fermentative H_2 production

Inoculum	Substrate	Nitrogen source	Nitrogen concentration		Optimal index	References
			Studied	Optimum		
Escherichia coli	Glucose	NH_4Cl	0–0.2 g N/L	0.01 gN/L	1.7 mol/mol glucose	Wang and Wan (2009a)
Enterobacter aerogenes HO-39		Polypepton	0–5 % (w/w)	2 % (w/w)	58 ml	Wang and Wan (2009a)
Clostridium acetobutylicum	Glucose	Yeast extract/ tryptone/peptone	13 g/L	13 g/L of yeast extract	308 ml H_2/g glucose	Kalil et al. (2009)
Compost	Glucose	Yeast extract	2–8 % (w/w)	4 % (w/w)	70 mmol	Wang and Wan (2009a)
Digested sludge	Glucose	Nitrate	0–10 g N/L	0.1 g N/L	313.1 ml/g glucose	Wang et al. (2009)
Grass compost	Food wastes	NH_4HCO_3	0–0.6 g N/L	0.4 gN/L	77 ml/g TVS	Wang and Wan (2009a)
Cracked cereals	Starch		0.1–2 g N/L	1 gN/L	146 ml/g starch	Wang and Wan (2009a)
Mixed culture	Cheese whey and vegetable–-fruit waste		C/N ratio: 7–46	C/N ratio: 21	449.8 ml/g COD	Gomez-Romero et al. (2014)

dark fermentation. The growth of hydrogen-producing bacteria is significantly affected by nitrogen, because it is one of the most important elements in protein structure, nucleic acids, and enzymes. However, it has been reported that the addition of an organic source of nitrogen to the culture seems to be more favorable, resulting in much more hydrogen production compared to the inorganic one (Kuan-Yeow and Duu-Jong 2013). Therefore, the addition of an appropriate concentration and a source of nitrogen are recommended to improve the growth rate of hydrogen-producing bacteria and hydrogen production efficiency (Chong et al. 2009). Several studies addressing the effect of nitrogen on dark fermentation process are listed in Table 7.8. Phosphate is also one of the important inorganic nutrients required for hydrogen production. Excess phosphate may favor hydrogen production over by-product solvent production, so phosphate supplementation may be needed, especially for carbohydrate-based feedstocks (Kuan-Yeow and Duu-Jong 2013).

7.8.6 Hydraulic Retention Time

The yield of hydrogen in anaerobic dark fermentation is obviously a function of microbial composition and diversity, which is affected by hydraulic retention time (HRT). In fact, HRT influences the rate and yield of H_2 production through its effect on metabolic pathways, biomass content, H_2-consuming microorganisms, oxidation–reduction potential, washing out of granular bacterial biomass at low HRT, and product inhibition due to the accumulation of VFA at high HRT (Ren et al. 2008). Typical specific growth rates for hydrogen-producing and methane-producing bacteria are about 0.17 and 0.017 h^{-1}, respectively. It means that by regulating the HRT or feed flow rate, methane-producing bacteria (H_2-consumers) will be washed out, while hydrogen-producing bacteria will be retained inside the reactor. Moreover, it has been reported that shortening the HRT can reduce the microbial diversity through the inhibition of propionate production (H_2-consuming reaction) without affecting the existence of dominant species, which, in turn, can increase the hydrogen yield (Kuan-Yeow and Duu-Jong 2013).

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Chapter 8
Biobutanol from Lignocellulosic Wastes

Hamid Amiri, Keikhosro Karimi, Sandip Bankar and Tom Granström

Abstract The perceived inability to economically provide conventional petroleum to meet the growing energy demands is facing a diverse and broad set of challenges. The major technical and commercial drawbacks of the existing biofuels (bioethanol or biodiesel) have prompted the continuing development of more advanced biofuels such as biobutanol. Acetone–butanol–ethanol (ABE) fermentation is an old process which recently attracted new interests for the production of butanol as an advanced biofuel. Efficient use of low cost lignocellulosic wastes as a carbon source for ABE fermentation can be a proper approach for the economical production of biobutanol. This chapter focuses on the utilization of lignocellulosic materials in ABE fermentation process. It explains the ABE fermentation process especially the processes that were economically used in the Soviet Union, China, and South Africa in the twentieth century. It also summarizes different technologies that have been suggested for the utilization of lignocelluloses for biobutanol production.

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8.1 Introduction

Butanol is a four carbon primary alcohol having several applications in different industries ranging from C4 feedstock for chemical synthesis (esters, ethers, acetates, and plasticizers) to solvents in chemical industry. However, its chemical properties as an advanced biofuel have fascinated the world since the beginning. First generation biodiesel although a popular biofuel, fails to replace petrol–diesel completely, unless the significant changes in the engine are configured (Kikuchi et al. 2009; Ranjan and Moholkar 2012). Bioethanol with a relatively low energy content (or heat of combustion) can be blended up to 10 % with the normal petrol for using in the current car engines. Hence, there is an extensive need for advanced biofuels with superior fuel properties. Biobutanol is regarded to be superior to bioethanol in terms of energy density, air/fuel ratio, heat of vaporization, and hygroscopicity (Dürre 2007). Butanol also offers some advantages over ethanol and methanol viz. (a) It can be blended to varied ratio with gasoline as well as diesel directly in the refinery without any additional infrastructure. (b) Easy transportation and less corrosion through pipelines because of low vapor pressure. (c) The air/fuel ratio for butanol is close to that of gasoline, which is permissible in existing vehicle engines. The complete replacement of gasoline by butanol would require an enhancement of the air/fuel ratio, but blends up to 20 % of butanol can be directly used in engines without any modifications. (d) The heat of vaporization of butanol is slightly higher than that of gasoline. Hence, the butanol-blended engine does not observe the cold start problem as seen in methanol or ethanol blended gasolines. (e) Low solubility of butanol in water reduces the potential for groundwater contamination (Bankar et al. 2013a; Ranjan and Moholkar 2012; Alasfour 1997).

Historically, the acetone–butanol–ethanol (ABE) production was prospered during the early twentieth century from molasses and starchy materials to be evolved as a second largest industrial fermentation process in the world after ethanol. However, due to progression of the petrochemical industry, low solvent yields, and increase in the feedstock cost, the ABE fermentation process had lost its competitiveness by 1960s. The continual depletion of fossil fuels and highly fluctuating market prices of crude oil reserves in the recent years again attracted an attention toward reviving this process (Karimi and Pandey 2014; Kumar and Gayen 2011). Low conversion rates of conventional fermentative technologies which result in the economically non-feasible large-scale production demand the modifications in the process development for its efficient and economic production. Substrate for biobutanol production plays a crucial role in the economics of the process. Hence, the utilization of relatively low cost biomass has recently been suggested for improving the economy of biobutanol production (Kumar and Gayen 2011). Biomass is the fourth largest source of energy in the world after coal, petroleum, and natural gas, and it is also a carbon neutral resource over its life cycle. The lignocellulosic biomass is the most abundant renewable resource on the earth for biofuel production. Several countries generate millions of tons biomass every year directly from plants, rice husk, sawdust, and bagasse. The potential of numerous lignocellulosic feedstock's such as wood forestry residues, corn

stover, wheat straw, corn fibers, barley straw, and switchgrass has been tested for ABE fermentation in several studies (Qureshi et al. 2008a, b, c, 2010; Qureshi and Blaschek 2001; Ezeji et al. 2007).

8.2 Butanol-Producing Microorganisms

The biosynthesis of butanol is restricted to species of genus *Clostridium,* a diverse group of gram-positive endospore-forming anaerobes, belonging to the phylum Firmicutes (Bahl and Dürre 2001). These bacteria are found most commonly in associated with living plant material rather than with decaying plant material or soil (Jones and Woods 1986). Potatoes, the roots of nitrogen-fixing legumes, and other root crops have been reported to be excellent materials for the isolation of these bacteria (Calam 1980). In addition, cereal crops, fruit such as gooseberries, and agricultural soil have also been reported to be successful sources of these bacteria (McCutchan and Hickey 1954; Jones and Woods 1986). Due to their ubiquitous nature and as a natural habitant of soil, Clostridium is capable of degrading a large range of carbohydrate substances derived from plant, animal, and microbial material sources (Jones and Woods 1986).

Although different species of solvent-producing clostridia isolated and industrially utilized in twentieth century, several patented industrial strains have been lost after the culmination of the process (Bahl and Dürre 2001). By the 1970s, the majority of survived industrial strains were referred as *Clostridium acetobutylicum* and *Clostridium beijerinckii* (Jones and Keis 1995; Kharkwal et al. 2009; Maddox 1989). However, along with the revaluation of clostridia in the beginning of 1990s, the industrial solvent-producing clostridial strains were divided into four groups of *C. acetobutylicum*, *C. beijerinckii, Clostridium saccharoperbutylacetonicum*, and *Clostridium saccharobutylicum* (Keis et al. 1995; Johnson et al. 1997).

The strain isolated from soil in Japan by Hongo and Nagata in 1959 was considered as a distinct species with the name of *C. saccharoperbutylactonicum* (Keis et al. 1995). This strain was industrially used for the production of acetone and butanol from molasses by the Sanraku Distiller's Company. However, phage infections of the original strain (N1-4), occurring 12 times a year, confined the performance of the process (Keis et al. 1995). Furthermore, the strains patented under the name of *C. saccharoperbutylactonicum-liquefaciens* by CSC in the USA were confirmed as a distinct species. This species was extensively used for commercial solvent production in the USA, Britain, and South Africa (Keis et al. 1995).

8.3 Biochemistry of ABE Fermentation

The most commonly considered substrates for the *clostridial* cultures include fibrous biomass containing hemicellulose and cellulose (e.g., wheat straw and rice straw); starchy biomass (such as ground corn and whey permeate); and fruits and

vegetables containing fructose, glucose, and xylose as the basic components. The growth of bacteria in strictly anaerobic conditions is controlled by the rate of energy yielding reactions, due to limited number of ATP molecules generated during fermentation. The bacteria use branched pathways of carbon and electron flow to optimize energy production under the energy-stressed conditions. The fermentation of a mole of glucose can generate between 1 and 4 mol of ATP depending on the pathways used.

Theoretical diversion of the carbon flow to acetate resulted in the production of relatively high amount of ATP (4 mol/mole glucose). The saccharolytic clostridia uses branched pathways which link the reduction of acetate, to produce ATP without additional consumption of reducing power for the production of either ethanol or butyrate, that allows the disposal of excess reducing equivalents (Fig. 8.1) (Minton and Clarke 1989). As a result, there is a direct relationship between the amount of Hydrogen produced and the amount of ATP which is regenerated (Minton and Clarke 1989).

The solvent-producing clostridia mainly produce CO_2, H_2, acetate, and butyrate during the initial growth phase in batch culture, named as acidogenic phase. Carbon flow initiates by uptake of sugars through phosphoenolpyruvate-dependent phosphotransferase system except galactose which may be transported by a non-phsphotransferase mechanism (Mitchell 1996). Pentose and hexose sugars are metabolized via the pentose phosphate and the Embden–Meyerhof–Parnas pathways, respectively. Each mole of hexose is converted to 2 mol pyruvate, with net production of 2 mol ATP and 2 mol NADH. The fermentation of 3 mol of pentose yields 5 mol pyruvate, 5 mol ATP, and 5 mol NADH. The pyruvate, ATP, and NADH are served as the sources for the carbon, energy, and electron flows, respectively. The pyruvate is cleaved in the presence of coenzyme (CoA) to produce carbon dioxide, acetyl-CoA, and reduced ferredoxin. Acetyl-CoA is subsequently condensed, reduced, or dehydrated to yield CoA derivatives, e.g., acetoacetyl-CoA and butyryl-CoA. The CoA derivatives are the central intermediates leading to both acid and ABE production.

After the exponential growth phase of acidogenesis, when the concentration of end products becomes inhibitory, both electron and carbon flow is shifted by the cell from acid-producing pathways to solvent-producing pathways. The factors affecting the transition between the two phases have not been the subject of extensive studies. In addition, different events have been reported to occur during the transition which may affect the transition (Fig. 8.2) (Minton and Clarke 1989). However, the mechanisms responsible for triggering solvent production have not been fully elucidated.

The level of intracellular undissociated butyric acid (UBA) is one of the suggested controlling factors in the shift from acid production to solvent production (Terracciano and Kashket 1986; Huang et al. 1986; Hüsemann and Papoutsakis 1988; Monot et al. 1984). The weak acids in their undissociated form are diffused across the membrane, resulting in a drop of pH of the external medium. Furthermore, the undissociated acids can act as uncouplers causing increased membrane permeability to protons, acidification of the interior of the cell, and collapse of the

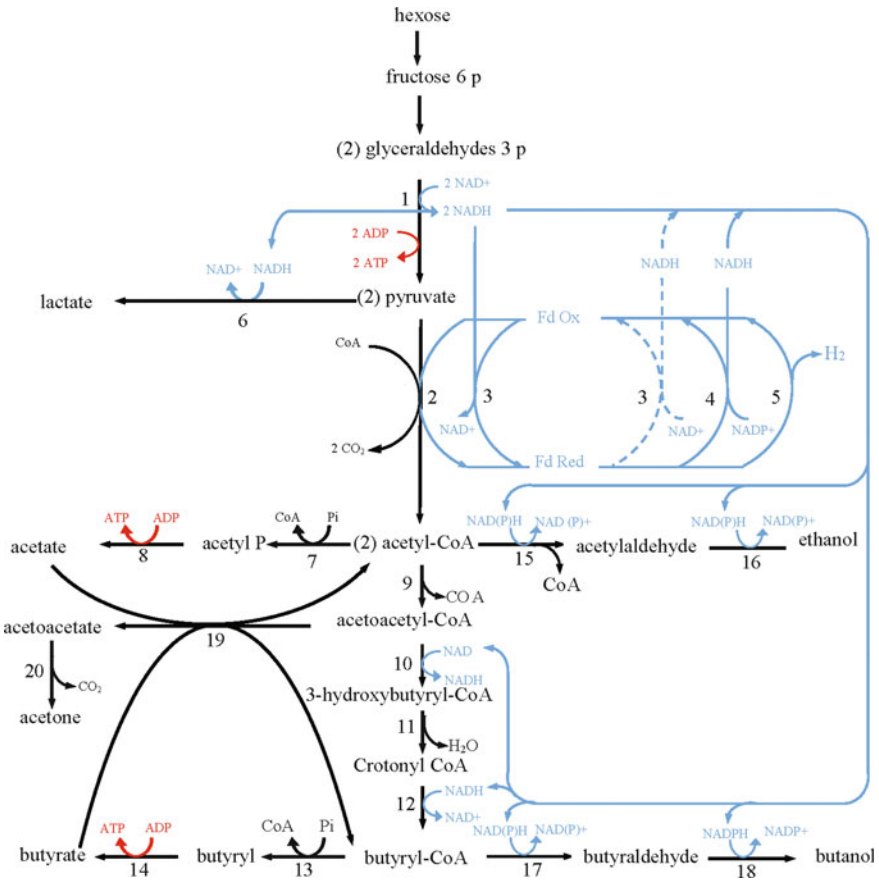

Fig. 8.1 Catabolic pathways used by the solvent-producing clostridia. The directions of carbon and electron flow are shown by *heavy* and *light arrows*, respectively. Enzymes indicated by numbers are as follows: (*1*) glyceraldehyde-3-phosphate dehydrogenase; (*2*) pyruvate-ferredoxin oxidoreductase; (*3*) NADH-ferredoxin oxidoreductase; (*4*) NADPH-ferredoxin oxidoreductase; (*5*) hydrogenase; (*6*) lactate dehydrogenase; (*7*) phosphate acetyltransferase (phosphotransacetylase); (*8*) acetate kinase; (*9*) thiolase (acetyl-CoA acetyltransferase); (*10*) 3-hydroxybutyryl-CoA dehydrogenase; (*11*) crotonase; (*12*) butyryl-CoA dehydrogenase; (*13*) phosphate butyltransferase (phosphotransbutyrylase); (*14*) butyrate kinase; (*15*) acetaldehyde dehydrogenase; (*16*) ethanol dehydrogenase; (*17*) hutyraldehyde dehydrogenase; (*18*) butanol dehydrogenase; (*19*) acetoacetyl-CoA: acetate/butvrate: CoA transferase; (*20*) acetoacetate decarboxylase (Minton and Clarke 1989)

membrane pH gradient, whereas low permeability of proton through the cell membrane is essential for maintaining the proton motive force of the cell (Kell et al. 1981; Terracciano and Kashket 1986). Continued acidogenic phase in the mutants of *C. acetobutylicum*, *cls*, which is unable to shift to solvent production, results

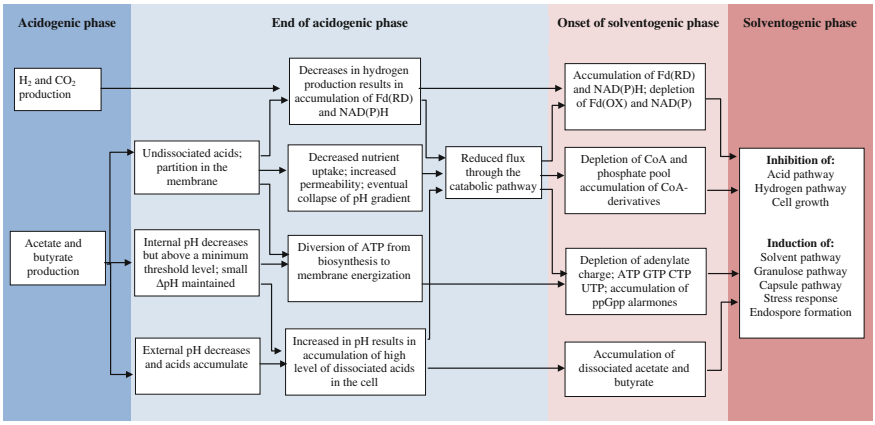

Fig. 8.2 The possible relationship between events which occur in the transition from acidogenic to solventogenic phase (Minton and Clarke 1989)

in the accumulation of up to 20 g/L acids in the external medium before all metabolism ceases, and cell viability is lost (Clarke 1987). Solvent-producing clostridia, however, switch to solvent production phase after decrease in the activity in most of the acid pathway enzymes (except butyrate kinase) and the induction of the solvent pathway enzymes. Even with solvent-producing species, fermentation might be ceased after acidogenesis, without controlling the pH known as "acid crash." It has been reported that solvent production by *C. acetobutylicum* began when the UBA level reached 13–18 mM (Terracciano and Kashket 1986). The overproduction of undissociated acids (more than 60 mM), with low pH, is considered as the main reason for acid crash (Maddox et al. 2000).

The auto adjustment of pH of the medium has been considered as an influencing parameter on the initiation of solventogenesis. The internal pH of the cell should be maintained above the threshold value of at least pH 5.5 to maintain cell functions (Terracciano and Kashket 1986). It has been observed that the cells divert ATP from biosynthesis to membrane energization when internal pH approaches the threshold level. This results in increase in the pH difference across the membrane. The concentration of acids within the cell would rapidly increase and inhibit the flux as a result of reversibility of the catabolic pathways (Gottwald and Gottschalk 1985). This leads to depletion of CoA and phosphate pools (Minton 1989). Besides, further reduction in the adenylate charge and accumulation of the reduced ferredoxin and NAD(P)H in the cells was also observed.

The sharp decline in Hydrogen production in the solventogenic phase suggested that disposing the excess reducing power to H_2 is no longer possible (Kim and Zeikus 1984). Therefore, the regeneration of the reduced ferredoxin is performed with NAD(P)H-ferredoxin oxidoreductase that accompanied with the formation of NAD(P)H. The shift to butanol production pathway is a strategy to dispose NAD(P)

H, enabling cells to regenerate 4 mol of $NAD(P)^+$ per mole of glucose metabolized. In this condition, the net amount of ATP reduces to 2 mol.

During solventogenic phase, dissociated acetate and butyrate which is formed in the acidogenic phase are also consumed by the cells, resulting in decrease in the intracellular concentration of acids and diffusion back to the cells. This process can be considered both as a detoxification mechanism for the reduction of acids concentration and a method for preparing CoA derivatives required for regenerating NAD^+. The uptake of acids is directly coupled to the formation of acetone (or isopropanol in some species) and depends on the continued metabolism of sugars (Hartmanis et al. 1984; Matta-el-Amouri et al. 1985).

Depending on the end products, catabolic pathways utilized by the solvent-producing clostridia result in different net amount of energy generation as well as production and disposal of reducing power (Table 8.1).

The solvents, especially butanol, are inhibitory to the cells. About 50 % of the cell growth is reduced by 11, 51, and 84 g/L butanol, ethanol, and acetone, respectively (Kotai 2013). Inhibition effects of these solvent are known as a solvent stress, and a large number of studies were performed by various researchers to explain this phenomenon (Liyanage et al. 2000; Tomas et al. 2004; Knoshaug and Zhang 2008).

Laboratory-based media for the culture growth have normally been semi-defined and defined wherein the main carbohydrate source is supported by various vitamins and minerals depending on the microbial cultures. The optimum temperature for the ABE fermentation is between 30 and 40 °C, while the initial pH of the fermentation broth is 6.8–7, and it drops down to 4.5–5 during acidogenic phase.

Table 8.1 Energy generation, production, and disposal of reducing power through different pathways of ABE fermentation (Minton and Clarke 1989)

	ATP produced	Reducing equivalent produced as NAD(P)H or reduced ferredoxin	Disposed reducing equivalent
Glycolysis via EMP	2	2	0
Pyruvate cleavage	0	2	0
Hydrogen production	0	0	0–4
Acetate production	2	0	0
Lactate production	1	0	2
Butyrate production	0	0	2
Ethanol production	0	0	2
Butanol production	0	0	4

8.4 Substrates Used for Butanol Production

8.4.1 Monosaccharides and Disaccharides

Solventogenic clostridia can utilize a large variety of substrates from monosaccharides including many pentoses and hexoses to polysaccharides. Mono-, di- or oligosaccharides, derived from breakdown of polymers or available as free sugars, are the substrates accumulated by cells to support growth. For each substrate, the cell synthesizes a specific membrane bound transport system and enzyme(s) which serve to convert the accumulated substrate to an intermediate of the principal metabolic pathways (Mitchell 1996; Jang et al. 2012b).

Most of monosaccharides and disaccharides are accumulated by the cells via phosphoenolpyruvate-dependent phosphotransferase system (PTS), which are involved in the transfer of a phosphate group from phosphoenolpyruvate (PEP) to the utilized sugars.

One of the carbon sources which has traditionally been used for industrial ABE fermentation is sucrose. Sucrose is utilized by *C. beijerinckii* and *C. acetobutylicum* through the sucrose phosphoenolpyruvate-dependent phosphotransferase system (Jang et al. 2012b). The activities of the enzymes of sucrose enzyme II complex, sucrose-6-phosphate hydrolase, and fructokinase facilitate the uptake of sucrose by these organisms.

Clostridia can secrete numerous enzymes that facilitate the breakdown of polymeric carbohydrates into sugar monomers for biobutanol production (Ezeji et al. 2007). However, acidic or enzymatic hydrolysis of lignocellulosic materials is essential to convert them into monosaccharides before using them as substrates in ABE fermentation. Clostridia are not cellulolytic bacteria and hence cannot utilize cellulose as a carbon source. However, cellulosomes, multienzyme complexes with high activity against crystalline cellulose, are being analyzed in clostridia (Bankar et al. 2013a, b).

8.4.2 Mixture of Hexose and Pentose Carbohydrates

Hexose and pentose sugars supported growth and fermentation by solventogenic clostridia. Even though the clostridia utilize cellobiose, galactose, mannose, arabinose, and xylose, glucose is its preferred carbon source. During the fermentation of mixed sugars, all the sugars were utilized throughout the fermentation concurrently but with different rates (Ezeji and Blaschek 2008). It has been reported that the order of sugar preference by *C. beijerinckii* BA101 is glucose > xylose > arabinose > mannose (Ezeji et al. 2007).

8.4.3 Other Substrates

Glycerol, which is a byproduct in the biodiesel production, can be utilized by solvent-producing clostridia (Li et al. 2014; Yadav et al. 2014; Khanna et al. 2013). *Clostridium pasteurianum* was the first solvent-producing strain reported to utilize glycerol as the sole carbon source. In the fermentation pattern of glycerol uptake, a part of the glycerol is converted into dihydroxyacetone phosphate (DHAP). Two pathways have been found for this conversion (Jang et al. 2012b). In the first pathway, glycerol is phosphorylated to glycerol 3-phosphate by glycerol kinase, and glycerol 3-phosphate is converted to DHAP by glycerol 3-phosphate dehydrogenase (Jang et al. 2012b). In the second pathway, glycerol is firstly oxidized to dihydroxy acetone and subsequently phosphorylated to DHAP by dihydroxyacetone kinase. Dihydroxyacetone phosphate is used in the standard glycolysis metabolism. The remaining glycerol is converted to 1, 3-propanediol. *C. acetobutylicum* ferment glycerol to butanol only in a mixture with glucose and cannot use glycerol as the sole carbon source.

Algae are also suggested as alternative substrates for ABE production with several advantages in comparison with other substrates, e.g., high growth rate, less water demand, high-efficiency CO_2 mitigation, more cost-effective farming (Demirbas 2010; van der Wal et al. 2013; Ellis et al. 2012). However, there are number of challenges associated with the utilization of algae that still need to be addressed. The biomass of algae is produced in a very dilute solution making the separation and downstream processing costly. Interestingly, isobutanol can also be produced directly from solar energy and CO_2 by some algae (Jang et al. 2012b; Atsumi et al. 2009).

8.4.4 Commercial Substrates

8.4.4.1 Maize and Potato Mash

Potatoes and starchy grains especially maize were the first carbohydrate sources used for industrial ABE production (Bahl and Dürre 2001). The industrial fermentation of the starchy substrates was being carried out at 37 °C and initial pH of 6.5 (McNeil and Kristiansen 1986). Since the starchy substrates can offer rich nutritional supplementation for the fermentation, the addition of other inducers is not necessary during fermentation (McNeil and Kristiansen 1986). Typical yields of fermentation products obtained from starchy materials are shown in Table 8.2.

Table 8.2 Typical yields of main products obtained from starchy materials (McNeil and Kristiansen 1986)

Products	Yield (kg/100 kg starch)
Butanol	22
Acetone	10.5
Ethanol	5.3
Carbon dioxide	62.4
Hydrogen	1.7

8.4.4.2 Molasses

Molasses is a dark-colored syrup by-product of sugarcane/beet industry which consists of water (approximately 50 %), total sugars (sucrose, glucose, and fructose), heavy metals, suspended colloids, vitamins, and nitrogenous compounds (Najafpour and Poi Shan 2003). Typical composition of molasses is listed in Table 8.3.

Molasses was one of the substrates used for commercial biobutanol production (Bahl and Dürre 2001; Zverlov et al. 2006). The ABE fermentation of molasses can be performed either directly with the addition of common nitrogen and phosphorous compounds or by supplementation with special additives, such as corn meal and rice bran with different strains (Olbrich 1963). Molasses as the substrate for ABE fermentation offers some advantages over starchy materials such as relatively low cost of handling and sterilization of the molasses, and easier cleaning of fermentation tanks (Olbrich 1963). Furthermore, the fermentation of molasses with relatively higher sugar concentrations is conducted at relatively lower temperatures which reduces the risk of contamination and has generally resulted in higher butanol yield and productivity (Olbrich 1963). Typical yields of products through ABE fermentation of molasses are listed in Table 8.4. However, as the petrochemical industry evolved during the 1960s, the production of acetone and butanol by fermentation was virtually ceased. Cost issues, relatively low-yield, sluggish fermentations, end product inhibition, and bacteriophage infections were some of the limitations associated with biobutanol that could not compete on a commercial scale with butanol produced synthetically (Brekke 2007). Moreover, the molasses

Table 8.3 Composition of molasses (Steg and Van Der Meer 1985)

	Percentage (%)	
	Sugarcane molasses	Sugar beet molasses
Sucrose	44	66
Fructose	13	1
Glucose	10	1
Betaine	–	6
Amino acids	3	8
Other substances	30	18

Table 8.4 Typical yields of main products obtained from sugarcane molasses (Olbrich 1963)

Products	Yield (kg/100 kg molasses)
Butanol	11.5
Acetone	4.9
Ethanol	0.5
Carbon dioxide	32.1
Hydrogen	0.8
Dry vinasse	28.6

quality was decreasing due to improved sugar processing technology, and the price of molasses also increased due to the fact that it was used as an additive animal feeds (Zverlov et al. 2006).

8.5 The Old Processes

8.5.1 Soviet Union's Industrial ABE Processes

The acetone butanol industry was first founded in the Union of Soviet Socialist Republics (USSR) between 1929 and 1935 (Zverlov et al. 2006). Along with development of the Weizmann process in England, Canada, and the USA during the First World War, the process was also founded in the USSR for production of solvents from wheat and rye starch by *C. acetobutylicum*.

The process developed in Soviet Union was operated in batch mode fermenters up to 275 m^3 working volume (Zverlov et al. 2006). In 1930, the application of fermenters in series with larger overall working volumes of 2,000 m^3 was achieved. In spite of general advantages of continuous process over a batch process, the latter is difficult to handle and was not used in industrial scales. However, in late 1950s, the continuous mode of operation for preparing and sterilizing of the fermentation broth as well as for downstream processing of the fermentation products was applied in USSR ABE plants. To continuously produce acetone and butanol, a system of linked batch fermenters was used in Dokshukino in 1962 (Jones and Woods 1986) (Fig. 8.3). Parallel batteries containing fermenters in series were used as "continual fermentation" by which the continuous inflow of syrup to the fermenters and outflow of products to the distillation unit were allowed. In this process, loading, fermentation, unloading, rinsing, and sterilization were performed in each fermenter at different times but in a specific order (Fig. 8.4). The productivity obtained by the continual fermentation was 31 % higher than that of batch fermentation (Zverlov et al. 2006).

In addition, the technology of using pentose containing hydrolysates from lignocelluloses for ABE production was developed in 1959 at Doshukino plant, which estimated to reduce the substrate cost to 45 % of that of traditional processes. Dilute sulfuric acid at moderately high temperature was used for obtaining pentose hydrolysates from lignocelluloses (Jones and Woods 1986).

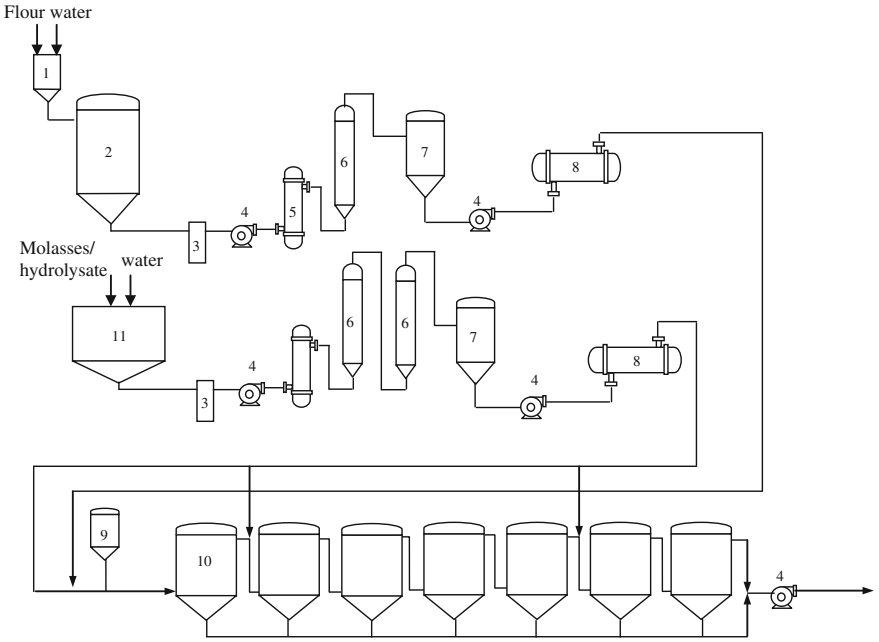

Fig. 8.3 Schematic process at the Dokshukino plant: (*1*) flour medium preparation mixing vessel; (*2*) preheating; (*3*) solid separation; (*4*) delivery pump; (*5*) column heater; (*6*) pressured incubation vessel for sterilization; (*7*) steam separator; (*8*) cooler; (*9*) seed fermenter; (*10*) fermenter; (*11*) molasses and hydrolysate preparation, mixing vessel (Zverlov et al. 2006)

In addition to acetone, butanol, and ethanol, a number of by-products were produced to improve the process economy. The insoluble sludge after distillation was subjected to thermophilic methanogenic fermentation for biogas production or used in the yeast–protein plant for fodder production. The biogas was used to provide the heat for sterilization and distillation units. In addition, vitamin B12 extracted from the archaebacteria, and 400–600 μg/L cobalamin obtained after the methanogenic fermentation, was a value added by-product of the process (Zverlov et al. 2006). The CO_2 and Hydrogen are the major by-products of the fermentation. About three moles of CO_2 and H_2 (with ratio of 1.5:1) were formed from each mole of fermented hexose. Therefore, in Russian ABE plant, along with production of each ton of solvents, about 1.7 t gas containing 1.649 t CO_2 (97 % w/w) and 51 kg H_2 (3 % w/w) was eluted. In Everemovo plant, about 8.7 million m^3 H_2 and 13.1 million m^3 CO_2 were produced annually. After separation of solvents from the gas stream (0.01 kg/m^3) by condensation, CO_2 was sold in the form of dry ice or liquid (Zverlov et al. 2006).

More than eight ABE plants were actively working in USSR by late 1980s. Bacteriophage infection which reported to be a major problem in the ABE plants were rarely reported in the Russian ABE industries (Zverlov et al. 2006). This advantage of Russian ABE industry owed to use phage-tolerant strains, rigorous sterilization scheme, e.g., hot steam sterilization, and relatively high temperature

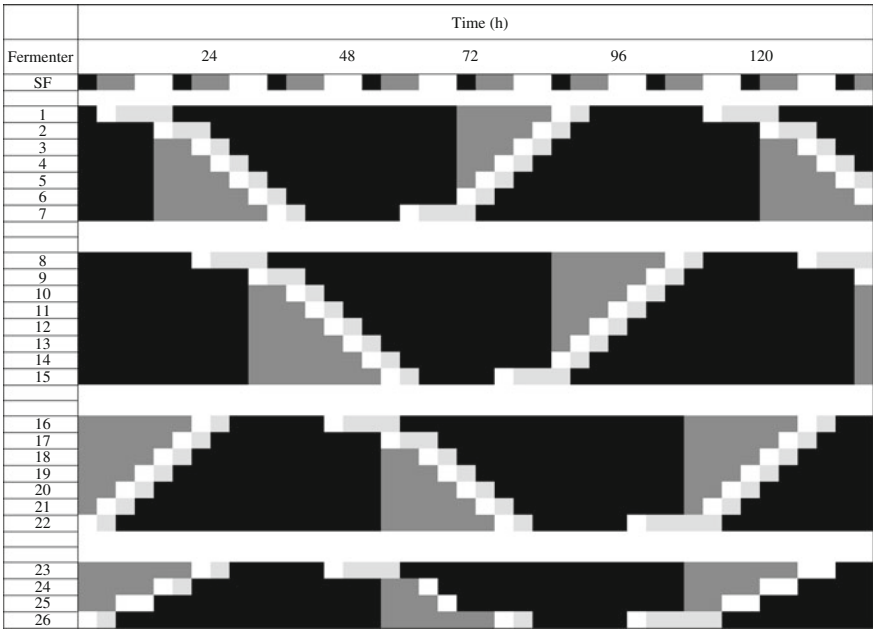

Fig. 8.4 The continual utilization of sterilization (*light gray*), loading and fermentation (*black*), and fermentation (*dark gray*) and unloading of each fermenter (*white*) in the four batteries at the Dokshukino plant (Zverlov et al. 2006)

fermentation (37 °C). The *C. acetobutylicum* strains which were independently isolated were also improved by a multi-cycle selection. One of the other typical difficulties in ABE industries which were controlled in Russian plants is the problem of foaming during fermentation. The high amount of gas production through fermentation is generally accompanied by foaming in the fermenter. In the Dokshukino plant, however, foaming was not a problem as a result of relatively flat fermenter geometry and steady incoming flow of broth to the vessels which thoroughly mixed the fermenter content. The advantages of Russian ABE plants over former Western plants includes the continual fermentation technology, selection and using phage-tolerant strains, strict sterilization of substrate and appliances, the use of pentose and hexose hydrolysates of agricultural waste materials instead of starch and/or molasses, and the production of different products, e.g., vitamin B12, fodder yeast, and dry ice, based on the biorefinery concept (Zverlov et al. 2006).

8.5.2 Chinese Industrial ABE Processes

Fermentative ABE production was one of the successful industries in China for several decades. Even after closure of most of ABE plants around the world in

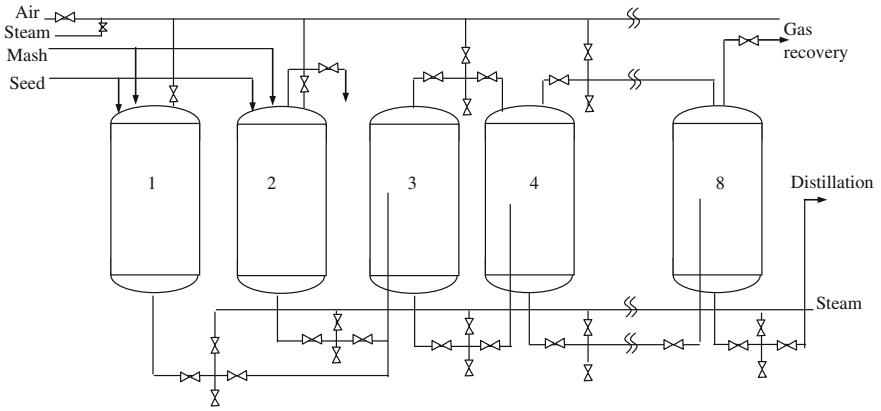

Fig. 8.5 The schematic flow diagram of continuous ABE fermentation (Ni and Sun 2009)

1950s, China continued biological ABE production using its own technology (Chiao and Sun 2007; Ni and Sun 2009). The real continuous ABE fermentation was developed in Chinese industries (Fig. 8.5).

In 1955, Shanghai Solvent Plant was launched in place of the Sino Ethanol Plant for ABE production from corn (Chiao and Sun 2007). Thereafter, in 1960s, the annual national production increased to 40,000–50,000 t ABE when more than ten ABE plants were founded in Beijing, Wuxi, Tianjin, Yunnan, Shanxi, Zhejiang, etc. (Chiao and Sun 2007). In addition, all ABE production plants in China gradually shifted their fermentation process from batch to continuous process. The Shanghai Solvent Plant with an annual output of 10,000 t was still a leading plant.

In 1980s, when fermentative ABE production was abandoned due to economic problems, Chinese ABE industry was in its heyday (Chiao and Sun 2007). In order to supply national demands, ABE plants relocated from southern to northern China to have an easy access to corn and coal production districts. In addition, a number of new plants with a yearly output of 5,000–10,000 t ABE were founded in Shandog, Hebei, and northeastern provinces and the annual solvent production increased to 170,000 t ABE (Chiao and Sun 2007).

Instead of using a set of batch fermenters to have the continual process (Russian plants), a real continuous fermentation was developed in Chinese ABE plants. In this process, fresh substrate continuously fed into the two activation tanks (200–500 m^3) to which seed culture broth was periodically added from seed culture tank (30–60 m^3) (Ni and Sun 2009). A set of linked in series tanks, 4–6 tanks with volume of 200–500 m^3, was fed by the activation tanks to perform the fermentation. The fermentation mash flowed through the tanks from the first tank with a pressure of $\sim$100 kPa to the final tank with a pressure of $\sim$50 kPa in the overflow. The fermentation was conducted for 170–480 h without sterilization and loading (Ni and Sun 2009).

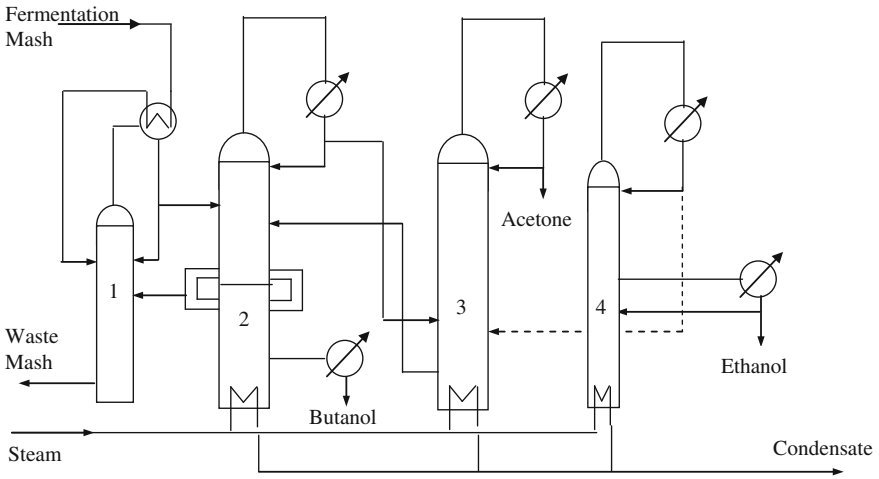

Fig. 8.6 The schematic flow diagram of a four-tower distillation system (Ni and Sun 2009)

The continuous fermenters in the Chinese solvent plants usually produced solvents with 20–50 % higher productivities in comparison with batch fermenters. The final fermentation broth containing about 2 % solvents fed to a four-tower continuous distillation unit (Fig. 8.6). The following stoichiometric reaction equation is used to estimate the Chinese ABE fermentation (Ni and Sun 2009):

$$12\ (\text{Glucose}) \rightarrow\ 6\ (\text{n-Butanol}) + 4\ (\text{Acetone}) + 2\ (\text{Ethanol}) + 18\,H_2 + 28\,CO_2 + 2\,H_2O$$

By the end of the 1990s, along with internationalization in China, the price of grain was increased to its global level which dramatically increased the production costs of fermentative ABE production (Chiao and Sun 2007). In addition, petrochemical industries equipped for production of acetone and butanol became an alternative for acetone and butanol production. Due to high cost of substrate and waste disposal, the fermentative ABE industries lost their feasibility in China and were closed by the end of the 1990s (Chiao and Sun 2007).

After closure of ABE plants in China, the petrochemical industry covered less than half of national butanol demand, and a considerable amount of butanol was imported. However, the fermentative ABE industry returned to economical competition once again in China after the increase in petroleum price. During 2007–2008, about five Chinese ABE plants restored to ABE production and about six new ABE plants were built (Table 8.5) (Ni and Sun 2009; Chiao and Sun 2007). At this time, the world's largest biobutanol plant, Jiangsu Lianhai Biological Technology Co., Ltd., was founded by Liantong Enterprise Co., Ltd., Shangyou Realty Co., Ltd., and Hong Ji Limited. The company plans to have an annual ABE production of 600,000 t, accompanied by 800,000 t carbon dioxide, 20,000 t hydrogen, and

Table 8.5 Some of the ABE plants in China (Ni and Sun 2009)

Plant	Starting date	Capacity (tons/year)	Location
Ji-An Biochemical Co. Ltd.	2007, 12	100,000	Jilin
Guiping Jinyuan Alcohol Industry Co. Ltd.	2007, 8	30,000	Guangxi
Cathay Industrial Biotech Co. Ltd. (Jilin plant)	2008, 3	30,000	Jilin
Jinmaoyuan Biochemical Co. Ltd.	2008, 3	30,000	Jiangsu
Lianyuang Lianhuan Chemical Product Co. Ltd.	2008, 1	40,000	Jiangsu
Jiangsu Lianhai Biological Technology Co. Ltd.	2008, 1	50,000	Jiangsu
Tonglia Zhongke-Tianyuan Chemical Co.	2007, 1	10,000	Inner Mongolia
Jilin Zhonghai Chemical Co. Ltd.	2007, 3	5,000	Jilin
Heilongjiang Haocheng Chemical Co. Ltd.	2007, 8	5,000	Heilongjiang
Tangshan Ji-Dong Solvent Co. Ltd.	2007, 9	5,000	Hebei
Hebei Jizhou Solvent Plant	2007, 9	3,000	Hebei

300,000 t other products. It is reported that the company achieved an annual ABE output of 300,000 t after completion of the third phase of construction in 2010 (www.lianhai.cn/en/).

8.5.3 South African's Industrial ABE Processes

The fermentative ABE industry was actively working in South Africa for several decades (Bahl and Dürre 2001). One of the long operating ABE industries was operated by the National Chemical Products (NCP) Company in South Africa using fermentation and distillation in batch modes (Bahl and Dürre 2001). In 1935, this ABE plant was constructed in Germiston, South Africa, as a part of a set of industries together with plants for ethanol, yeast, vinegar, and dry ice production. The process with twelve 90,000 L fermenters was designed and established by Ets. Barbet, a French engineering company, using the licensed technology developed by Usines de Melle in France (Bahl and Dürre 2001). After 1944, when NCP became a public company, the Distillers Company of London became the major stockholder. In 1965, another British company, BP Chemicals, took the share, but a South African company, Sentrachem, purchased all BP chemicals shares in NCP within two years (Bahl and Dürre 2001).

The NCP ABE fermentation process was firstly designed for the production of ABE from maize using the Melle strain, but a number of changes were made during ups and downs of the process in its lifetime. During the years 1943–1945, the factory encountered with serious challenges which accompanied with a number of significant changes in the process (Bahl and Dürre 2001).

In 1943, the bacteriophage infection has been experienced by the factory (Jones et al. 2000). By continuing this problem along with increasing request for maize, the company management decided to use blackstrap molasses instead of maize. The blackstrap molasses was a by-product of a local cane sugar industry that was readily available to be utilized as the carbohydrate source for ABE fermentation. The first trials of using molasses in the fermentation by the Melle culture gave poor results (Bahl and Dürre 2001). In 1945, in order to improve the fermentation of molasses, the Melle culture was successfully substituted with industrial strains supplied by Commercial Solvent Corporation (CSC) in the USA (Bahl and Dürre 2001).

In this process, the blackstrap molasses as the carbohydrate source was supplemented with nitrogen, phosphorous trace elements, and buffering capacity agents. The organic nitrogen content of molasses was increased by addition of ammonium sulfate. The high buffering capacity was obtained by the addition of calcium carbonate (Bahl and Dürre 2001). In addition, 25 % ammonia liquor was fed into the fermentation vessel during the transition phase (acidogenic to solventogenic) to control the pH and as an additional nitrogen source. (Bahl and Dürre 2001). Furthermore, corn steep liquor at concentration of 1.5 % was added to the mash as an additional source of organic nitrogen, amino acids, polypeptides, growth stimulators, and vitamins (Bahl and Dürre 2001). The molasses mash was diluted by the recycled water to give a sugar concentration between 5 and 8 % (Bahl and Dürre 2001).

The low sugar to ash ratio of the molasses had inhibitory effect on the culture used for ABE fermentation limited the process efficiency which further led to poor solvent yields.

The process was efficiently in use until 1960, when one of the factory fermenters was exploded. After restarting the process, the performance of the fermentation was considerably diminished. The presence of a phage with a contractile tail confirmed by electron microscopy was reported as the reason for the poor fermentation (Bahl and Dürre 2001; Jones et al. 2000). In 1976, the bacteriophage contamination was also reported to be the main problem. Using unfiltered nitrogen instead of carbon dioxide for purging the fermenter was reported to be one of the reasons for the contaminating (Jones et al. 2000). After confirmation of the phage infection, the plant was closed to perform cleaning and disinfection strategies for 10 days. Similar problem was recorded in 1979, when contamination with a pseudo-lysogenic phage became a widespread problem (Jones et al. 2000). Even though these problems were mostly overcome in the NCP plant, the production of solvents completely ceased due to shortage of molasses in 1981.

8.6 Suggested Processes for ABE Production from Lignocellulose

From the three carbon-based polymers provided by the lignocelluloses, cellulose and hemicellulose have been utilized as a carbon source for ABE production. In contrast with the most ethanol-producing microorganisms, solvent-producing

clostridia have an ability to produce solvents from hemicellulose-derived pentoses as well as from hexoses. However, in the process of obtaining fermentable sugars from lignocelluloses, some compounds with high inhibitory effect on ABE fermentation will be released or formed causing major hindrances during the fermentation (Ezeji et al. 2007).

Sugar polymers in the lignocelluloses may be utilized for ABE fermentation after hydrolysis of the fermentable sugars. The hydrolysis of lignocelluloses for ABE production can be carried out by acid or enzymatic process, or combination of both (Amiri and Karimi 2013).

8.6.1 Fermentation of Hemicellulosic Hydrolysate

8.6.1.1 Using Acid Hydrolysis

Acid hydrolysis is a traditional process for obtaining sugars from lignocellulosic materials which was industrially used for ABE production in Russian plants. Acid hydrolysis of lignocelluloses can be performed with concentrated or diluted mineral acids. Even though concentrated acid processes generally give higher sugar yield, its application has been restricted due to high investment and maintenance costs (Taherzadeh and Karimi 2007a).

Dilute acid process that has been used for ABE production is quite different from that developed to be used for ethanol production. Through the acid treatment of lignocelluloses, both cellulose and hemicellulose are partially hydrolyzed to monomeric sugars (Wyman et al. 2005). Depending on the hydrolysis conditions, e.g., temperature, time, acid concentration, and different amounts of hexoses and pentoses are released which may be degraded afterward (Amiri et al. 2010; Wyman et al. 2005). The hydrolysis processes developed for ethanol production by traditional yeast fermentation aimed at production of "hexose hydrolyzate" which is accompanied with large degradation of pentoses (Zverlov et al. 2006). Solvent-producing clostridia, however, can efficiently uptake hemicellulosic pentoses. Partial acid hydrolysis of lignocelluloses to "pentose hydrolyzate" not only is more easily attainable but it may also give better fermentation results in comparison with the complete hydrolysis. In 1960, the partial hydrolysis of lignocelluloses by dilute sulfuric acid treatment for ABE production was developed at the Doshukino plant research laboratory (Zverlov et al. 2006). In this process, the grinded lignocelluloses, e.g., hemp waste, sunflower shell, and corncobs, in the powder form were subjected to 1 % (v/v) sulfuric acid at 115–125 °C for 1.5–3 h. The hydrolysates were then neutralized by the addition of lime ($CaOH_2$) (Zverlov et al. 2006). The type of substrate and the hydrolysis conditions were reported to be effective on the fermentation yield. The fermentation of pentose syrup resulted in relatively the same yield of 32 % (35–36 % from flour starch) but with longer fermenting time (Zverlov et al. 2006). The fermentation of pentose hydrolysate accompanied with flour starch had the same rate and yield as the traditional fermentation. It was

reported that using pentose hydrolysates instead of pure starch resulted in 15–30 % higher ethanol production probably due to fermentation of calcium acetate (Zverlov et al. 2006).

Utilization of dilute acid hydrolysis for production of acetone, butanol, and ethanol from the hemicellulosic hydrolysate of lignocellulosic materials was the subject of several studies (Table 8.6).

8.6.1.2 Other Methods for Obtaining Hemicellulosic Hydrolysate

The polysaccharides in hemicellulose can be readily hydrolyzed to fermentable sugars with dilute mineral acids at modest temperature (Taherzadeh and Karimi 2007a). However, dilute acid hydrolysate of hemicellulose contains inhibitors that prevent efficient ABE fermentation. Ezeji et al. (2007) investigated the effect of some lignocellulosic hydrolysate inhibitors associated with *C. beijerinckii* BA101 growth and fermentation. Thus, in order to have less inhibitory components, some other methods, e.g., hot water extraction (Sun and Liu 2012), steam explosion (Ranjan and Moholkar 2013), and SO_2-ethanol-water (SEW) (Survase et al. 2011), have been evaluated for obtaining hemicellulosic hydrolysate from lignocelluloses for ABE production.

Hydrothermal processing of lignocelluloses, e.g., hot water extraction or autohydrolysis, steam explosion, and wet explosion, is a method for obtaining hemicellulosic hydrolysate using only water/steam and lignocellulosic material as the reagents. Through the steam explosion, the materials are deconstructed mainly as a result of sudden pressure drop. On the other hand, autohydrolysis and wet explosion of the lignocelluloses are based on the depolymerization of hemicellulose by hydronium ions provided from water autoionization in the initial stages and in situ-generated compounds (e.g., acetic, uronic, and phenolic acids). The heterocyclic ether bonds of hemicelluloses are attacked, leading to both generation of oligosaccharides and the splitting of the acetyl groups from the hemicellulosic fraction of the raw materials. Sun and Liu (2012) used the hemicellulosic hydrolysate obtained from hot water extraction or autohydrolysis of sugar maple (at 160 °C, for 120 min) for ABE fermentation. To convert polysaccharide content of hydrolysate into sugar monomer, the wood extract was hydrolyzed with dilute sulfuric acid. However, the untreated hydrolysate completely inhibited the growth of the *C. acetobutylicum* ATCC 824. Therefore, the hydrolysate obtained after the hot water extraction was treated by multistage process containing microfiltration, nanofiltration, secondary hydrolysis with dilute acid, and overliming. These stages were selected for two main purposes: (1) increasing the concentration of fermentable sugars by nanofiltration and secondary acid hydrolysis and (2) decreasing the concentration of inhibitory compounds achieved by nanofiltration and overliming. Nanofiltration apparently played the two roles by increasing the total concentration of fermentable sugars and removing a part of inhibitors, such as phenolic compounds. On the other hand, the suspended solid particles removed by microfiltration did not show any inhibitory effect on butanol production. In another study, Ranjan and Moholkar

Table 8.6 ABE production from hemicellulosic hydrolysate obtained by dilute acid hydrolysis

	Dilute acid conditions											
Substrate	T (°C)	t (min)	Acid (%)	Solid loading (g/L)	Culture	Treatment	Initial sugar (g/L)	ABE (g/L)	Yield (g ABE/ g sugar)	Yield (g ABE/kg raw substrate)	Productivity (g/L/h)	References
Brewing bagasse	96	300	1.6 (0.3 M)	156	*C. acetobutylicurn* ATCC 824	–	45.5	1.3	0.11	8.3	0.002	Juanbaró and Puigjaner (1986)
Corn stover supplemented with glucose	160	20	1	86	*C. beijerinckii* P260	Overliming	60	26.27	0.44	305	0.31	Qureshi et al. (2010)
Switchgrass supplemented with glucose	120	60	1	86	*C. beijerinckii* P260	Overliming	60	ND	–	–	–	Qureshi et al. (2010)
Wheat barn	121	45	0.75	90	*C. beijerinckii* 55025	–	53.1	11.8	0.32	131	0.16	Liu et al. (2010)
Rice straw	60	24 h	1	80	*C. acetobutylicum* NCIM 2337	–	39.9	20.6	–*	257	<0.08	Ranjan et al. (2013)

*Due to loss of water during the long fermentation (12 days), the presented yield was not precise

(2013) used untreated hemicellulosic hydrolysate obtained by hydrothermal processing of rice straw at 121 °C, for 30 min for ABE fermentation. They produced ABE with concentration of 1.92 g/L (1.6 g/L butanol) after the fermentation of the hemicellulosic hydrolysate for 120 h. In comparison with dilute acid hydrolysis, the hydrothermal processing has the advantages of limited corrosion to process equipment, little or no sludge generation, lower capital and operational costs, and less cellulose degradation (Garrote et al. 1999).

Furthermore, some pulping processes, such as SO_2-ethanol-water pulping, formerly developed for separating cellulose fibers from lignocellulosic materials and were used for the production of fermentable hemicellulosic hydrolysate for clostridia (Retsina and Pylkkänen 2007). SEW pulping is a hybrid of acid sulfite and organosolv pulping process, hydrolyzing hemicellulose with a high yield. However, it was reported that, without a proper conditioning, the spent liquor is not fermentable by clostridia species. Similarly, the fermentation of the liquor obtained by ethanol organosolv pretreatment of rice straw was failed even after evaporation of the ethanol and dilution after different ratios (Amiri et al. 2014). Survase et al. (2011) used a sequence of conditioning steps for making SEW spent liquor from spruce fermentable by *C. acetobutylicum* DSM 792. The SEW spent liquor was subjected to evaporation, steam striping, liming, catalytic oxidation, and anion exchange treatment (Amberlite XAD-4) for obtaining a fermentable hydrolysate. After conditioning, the spent liquor was subjected to fermentation by *C. acetobutylicum* DSM 792. Total ABE concentration of 3.09 g/L was obtained with fourfold-diluted liquor, whereas twofold and eightfold dilution of the liquor resulted in ABE concentrations of 1.48 and 0.51 g/L, respectively. However, using eightfold-diluted liquor for inoculum preparation increased the ABE production from twofold diluted to give ABE concentration of 5.22 g/L.

8.6.2 Fermentation of Cellulosic Hydrolysate

More than 40 % of the common lignocellulosic materials composed of cellulose that can selectively be converted to glucose as one of the most preferred carbon source of clostridia. Among the main butanol-producing clostridia, *C. acetobutylicum* has shown to naturally produce a cellulosome, a multienzyme complex consisting of several catalytic components surrounding a scaffold protein for converting cellulose (Jang et al. 2012b). However, it has no cellulolytic activity suggesting that some element of the cellulosome is missing or not expressed. Several studies have been conducted to characterize the existing cellulase gene cluster in *C. acetobutylicum* and to discover the reason for the lack of its cellulolytic properties (Nölling et al. 2001; Sabathé and Soucaille 2002). However, the cellulose can indirectly be utilized by solvent-producing clostridia after converting it into fermentable sugars of glucose and cellobiose. Therefore, like the technologies developed for bioethanol production from lignocellulosic materials, a chemical or enzymatic hydrolysis can be used after a proper pretreatment for preparing a

fermentable hydrolysate. Different methods have been developed for obtaining a cellulosic hydrolysate rich in fermentable sugars for ABE production.

A number of pretreatment technologies have been used prior to enzymatic hydrolysis of lignocelluloses to obtain a cellulosic hydrolysate suitable for ABE production (Marchal et al. 1992; Wang and Chen 2011; Cheng et al. 2012; Gao et al. 2012). The cellulosic hydrolysates, in comparison with the hemicellulosic ones, generally contain higher amount of preferred sugars glucose and cellobiose and fewer amount of inhibitors because of the selective enzymatic hydrolysis of the pretreated materials.

8.6.2.1 Large-Scale ABE Production from Cellulosic Hydrolysate

The first large-scale process for the production of ABE from cellulosic hydrolysate derived from lignocellulosic materials was developed at Institut Français de Pétrole (IFP) in 1980 (Marchal et al. 1992) (Fig. 8.7). The steam explosion pretreatment, hydrolysis, and ABE fermentation processes were optimized for ABE production from corncobs.

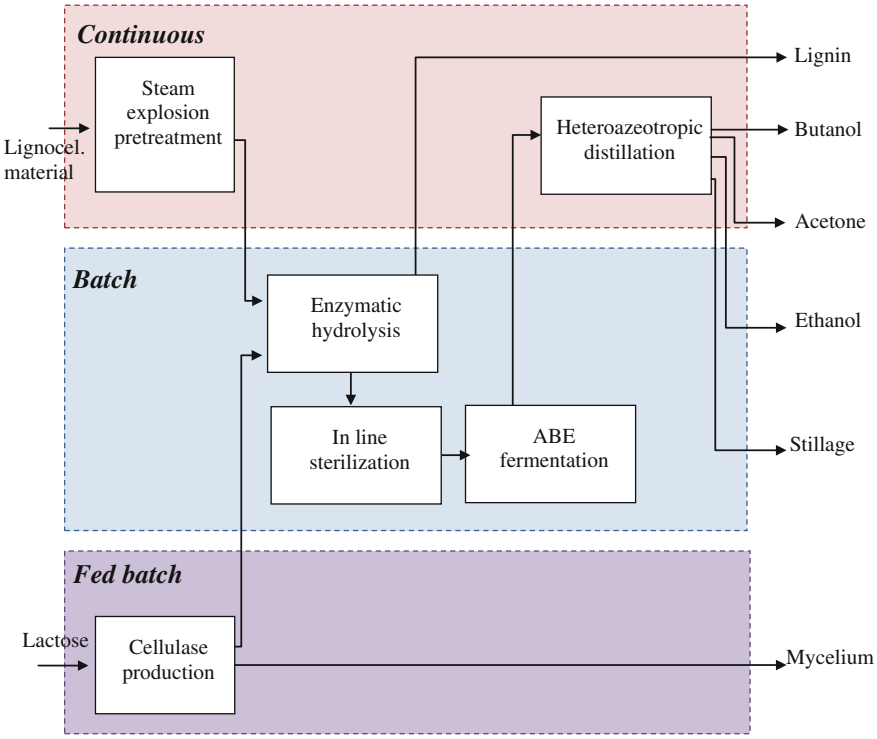

Fig. 8.7 Process scheme for ABE from cellulosic part of lignocelluloses developed by IFP (Marchal et al. 1992)

Steam explosion is thermochemical pretreatment in which the lignocellulosic material is subjected to high pressure and temperature for a few seconds to several minutes and suddenly undergoes an explosive decompression by reducing the pressure. Removing most of the hemicellulose from the material into a liquid stream is the main feature of this pretreatment that improves the enzymatic digestion of cellulose. In the process of ABE production, a continuous steam explosion pretreatment with the capacity of 2–4 t of raw material/h was used for the pretreatment of corncobs (Marchal et al. 1992). Even though a hemicellulosic liquid stream, obtained by the pretreatment, was rich in soluble oligomeric hemicellulosic sugars, it was separated from the pretreated materials and was not used for ABE production due to its inhibitory effects on the fermentation.

The water-washed pretreated substrate was subjected to hydrolysis for cellulosic hydrolysate production which was subsequently fermented to ABE. In this process, the hemicellulosic liquid stream was used as an alternative to lactose for cellulase production by *T. reesei* (Marchal et al. 1992). The enzyme prepared in hemicellulosic hydrolysate was particularly suitable for the hydrolysis of corncobs, pretreated by steam explosion because of other enzymes activities such as xylanase. The enzymatic hydrolysis of the water-washed pretreated corncobs was conducted in a 25 m^3 batch stirred reactor at 50 °C and pH regulated at 4.8 that resulted in the production of 57.2 g/L total sugar (Marchal et al. 1992).

The obtained hydrolysate was detoxified by heating to 70 °C at neutral pH in the presence of calcium hydroxide through which the concentration of some inhibitors specially *p*-coumaric were reduced. On-line sterilization of the hydrolysate was performed at 130–140 °C for 30 s in order to control microbial contaminations without degrading the sugars. After the addition of nutrients, viz. $(NH_4)_2SO_4$, KH_2PO_4, $MgSO_4$, $FeSO_4$, yeast extract, and antifoam, the hydrolysate was fed to a 50 m^3 sterilized fermenter, and the fermentation was conducted at 35 °C by addition of 4–5 % inoculum (Marchal et al. 1992).

Ropars et al. (1992) optimized the steam explosion pretreatment for ABE production with respect to the severity of pretreatment. The "severity index" (Ro) associates the effects of time (t, min) and temperature (T, °C) of the pretreatment was defined as $Ro = t \exp[(T-100)/14.75]$. It was shown that success in the enzymatic hydrolysis which described by high sugar production does not necessarily guarantee the appropriate ABE production through the fermentation. Using the continuous pretreatment process, the highest sugar concentration of about 53 g/L was obtained by the hydrolysis of the materials pretreated with severity index between 2,500 and 4,000. However, the highest ABE concentration was obtained by using the pretreatment at a relatively lower severity index of 979 (1 MPa, 5 min) (Marchal et al. 1992). Therefore, the pretreatment conditions should be optimized based on the final ABE production and the hydrolysate with the highest total sugar concentration.

8.6.2.2 Advances in ABE Production from the Cellulosic Hydrolysate

The cellulose content of lignocellulosic materials can be utilized for ABE production after converting it into fermentable sugars. In this process, the first stage is treating the materials for an efficient hydrolysis process, i.e., the second stage. The hydrolysate obtained after these two stages is subjected to the fermentation by solvent-producing clostridia for ABE production. The pretreatment stage plays a crucial role in ABE production from lignocelluloses (Jang et al. 2012a; García et al. 2011; Jurgens et al. 2012). The quality of pretreatment given to the feedstock highly affects the amount of sugar produced through the hydrolysis. However, this is not the only effect of pretreatment on ABE production process. ABE fermentation is highly sensitive to some chemicals in the hydrolysate that may act either as an inhibitor or a stimulator of the fermentation (Qureshi et al. 2013). Therefore, the pretreatment also manipulate ABE production by affecting the composition of the hydrolysate. In addition, selected pretreatments may offer some additional benefits for improving the economy of ABE production, e.g., separation of pure lignin as a value added by-product (Amiri et al. 2014; Mirmohamadsadeghi et al. 2014).

In the process of obtaining cellulosic hydrolysate, the liquid stream obtained by pretreatment containing lignin and/or hemicellulosic oligomers is separated, and the pretreated materials are subjected to enzymatic hydrolysis preferably after washing. Different treatments including steam explosion (Marchal et al. 1992; Wang and Chen 2011), alkaline with NaOH (Gao et al. 2012; Moradi et al. 2013), alkaline peroxide (Wang and Chen 2011; Cheng et al. 2012), dilute acid (Gottumukkala et al. 2013), phosphoric acid (Moradi et al. 2013), and ethanol organosolv (Amiri et al. 2014) pretreatments have been used prior to enzymatic hydrolysis for the production of cellulosic hydrolysate used for ABE production (Table 8.7).

8.6.3 Fermentation of Hydrolysates from Both Cellulose and Hemicellulose

Chemical treatments, such as dilute acid pretreatment, catalyze the hydrolysis of hemicellulose and pretreat the rest of materials for the following enzymatic hydrolysis. Therefore, a possible method for obtaining hydrolysates with relatively higher total sugar concentration is to conduct the enzymatic hydrolysis of pretreated materials in the liquid stream obtained by the pretreatment. Although the yield of enzymatic hydrolysis of cellulose decreased due to inhibitory effect of sugars in the media, the preparation of hydrolysates with higher total sugar concentration by this method has been suggested for improved ABE production from lignocellulosic materials (Qureshi et al. 2008a).

The pretreatments of dilute acid hydrolysis (Qureshi et al. 2008a), alkaline peroxide (Qureshi et al. 2008b), prehydrolysis with SO_2 (Parekh et al. 1988), and wet disk milling (Zhang et al. 2013) have been previously used in combination with

Table 8.7 ABE production from cellulosic hydrolysate obtained by enzymatic hydrolysis of pretreated lignocellulosic materials

	Pretreatment		Enzymatic hydrolysis			Fermentation	ABE production				
Substrate	Type	Conditions	Solid loading (g/L)	Initial sugar (g/L)	Detoxification of hydrolysate	Culture	ABE (g/L)	Yield (g ABE/g sugar)	Yield (g ABE/kg raw substrate)	Productivity (g/L/h)	References
Corncobs	Steam explosion	1.3 MPa, 3 min	111.5	57.2	Heating at neutral pH in the presence of calcium hydroxide	*C. acetobutylicum* IFP 913	20.5	0.31	~137	0.45	Marchal et al. (1992)
Corn stover	Steam explosion	1.1 Mpa, 4 min	100	53.5[a]	(1) concentrating by rotary vacuum evaporator; (2) treatment by activated charcoal	*C. acetobutylicum* ATCC 824	12.4	0.30	<124[b]	0.17	Wang and Chen (2011)
Corn stover	Alkaline peroxide	4 % H_2O_2 and 1 % NaOH, 24 h	100		Ditto	*C. acetobutylicum* ATCC 824	12.1	0.30	<121[b]	0.17	Wang and Chen (2011)
Bagasse	Alkaline peroxide	2 g/L H_2O_2 and 15 g/L NaOH	20	13.4	–	Mixed culture[c]	2.7	0.25	<135[b]	0.05	Cheng et al. (2012)
Corn stover	Alkali	120 °C, 2 % NaOH, 30 min	100	49.5	–	*C. acetobutylicum* ATCC 824	15.4	0.41	<154[b]	0.21	Gao et al. (2012)
Rice straw	Alkaline peroxide	2 g/L H_2O_2 and 15 g/L NaOH	20	16.6	–	Mixed culture[c]	3.8	0.27	<190[b]	0.07	Cheng et al. (2012)
Rice straw	Alkali	0 °C, 12 % NaOH, 180 min	20	10.8	–	*C. acetobutylicum* NRRL B-591	2.8	0.26	64.1	0.04	Moradi et al. (2013)
Rice straw	Dilute acid	121 °C, 4 % H_2SO_4, 60 min	100	52.08	Anionic resin Seralite SRA400 (SRL, India)	*C. sporogenes* BE01[d]	5.52	~0.21	<55[b]	0.05	Gottumukkala et al. (2013)
Rice straw	Phosphoric acid	50 °C, 85 % H_3PO_4, 30 min	20	8.6	–	*C. acetobutylicum* NRRL B-591	2.1	0.24	63.0	0.02	Moradi et al. (2013)

(continued)

Table 8.7 (continued)

	Pretreatment		Enzymatic hydrolysis			Fermentation	ABE production				
Substrate	Type	Conditions	Solid loading (g/L)	Initial sugar (g/L)	Detoxification of hydrolysate	Culture	ABE (g/L)	Yield (g ABE/g sugar)	Yield (g ABE/kg raw substrate)	Productivity (g/L/h)	References
Rice straw	Ethanol organosolv	180 °C, 75 % EtOH, 1 % H_2SO_4, 30 min	50	22.3	–	*C. acetobutylicum* NRRL B-591	7.9	0.35	123.9	0.12	Amiri et al. (2014)
Rice straw	Ethanol organosolv	180 °C, 75 % EtOH, 1 % H_2SO_4, 30 min	80	23.6	–	*C. acetobutylicum* NRRL B-591	10.5	0.44	103.2	0.20	Amiri et al. (2014)

[a] The hydrolysate was concentrated in a rotary vacuum evaporator at 60 °C

[b] The yield was calculated based on reported data assuming with some assumptions, e.g., solid recovery of 100 %

[c] The mixed culture contained *C. saccharoper butylacetonicum, C. butylicum, C. beijerncki, and C. acetobutylicum*

[d] A non-acetone producing strain was used, so butanol concentration, yields, and productivity were reported

the enzymatic hydrolysis for preparing a combined hydrolysate containing high concentration of hemicellulosic and cellulosic sugars for ABE production (Table 8.8).

Utilization of the hemicellulosic sugars obtained by the pretreatments mainly increases the concentration of sugars in the final hydrolysate; however, the harsh conditions used in the pretreatments produce wide range of chemicals that may have inhibitory effects on the fermentation. It is reported that syringaldehyde, ferulic, and *r*-coumaric acids in dilute acid hydrolysates have inhibitory effects on ABE fermentation by *C. beijerinckii* BA101 (Ezeji et al. 2007). Fermentation of the hydrolysate obtained from corn fiber inhibited cell growth and butanol fermentation by *C. beijerinckii* BA101 (Qureshi et al. 2008a). Treatment of the hydrolysate with XAD-4 resin improved the yield and concentration of ABE production (9.3 g/L ABE) (Qureshi et al. 2008a). Similarly, it has been observed that the alkaline peroxide pretreatment generates salts that have inhibitory effect on the fermentation by *C. beijerinckii* P260 (Qureshi et al. 2008b). ABE fermentation was improved after removing salts from the hydrolysate by electrodialysis (Qureshi et al. 2008b).

Although the inhibitors can be removed by the detoxification processes, sugar loss through these processes is commonly unavoidable, leading to additional capital investment (Qureshi et al. 2010; Amiri et al. 2014). Therefore, various efforts are being made to find pretreatment methods to obtain hydrolysates with less inhibitors for efficient ABE production. One of these methods is steam pretreatment with SO_2 catalyst that has been used for ABE production (Parekh et al. 1988). In addition, wet disk milling (WDM) has been recently suggested to produce hydrolysates with low levels of inhibitors (Zhang et al. 2013).

8.6.4 Process Integration

Process integration combines more than one unit operations into a single unit, generally suggested to reduce both capital and operational costs. In the process of ABE production from lignocellulosic materials, the process integration has the additional advantages of reducing the inhibitory effect of end products in both hydrolysis and fermentation. Process integration for biobutanol production was firstly suggested in late 1980s where butanol fermentation was integrated with product separation (Qureshi and Maddox 1990; Ennis et al. 1986).

Integration of the main processes of hydrolysis, fermentation, and recovery has been suggested for improvement of ABE production from lignocellulosic materials (Qureshi et al. 2013).

8.6.4.1 Simultaneous Saccharification and Fermentation (SSF)

In simultaneous saccharification and fermentation (SSF), sugars produced by hydrolytic enzymes are consumed immediately by the fermenting microorganism.

Table 8.8 ABE production from hydrolysate obtained from both cellulose and hemicellulose through combination of a pretreatment with enzymatic hydrolysis

Substrate	Pretreatment		Enzymatic hydrolysis		Detoxification of hydrolysate	Fermentation	ABE production				References
	Type	Conditions	Solid loading (g/L)	Sugar (g/L)		Culture	ABE (g/L)	Yield (g ABE/g sugar)	Yield (g ABE/kg raw substrate)	Productivity (g/L/h)	
Corn fiber	Dilute sulfuric acid	0.5 % sulfuric acid, 121 °C, for 60 min	70	29.8	–	*C. beijerinckii* BA101	1.7	–	–	0.03	Qureshi et al. (2008a)
Corn fiber	Dilute sulfuric acid	0.5 % sulfuric acid, 121 °C, for 60 min	70	29.8	XAD-4 resin	*C. beijerinckii* BA101	9.3	0.39	133	0.10	Qureshi et al. (2008a)
Wheat straw	Alkaline peroxide	35 °C, for 24 h	88.6	60.1	–	*C. beijerinckii* P260	2.59	0.32	29	0.04	Qureshi et al. (2008b)
Wheat straw	Alkaline peroxide	35 °C, for 24 h	88.6	59.6	salts were removed by electrodialysis	*C. beijerinckii* P260	22.2	0.42	250	0.55	Qureshi et al. (2008b)
Pine	Prehydrolysis with SO_2	3 % SO_2, 30 min, 0.5 MPa, 160 °C	120–160	53	–	*C. acetobutylicum* P262	14.0	0.26	231	–	Parekh et al. (1988)
Aspen poplar	Prehydrolysis with SO_2	3 % SO_2, 30 min, 0.5 MPa, 160 °C	120–160	78	–	*C. acetobutylicum* P262	20.8	0.27	216	–	Parekh et al. (1988)
Corn stover	Prehydrolysis with SO_2	3 % SO_2, 30 min, 0.5 MPa, 160 °C	120–160	74	–	*C. acetobutylicum* P262	21.9	0.27	233	–	Parekh et al. (1988)
Corncob	Wet disk milling	–	50	39.7	–	*C. acetobutylicum* SE-1	14.1	0.36	282	0.20	Zhang et al. (2013)

As a result, the inhibitory effects of cellobiose and glucose on the activity of cellulases enzymes are minimized by keeping a low concentration of these sugars. After the pretreatment of lignocelluloses, enzymes are added to the reactor while the reactor is inoculated with a butanol-producing strain.

It was shown that the presence of *T. reesei* cellulase has no inhibitory effects on growth and solvent production by *C. acetobutylicum.* On the other hand, *C. acetobutylicum* does not significantly degrade the cellulases (Marchal et al. 1984). In fact, the SSF process with solventogenic clostridia is a method for direct bioconversion of pretreated lignocellulosic materials to ABE, which is comparable with direct conversion of starch or molasses to ABE through ABE fermentation. Marchal et al. (1984) used SSF process for ABE production from wheat straw. After the pretreatment of straw with 10 % NaOH at 80 °C for 30 min, the hydrolysis of pretreated material with cellulase from *T. reesei* and fermentation with *C. acetobutylicum* IFP 921 was simultaneously carried out at 37 °C and pH 6.5, 17.3 g/L ABE was produced after 36 h with this process (Marchal et al. 1984). It has been shown that the SSF of the alkali-pretreated wheat straw with *C. acetobutylicum* is a promising process for direct bioconversion of the straw to ABE with a rate of the same order of magnitude as in the fermentation of molasses (Marchal et al. 1984). SSF process with *C. beijerinckii* P260 was also evaluated for ABE production from the dilute acid-pretreated wheat straw (Qureshi et al. 2008c). In this process, SSF was carried out by addition of enzymes and actively growing culture to the slurry of the pretreated straw in the hydrolysate obtained in the dilute acid hydrolysis process (Qureshi et al. 2008c). Therefore, along with hydrolysis of the pretreated materials in the early stage of the process, the initial sugar content of the culture provided from the acid hydrolysates was utilized by the clostridia as a carbon source (Qureshi et al. 2008c). The SSF process of dilute acid-pretreated wheat straw (at 35 °C, pH 6.5) in a culture with initial sugar concentration of 25.6 (5.2 g/L glucose) resulted in the production of 11.9 g/L ABE, which was 27 % higher that of obtained in the separate hydrolysis and fermentation (SHF) process (Qureshi et al. 2008c).

The differences between optimum conditions of the hydrolyzing enzymes and fermenting microorganism have been reported to be a drawback of SSF for ethanol production by yeasts (Taherzadeh and Karimi 2007b). The optimum temperature for cellulases is usually between 45 and 50 °C, whereas the fermentation temperature is in the range of 30–35 °C for *Saccharomyces cerevisiae* (Qureshi et al. 2008c). On the other hand, solvent-producing clostridia have a higher optimum temperature of 37 °C close to that of cellulases (Qureshi et al. 2008c). Therefore, the condition in which the SSF is conducted is fairly close to the optimum condition of both hydrolysis and ABE fermentation.

In the SSF process of pretreated woody materials, hydrolysis has been reported to be the rate-limiting step (Shah et al. 1991). As a result, SSF proceeded mostly under glucose-limited conditions. SSF of aspen wood pretreated with supercritical CO_2–SO_2 or monoethanolamin (MEA) with *C. acetobutylicum* ATCC 824 at 37 °C resulted in the production of 131–156 g ABE per kg initial dry wood (Shah et al. 1991).

8.6.4.2 Simultaneous Saccharification, Fermentation, and Recovery (SSFR)

Butanol, the dominant product of solvent-producing clostridia, is toxic to the cells. To obtain a high concentration of butanol with an appropriate yield, butanol should be continuously removed from the fermentation broth, referred to as "in situ butanol recovery."

Product removal techniques, such as adsorption (Yang et al. 1994), liquid–liquid extraction (Qureshi and Maddox 1995), perstraction (Qureshi and Maddox 2005), pervaporation (Li et al. 2011), and gas stripping (Qureshi and Blaschek 2001), have been developed earlier for in situ butanol recovery. Each recovery method has its own advantages and disadvantages that have been reviewed previously (Ezeji et al. 2010). Groot et al. (1992) reported the pervaporation and liquid–liquid extraction as the methods with greatest potential to be used for in situ butanol recovery. Even though yield and productivity of solvent production were improved using these methods, the additional cost from capital investment on facilities and energy consumption is higher than the benefit contributed by improvements and energy saving (Xue et al. 2013).

In order to improve the economy of ABE production, another compact process has been suggested to simultaneously perform the hydrolysis, fermentation, and recovery of butanol in a single process. This process has the advantages of both SSF and in situ butanol recovery. The overall process benefits from this system as all three unit operations are performed in a single reactor.

The studies on butanol production employing a SSFR process improved the yield of ABE production from lignocellulosic materials (Qureshi et al. 2013). SSFR process of wheat straw pretreated with dilute sulfuric acid (at 121 °C for 1 h) with *C. beijerinckii* P260 (35 °C, pH 6.5) resulted in the production of 21.4 g/L ABE with productivity and yield of 0.31 g/L/h and 0.41, respectively (Qureshi et al. 2008c). Furthermore, the SSFR process of corn stover pretreated with dilute sulfuric acid (at 160 °C for 20 min) was conducted after overliming as a detoxification process (Qureshi et al. 2014). In this process, about 14 g/L ABE was produced form corn stover with productivity and yield of 0.34 g/L/h and 0.39, respectively (Qureshi et al. 2014).

Even though SSFR improved ABE production from some lignocellulosic materials, this method has some serious drawbacks (Qureshi et al. 2013). The utilization of pretreated lignocelluloses in the fermentation process is accompanied by some difficulties. The transfer of pretreated solids to fermenter should be in highly aseptic conditions to prevent the possible contamination (Qureshi et al. 2013). Furthermore, the axial agitation of biomass and cell broth should be avoided because it negatively affected the fermentation (Qureshi et al. 2013). Butanol removal from the broth by gas stripping which has been used in this process has a relatively low rate, requiring a large amount of gas recycle (Qureshi et al. 2013).

8.7 Concluding Remarks

Biological production of butanol, an advanced biofuel, has gained renewed interest as an approach to sustainable development. The efficient utilization of lignocellulosic wastes has been suggested as one of the main strategies for improving the economy of biobutanol production to be a substitution for current petrochemical routes of butanol production.

The butanol-producing species, genus *Clostridium,* are capable of using a large range of carbohydrate substances, including hexose and pentose sugars. Therefore, cellulose and hemicellulose content of lignocellulosic materials can efficiently be converted to biobutanol after hydrolysis to their pentose and hexose sugars contents. Efficient conversion of the complex structure of lignocellulosic materials to hydrolysates with proper characteristics, e.g., appropriate concentration of total sugars, inhibitors, and stimulators, is the main challenge for economical biobutanol production based on lignocellulosic wastes. From 1960 to 1980, the first industrial plants of ABE production from hemicellulosic and cellulosic hydrolysates were utilized in Dokshukino plant, Russia, and Institut Français de Pétrole plant, France, respectively. By recognition of biobutanol as a biofuel with superior characteristics, an extensive research was conducted for improving ABE production from lignocellulosic materials.

Processes comprising different pretreatment and hydrolysis technologies were evaluated for obtaining appropriate hemicellulosic hydrolysates, cellulosic hydrolysates, or both of them for production of ABE through the subsequent ABE fermentation. In addition, the process integrations of SSF, in situ butanol recovery, and SSFR were employed to reduce the inhibitory effect of end products in hydrolysis and/or fermentation. Significant progress has been achieved in applied technologies to use lignocellulosic materials for ABE production. However, many challenges are still ahead due to the complexity of both the fermentation and the bioconversion of lignocellulosic materials, and research is in progress toward developing technologies for ABE production from lignocellulosic wastes.

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