Chapter 8
Microbial Lipid Production from Lignocellulosic Biomass Pretreated by Effective Pretreatment

Cui-Luan Ma and Yu-Cai He

8.1 Introduction

A worldwide concern has recently aroused about the soaring depletion of natural resources and degradation of environmental conditions, which is leading to an increased interest in alternative and renewable energy sources [1, 2]. Biomass, consisting of inedible plant material that does not compete with our food production, is regarded as a suitable renewable feedstock [3–6]. From the last decade, there has been an increasing interest in the value-added utilization of lignocellulosic biomass, which can be used as the most abundant, inexpensive, and renewable source for production of platform organic molecules, functional materials, liquid fuels, and value-added chemicals [7–21].

Bioresources comprising over 2.0 × 10^{12} tons of annual production are potentially the world's largest sustainable and safe source of energy. Very recently, much research has been focused on developing new chemical strategies for the valorization of biomass into liquid biofuels and chemicals [4, 22–31]. Lipids are one kind of value-added energy-rich compounds, which can produce by oleaginous microorganisms using biomass and/or biomass-hydrolysates [16, 32–35]. Microbial lipids are composed of saturated and unsaturated fatty acids with potential use as nutraceuticals, food additives, and biofuels [4, 36–38]. Microbial lipids include

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triacylglycerols (TAGs), glycolipids, phospholipids, and steryl ester, which have many similarities with plant oils [39, 40]. Fatty acid of microbial lipid is composed of Palmitic acid (C16:0), Palmitoleic acid (C16:1), Stearic acid (C18:0), Oleic acid (C18:1), Linoleic acid (C18:2), Linolenic acid (C18:3), etc. The percentage of these saturated and unsaturated fatty acids very much depends on the type of oleaginous microorganisms and growth conditions [40–43].

Biomass sources like energy crops, agriculture and forest residues, sewage sludge, animal and food waste, municipal solid waste, etc. are generally used for energy production [9, 17, 44–46]. Typically, biomass valorization processes to produce both biofuels and/or bio-based chemicals are mainly consisted of three steps: biomass pretreatment, enzymatic saccharification, and fermentation [43, 47]. Pretreatment is considered as a crucial step in lignocellulosic biomass valorization (Fig. 8.1), which can be used for disrupting recalcitrant lignocellulosic structures and removing lignin and hemicelluloses to make cellulose more accessible to the enzymes for efficient conversion into fermentable sugars [48–53]. Although different pretreatments including physical, chemical, physicochemical, biological, or their combination are available [54–58], the development of a suitable pretreatment to avoid or reduce the formation of inhibitors (furfural and/or hydroxymethyl furfural) deserves the great challenge in biofuel production [59, 60].

In this chapter, various biomass pretreatments for effectively improving the enzymatic saccharification of lignocellulosic biomass are introduced. Furthermore, microbial lipid production from lignocellulosic biomass pretreated by effective pretreatment is discussed.

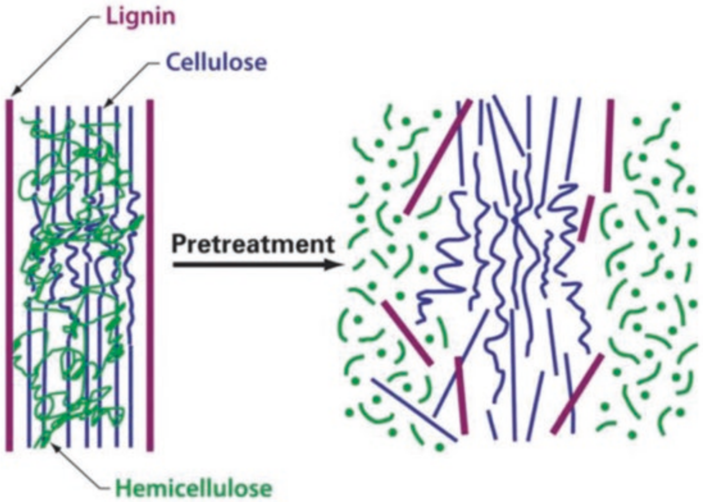

Fig. 8.1 Disruption of lignocellulose structure via pretreatment. (Adapted from Ref. [61, 62])

8.2 Main Components of Lignocellulosic Biomass

Hemicellulose $(C_5H_{10}O_5)_m$, cellulose $(C_6H_{10}O_5)_n$, and lignin $(C_{10}H_{11}O_{3.5})$ are the three major components of lignocellulosic biomass along with small number of other organic compounds such as pectin and protein. Chemical compositions of some lignocellulosic materials are provided in Table 8.1. Most of the biomass is composed of lignocellulose, which is a complex carbohydrate polymer of cellulose, hemicellulose, and lignin [63–65]. The percent composition of cellulose, hemicellulose, and lignin in biomass are in the ranges 30–50%, 20–35%, and 10–20%, respectively.

Cellulose is a linear polysaccharide consisting of β-1,4-linked-*D*-glucose residues, which is made up of crystalline and amorphous region [75–77]. Hemicelluloses (also known as polyose), the second most abundant constituent of lignocellulosic biomass, are polysaccharides in plant cell walls that have β-(1→4)-linked backbones with an equatorial configuration [9, 15, 18, 29]. Hemicelluloses, which have linear and branched structures, include glucomannans, xylans, xyloglucans, mannans, and β-(1→3,1→4)-glucans. It is more energy dense than carbohydrates (hemicelluloses and cellulose) because of its higher carbon-to-oxygen ratio [35, 78]. Lignin is a poly-aromatic non-sugar component typically found in biomass, which confers high mechanical strength and hydrophobicity to plant walls [79]. It is a

Table 8.1 Main components (cellulose, hemicellulose, and lignin) in common lignocellulosic biomass[a]

Lignocellulosic biomass	Cellulose, wt%	Hemicellulose, wt%	Lignin, wt%
Bamboo shoot shell	38.5	23.1	11.4
Cotton seed hairs	80–95	5–20	0
Corncob	45	35	15
Corn straw	42.6	21.3	10–20
Grass	25–40	35–50	10–30
Hardwood stem	40–50	24–40	18–25
Leaves	15–20	80–85	0
Maize stover	37.5	30	10.3
Nutshell	25–30	25–30	30–40
Newspaper	40–55	25–40	18–30
Oat straw	39.4	27.1	20.7
Paper	85–99	0	0–15
Rice straw	32–47	18–28	5.5–24
Rice husk	34.4	29.3	19.2
Softwood stem	45–50	25–35	25–35
Solid cattle manure	1.6–4.7	1.4–3.3	2.7–5.7
Sugarcane bagasse	32–48	19–24	23–32
Switchgrass	45	31.4	12
Wheat straw	33–45	20–32	8–20

[a] Adapted from Refs. [66–74]

highly cross-linked complex aromatic biopolymer formed by polymerization of 4-hydroxyphenylpropanoid monomer units such as syringyl (S), guaiacyl (G) and *p*-hydroxyphenyl (H) units and linked by ether or C–C bonds [61, 79, 80, 81]. The lignin biopolymers are attached to hemicelluloses by covalent bonds creating protection against chemical and biological degradation, inhibiting usability of raw biomass for producing biofuels and biobased chemicals.

8.3 Pretreatments of Lignocellulosic Materials for Enhancing the Production of Microbial Lipids

Various biomass pretreatments including physical (chipping, irradiation, grinding, milling, and pyrolysis) [76, 82–90], chemical (concentrated acid, concentrated alkali, deep eutectic solvent, dilute acid, dilute alkali, ionic liquid, *N*-methylmorpholine-*N*-oxide, ozonolysis, organic solvent, and oxidizing agent) [43, 67, 76, 91–97], physico-chemical (Ammonia fiber explosion, CO_2 explosion, liquid hot water, oxidative pretreatment, sulfite pretreatment, and steam pretreatment) [62, 95, 97, 98–103], biological [67, 75, 104–110], or their combination [99, 110–113] (Fig. 8.2) have been developed for enhancing enzymatic saccharification of biomass and sequential biofuel production (e.g., microbial lipids). The choice of pretreatment technologies that increase the digestibility of cellulose and hemicelluloses to help in cost-effective and eco-friendly conversion of lignocellulosic materials to microbial lipids depends on the compositions of biomass and the generated by-products after pretreatments.

8.3.1 Physical Pretreatment

8.3.1.1 Mechanical Pretreatment

Reduction of biomass particle sizes is a necessary procedure for converting biomass to biofuels. Milling, grinding, and chipping are known as the common mechanical pretreatment techniques for reducing particle sizes [76, 84–88, 90, 114, 115]. Grinding and milling can reduce biomass to 0.20 mm, while chipping can reduce biomass to 10–30 mm [102]. Various milling methods (e.g., knife, ball milling, hammer milling, and attritor milling) can be used for significantly reducing the polymerization degrees of cellulose and lignin, which aid in enzymatic sugar release and subsequent sugar fermentation into biofuels [116–122], wet disk milling (WDM) is used as a popular mechanical pretreatment for treating lignocellulosic biomass because of its low energy consumption [123, 124]. The energy requirements of milling increase with the reduction of biomass particle sizes [82, 125, 126]. This pretreatment is environmentally friendly because no chemicals or reagents were used during the pretreatment; however, it is generally needed to

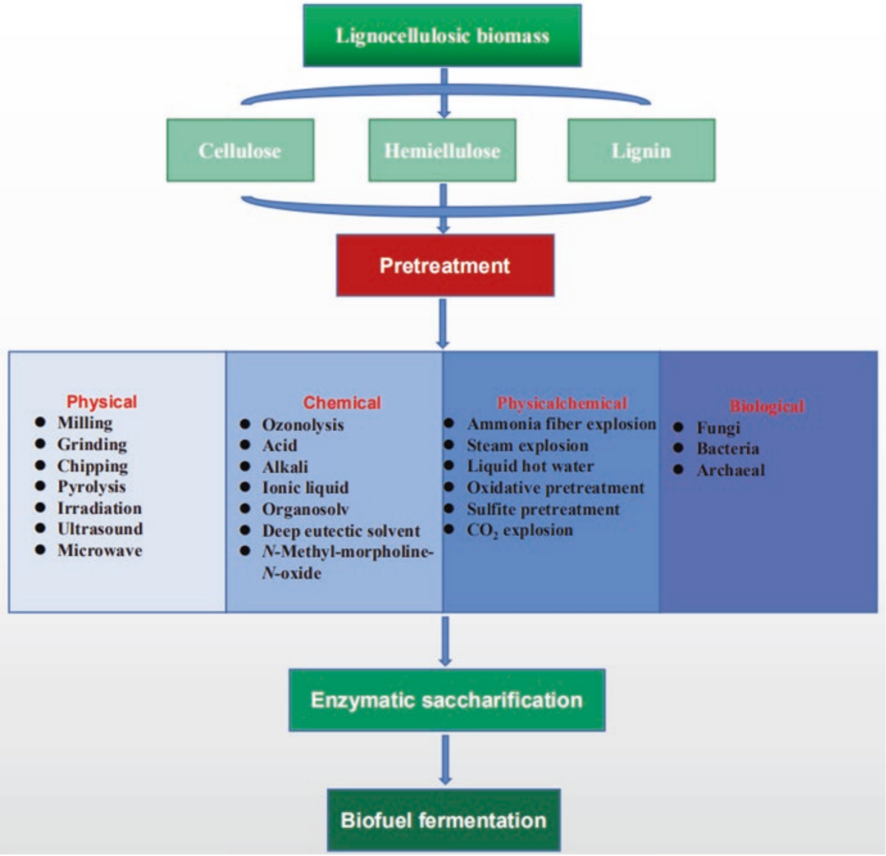

Fig. 8.2 Pretreatment technologies including physical, chemical, physico-chemical, biological, or their combination for enhancing enzymatic saccharification and microbial lipid fermentation

consume a lot of energy. In addition, although the milling can enhance the enzymatic hydrolysis of lignocellulose to a certain extent, it is difficult to thoroughly improve the enzymatic saccharification of biomass because the milling cannot remove lignin effectively, which limited the access of cellulase to cellulose. In most of the cases, a combination of milling and other pretreatments was used to effectively pretreat biomass for enhancing enzymatic saccharification and biofuel production. The milled sorghum stalks were pretreated with 1.25% (w/v) NaOH at 121 °C for 30 min. Commercial cellulases (Cellic C-Tec2 plus Cellic H-Tec2) were complexed for the hydrolysis of pretreated biomass for 48 h. The total lipid of 13.2 g/L and lipid yield of 0.29 g/g were obtained by *Trichosporon oleaginosus* using sorghum stalk hydrolysates as carbon sources [127].

8.3.1.2 Irradiation

Various pretreatments under the irradiation including electron beam, microwave heating, γ-rays, ultrasound, and UV are widely used to treat biomass for improving its saccharification [89, 128–132]). Raw lignocellulosic biomass can be pretreated to decrease the polymerization degree of cellulose, loose cellulose structure, and enhance the enzymatic saccharification under the high-energy irradiation (e.g., UV or γ-rays) [133]. However, high-energy electron irradiation is high-cost consumption, which restrict its application on the large-scale [134]. Microwaves are a type of non-ionizing electromagnetic radiation with wavelengths ranging from as long as one meter to as short as one millimeter [135]. Microwave heating is a volumetric and rapid heating technique with high efficiency and a minimal thermal gradient, which has been widely used to pretreat lignocellulosic biomass because of its high-heating capacity in a short time, low-energy consumption, easy to operation, and minimum formation of inhibitors [102, 136–139]. Microwave-assisted alkali pretreatment of coastal bermudagrass and switchgrass could yield >70% reducing sugars [140, 141]. Ultrasound waves produce cavitation and acoustic streaming, which can alter the morphology of biomass and rupture the carbohydrate fractions in lignocellulosic biomass thereby enhancing enzymatic its saccharification [102, 142–146]. Ultrasound (10–100 kHz) can be used for breaking cell walls and degrading of polymers [142]. Microwave and ultrasound could be used for enhancing the production of microbial lipids. For example, the total lipid of 38.3 g/100 g CDW was obtained from algae *Nannochloropsis* sp. via microwave-assisted pretreatment compared to 23.0 g/100 g CDW obtained from the water-bath system [139]. By increasing the microwave pretreatment temperature from 80 to 95 °C, the oil extraction was obtained from 24% to 33% (dry weight basis) for 30 min of microwave irradiation [147]. Different intensities of ultrasound power (0.1–0.5 W/mL) were used at a frequency of 30 kHz and for 5–60 min to treat mixed microalgal culture. Ultrasound could affect the cell disruption. Moreover, the lipid extraction efficiency could be enhanced under the ultrasound irradiation [148]. At 0.4 kW h/L, up to twofold increase in lipid extraction yields were obtained.

8.3.1.3 Pyrolysis

Pyrolysis is a complex thermochemical conversion process by which a solid or liquid undergoes the thermal degradation into smaller volatile molecules, without interacting with O_2 or any other oxidants [83, 149, 150]. Pyrolysis has also been used for pretreating lignocellulosic biomass in biorefinery processes. The biomass pyrolysis processes typically result in the generation of various products including solids, liquids, and gases [102]. Pyrolysis is found to be more efficient when conducted in the presence of O_2 at lower temperatures [149]. H_2SO_4 (1.0 M) was employed for the pyrolysis of biomass within 2.5 h at 97 °C, and the saccharification was obtained at 85% yield [151]. Coupling lipid fermentation with pyrolysis has been used for converting biomass into lipid. Carboxylic acids, which generated from pyrolysis, were fermented into lipids by *Cryptococcus curvatus* [152]. In pyrolytic liquor, 20 g/L acetate was fermented with *C. curvatus* after neutralization and detoxification to produce ~7.0 g/L dry biomass and 2.0 g/L lipid.

8.3.2 Chemical Pretreatment

8.3.2.1 Alkalic Pretreatment

Alkali-based pretreatment is essentially used for reducing the crystallinity degree, swelling fibers, and removing lignin in corn stover, switchgrass, rice straw, wheat straw, and softwood [95, 153–155]. Alkali can saponify uronic ester linkages between 4-*O*-methyl-*D*-glucuronic acid units in xylan and cleave hydrolysable linkages in lignin and glycosidic bonds of polysaccharides, which causes a reduction in the degree of polymerization and crystallinity, swelling of the fibers, as well as disruption of the lignin structure. The alkali-pretreated lignocellulosic materials are loose, swollen, and porous, which facilitates the cellulose accessibility to enzymes for enhancing their enzymatic saccharification [91, 95]. Various alkaline reagents including oxidative alkali, sodium hydroxide (NaOH), potassium hydroxide (KOH), calcium hydroxide ($Ca(OH)_2$), and ammonia ($NH_3 \cdot H_2O$) have been widely used to pretreat lignocellulosic materials for enhancing enzymatic saccharification at ambient temperature and pressure [86, 91, 153]. NaOH–CH_3OH solution (NaOH 4.0 g, CH_3OH 80 mL) was used to pretreat 40 g raw corn stover at high solids loading for effectively enhancing its enzymatic saccharification [156]. The hydrolysates were used for lipid production by *Cutaneotrichosporon oleaginosum.* Biomass, lipid content, and lipid yield were 50.7 g/L, 61.7%, and 0.18 g/g, respectively. Dilute alkali (NaOH, 2 wt%) pretreatment of corn stover (10 wt%) was conducted a high-pressure reactor at 121 °C for 20 min, and lipid of *Trichosporon dermatis* 32,903 could reach 20.36 g/L with sugar to lipid yields improved to 0.186 g/g [43]. Compared to mineral acid pretreatment, alkali-based pretreatment is required for a relatively long pretreatment time. Less inhibitors (e.g., acetic acid, hydroxyl acids, and minor amounts of furan aldehydes) form when compared to acid pretreatments [91]. High cost of alkali seriously restricts its application. Aqueous ammonia (AA) can be used for pretreatment of biomass at ambient condition, and the leftover ammonia is an important nitrogen source for the growth of energy microorganisms [157–161]. AA can selectively remove lignin from lignocellulosic materials while most of carbohydrates (hemicelluloses and cellulose) remain in lignocellulose [158, 159, 162, 163]. High enzymatic saccharification was obtained at 73.6% when Pecan Nutshell (PS) pretreated with ethylene glycol–H_2SO_4–water (78:2:20, wt:wt:wt) was further treated by AA (25 wt%) for 1 d at 50 °C. Using hydrolysates (20 g/L) as carbon source, the lipid content 0.44 g lipid/g DCW was achieved in *Rhodococcus opacus* ACCC41043 cells [4].

Alkalic salts (e.g., Na_2CO_3, Na_3PO_4, Na_2S, Na_2SO_3) with its low corrosivity have been employed to pretreat biomass for improving its enzymatic saccharification

[159, 164–169]. Alkalic salts containing sulfite (SO_3^{2-}) can cleave β-benzyl ether, α-benzyl ether, and α-alkyl ether linkages on phenolic lignin units [170]. H_2O_2/Na_2CO_3 (15% H_2O_2, 40% Na_2CO_3) and Na_2S/ Na_3PO_4(4% Na_3PO_4, 10% sulfidity) were employed to pretreat corn stover at 120 °C for 40–60 min [171, 172], most of carbohydrates (hemicelluloses and cellulose) in pretreated biomass could be saccharified to fermentable sugars. However, it has several disadvantages include the degradation of biomass-derived sugar, large amount of water for post-pretreatment washing of biomass, and high cost for recovering pretreatment chemicals [102]. Using dilute alkali salts (0.4 wt% Na_3PO_4, 0.03 wt% Na_2SO_3) as pretreatment chemicals for treating sugarcane bagasse in an autoclave within 40 min at 110 °C, enzymatic in situ saccharifications of biomass were carried out at 50 °C [173], which avoided the steps for post-pretreatment washing of biomass and recovery of pretreatment chemicals. High saccharification was obtained at 67.6%. Combination pretreatment (BP-AP) by sequential biological treatment with *Galactomyces* sp. and dilute Na_2CO_3 (0.82 wt%) was employed to treat corn stover for improving its enzymatic saccharification. The fermentable sugars containing 25.6 g/L glucose without removal of Na_2CO_3 could be effectively fermented into microbial lipid by *Rhodococcus pyridinivorans* CCZU-B16. Fatty acids rich in C16 and C18 including oleic acid, stearic acid, palmitoleic acid, and palmitic acid were detected in whole-cells [40].

8.3.2.2 Acid Pretreatment

Industrially, various mineral and organic acids are widely used for pretreating biomass [34]. Acid pretreatment can hydrolyze hemicelluloses into monomeric sugars by destroying the polymeric bonds, increasing the availability of cellulose, and thereby enhancing the saccharification. Inorganic acids (e.g., H_2SO_4, HCl, HF, HNO_3, H_3PO_4) are common acid catalysts for acid-catalyzed lignocellulose biomass pretreatment. Concentrated and diluted acids can be employed to destroy the rigid structure of the lignocellulosic material. Out of these acids H_2SO_4 is mostly used because of its low cost and high efficiency in lignin removal [95]. It is performed at 120–210 °C with H_2SO_4 (<4 wt%) at the different pretreatment time from minutes to hours. Although acid pretreatment is cost-effective, it has some of the drawbacks of high reactor cost for their usage, gypsum formation during neutralization after pretreatment, and formation of inhibitory by-products (HMF, furfural, aliphatic carboxylic acids, etc.). Recently, organic carboxylic acids (e.g., acetic acid, fumaric acid, maleic acid, oxalic acid, succinic acid) are considered as alternatives to inorganic acids. Organic acid pretreatment has the advantages including low energy consumption for acid recovery and low equipment corrosions. High recovery of cellulose components in biomass can be obtained by organic acid pretreatment; however, hemicelluloses are recovered at low yields [95]. Dilute acid (H_2SO_4 1 wt%) pretreatment of corn stover (10 wt%) was conducted a high-pressure reactor at 160 °C for 10 min, and lipid of *T. dermatis* 32,903 could reach 11.4 g/L with sugar to lipid yields improved to 0.16 g/g [43]. Corn fiber and sweet sorghum bagasse were pretreated with dilute H_2SO_4 at a severity factor of 1.06 and 1.02. The sweet sorghum bagasse hydrolysates, which were derived from pretreatment at the severity factor of 1.02, were used for microbial lipid of *C. curvatus* at 10.8 g/L with a lipid content of 40% (w/w) [174]. After the pretreatment with 0.25 wt% H_2SO_4 and 2 h, 11.5 g/kg of microbial lipid was obtained with glucan and xylan recovery rate of 82% and 62% [175], respectively.

8.3.2.3 Ionic Liquid Pretreatment

Ionic liquids (ILs), one kind of salts with low melting points (< 100 °C) and high vapor pressure [176, 177], are regarded as being green solvents because of their high thermostability, low toxicity, excellent solvency, nonvolatility, and recyclability [178–187]. Swatloski et al. [188] reported for the first time that imidazolium-based ILs (e.g., [Bmim][C1]) can dissolve cellulose very well. However, many chlorine-free ILs have been developed because chloride-based ILs can be toxic and corrosive [189]. Acetate-based ILs have higher capability to solubilize cellulose [190] and are less toxic and corrosive [112, 177, 191–196]. ILs have tunable capability to dissolve lignocellulosic materials, resulting in reduction of lignin content, increase of surface area, and enhancement of enzymatic saccharification [92, 95, 197, 198]. However, IL pretreatment is costly because of its high prices compared to commercial solvents, which restricts its application on large-scale in the pretreatment of lignocellulose [95, 179, 187, 199, 200]. Hydrolysates from enzymatic saccharification of IL-treated biomass could be effectively employed as carbon source to produce microbial lipids. Simultaneous saccharification and enhanced lipid production (SSELP) were used for converting IL 1-ethyl-3-methylimidazolium acetate (EmimOAc)-pretreated corn stover into lipids. At 5% (w/v) of solid loading, lipid titer could reach 6.2 g/L after 2 d of fermentation by *C. curvatus* cells, and lipid coefficient was 112 mg/g regenerated biomass, or 81 mg/g raw biomass [201]. IL *N*-methylpyrrolidone (NMP)-1-ethyl-3-methyl imidazolium acetate (EmimAc) could dissolve ≥10 wt% corn stover at 140 °C in 1 h. Enzymatic hydrolysis of pretreated corn stover afforded an 83% total reducing sugars yield and 61% glucose yield within 1 d. The hydrolysates without detoxification could be used as carbon sources for producing microbial lipid by *Rhodosporidium toruloides* Y4 [202].

Very recently, ecologically friendly deep eutectic solvents (DESs) are effectively used for pretreating lignocellulosic materials [203, 204], which, can be synthesized by mixing hydrogen bond acceptors (HBAs) and hydrogen bond donors (HBDs) at the appropriate molar ratio and heating this mixture at the moderate temperature (60–100 °C) under stirring for a few hours until a homogeneous clear DES liquid form (100% atom economy). Dissolution of lignocellulosic materials with DESs can be conducted by using glycerol, ethylene glycol, lactic acid, malic acid, malonic acid, formic acid, nicotinic acid, and oxalic acid as hydrogen bond donors combined in a variety of molar ratios with hydrogen bond acceptors alanine, betaine, choline chloride, proline, histidine, and glycine [67, 203, 204]. Lignin and hemicelluloses in biomass can be effectively removed with various DESs [204, 205]. Glucose yields over 80% were obtained after the enzymatic saccharification of biomass pretreatment with cholinium lysinate ([Ch][Lys]). The hydrolysates were fermented directly by *R. toruloides*—with glucose, xylose, acetate, and lactate fully consumed during fermentation [206].

8.3.2.4 Organosolv Pretreatment

Organosolv pretreatment can be effectively used for the extraction of lignin in biomass, which works by breaking the noncovalent bonds between the lignocellulosic components and disrupting the recalcitrant structures [207–209] Recalcitrance. It has the ability to fractionate lignocellulosic biomass into hemicellulose, cellulose, and lignin with high purity, easy solvent recovery, and solvent reuse. Various organic solvents (e.g., acetone, alcohol, amines, dioxane, esters, formaldehyde, phenol, propionic acid) or aqueous-organic solvent system with and without catalyst have been used for pretreating biomass at temperatures ranging from 100 to 250 °C [210]. Low-molecular-weight alcohols (e.g., methanol and ethanol) are often used as solvents for organosolv pretreatment due to their low boiling points and ease of removal. However, the low-boiling-point alcohols can seriously hinder the development of biomass pretreatment process due to their high volatility and flammability under the high-pressure operation [211, 212]. To avoid these drawbacks of low-boiling-point alcohol pretreatments, high-boiling solvents are of great interest. Glycerol and ethylene glycol (EG) are the most widely used high-boiling solvents for treating lignocellulosic biomass [195, 196, 210, 213–215]. Under the microwave irradiation (200 W) at 100 °C for 5 min, the lignin in corn stover could be effectively removed with EG-$HClO_4$-water (88.8:1.2:10, w/w/w) media [211]. Combination pretreatment with EG-H_2SO_4-water (78:2:20, w/w/w) at 130 °C for 0.5 h and AA (25 wt%) at 50 °C for 1 d was employed to improve the enzymatic hydrolysis of Pecan nutshell. The hydrolysates could be effectively fermented into microbial lipids at 17.1 g lipid/g reducing sugars by *R. opacus* ACCC41043 [4].

To help meet the challenge of biomass conversion, *N*-methyl-morpholine-*N*-oxide (NMMO) has attracted substantial research interest for pretreating biomass. NMMO is a hygroscopic compound crystalline at room temperature [216, 217]. It has melting point at 170 °C, implying nonvolatility and nonflammability. NMMO molecules are capable of softening and dissolving cellulose in biomass because of their high polarity of N–O bonds, which disrupt the hydrogen bonds of the cellulose and further form new hydrogen bonds with the solutes [218]. The operation conditions for these pretreatments are much milder (< 100 °C and atmosphere pressure) as compared to the conventional pretreatment methods. NMMO retains all the advantages of the ionic liquids-ability to dissolve a variety of lignocellulosic substrates (up to 20% by weight) without the need to chemically modify them and more than 99% of the solvent can be recovered due to its low vapor pressure [93]. It is also non-toxic and biodegradable as proven by the work of Lenzig researchers (Ramakrishnan et al. 2010). Cellulose withdrawn from NMMO solutions has also generated increased rates of hydrolysis by cellulases thus implying its potential use in pretreating lignocellulosic biomass for biofuels [218].

8.3.3 *Physical-Chemical Pretreatment*

8.3.3.1 Ammonia Fiber Explosion (AFEX)

AFEX is one kind of alkaline thermal pretreatment with aqueous ammonia ([88, [219–221]). The merit of this pretreatment process is that it does not require small particle size for efficiency, and further, inhibitors are not formed during the pretreatment [149]. AFEX can be carried out at ambient temperature. High saccharification rates (> 90%) based on the carbohydrate content in AFEX-treated biomass can be obtained. During the AFEX pretreatment, ammonia molecules can cause swelling, removal of lignin, and phase change of cellulose crystallinity in lignocellulosic biomass, which promotes in the reactivity of hemicelluloses and cellulose in pretreated biomass [102]. During AFEX pretreatment, no highly inhibitory products (e.g., phenols, furfural, and HMF) generate, which avoids the detoxification step. In addition, aqueous ammonia is easily recycled for reducing overall performance cost. Thus, AFEX is a cost-effective pretreatment technique for treating lignocellulosic biomass if aqueous ammonia can be recovered and recycled for repeated pretreatment. Microbial lipid production from AFEX pretreated and hydrolyzed corn stover using an oleaginous yeast *Lipomyces tetrasporus.* 36.7 g lipids were produced from 1 kg AFEX-pretreated corn stover via SHF at a titer of 8.4 g/L [222].

8.3.3.2 CO_2 Explosion

CO_2 explosion is similar to AFEX. It is a supercritical CO_2-based pretreatment of lignocellulosic biomass [223]. Supercritical CO_2 is considered as one kind of green solvent due to its abundance, low-cost, non-toxicity, non-flammable, and ease to recover [99]. Its critical pressure and critical temperature are 1071 psi and 31 °C, respectively [224]. Various parameters including extraction bed size, performance pressure, performance temperature, and solvent flow can be set to obtain the high yields of specific compounds [225]. The supercritical CO_2 molecules enter lignocellulosic materials at the required temperature and time under the high-performance pressure [99]. Subsequently, the formed H_2CO_3 can hydrolyze hemicelluloses in biomass. The CO_2 release after CO_2 explosion can break the structures of cellulose and hemicelluloses, thereby increasing the surface area of biomass for enhancing its hydrolysis [223, 226]. For CO_2 explosion, no toxin formation makes it an attractive for the pretreatment of biomass [110, 227]. Pretreatment can be used for improving lipid recovery from biomass by disrupting wet cell walls prior to extraction. The extraction of lipid was carried out under the pressurized CO_2 (3500 kPa) [228]. The solubility of CO_2 in *Rhodotorula glutinis* was higher than that of sugar broth media and spent media due to the presence of lipid in *R. glutinis*.

8.3.3.3 Liquid Hot Water (LHW) Pretreatment

LHW pretreatment, commonly known as autohydrolysis, uses pressure to keep water in the liquid state at elevated temperature [95]. It is regarded as an effective pretreatment strategy for selectively recovering hemicelluloses in the liquid stream [54]. In the solid phase, lignin components can be easily recovered with minor losses. No additional chemicals are needed [75]. LHW pretreatment can remove up to 80% of the hemicellulose and to enhance the enzymatic saccharification of pretreated biomass [229]. LHW can be carried out at relatively low pretreatment temperature in the low cost of pretreatment solvent; however, a large amount of water is needed to be recovered in downstream processing [75]. Hot water was used to enhance the microbial lipid production by oleaginous *R. opacus* PD630 and DSM 1069. *R. opacus* PD630 could accumulate lipid from detoxified sweet gum autohydrolysate with the lipid yield of 0.25 g/L of its cell dry weight in lipids while growing on that translates to 0.25 g/L lipid yield, while *R. opacus* DSM 1069 could accumulate lipid from detoxified pine autohydrolysate with the lipid yield of 0.3 g/L [230].

8.3.3.4 Oxidative Pretreatment

Delignification of lignocellulosic biomass can be conducted by using oxidizing agents such as O_2, O_3, H_2O_2, air, or per acetic acid [76, 231–238]. Various reactions including side chain displacements, electrophilic substitution, and oxidative cleavage of aromatic nuclei or cleavage of alkyl aryl ether linkages may happen during the oxidative pretreatment of biomass. The delignification effectiveness is attributed to the high reactivity of oxidizing agents with aromatic rings of lignin in lignocellulosic biomass. The lignin polymers can be oxidized into carboxylic acids, which act as potential inhibitors in the biotransformation or fermentation steps. These inhibitors must be neutralized or removed by additional processes. Oxidative pretreatment can also influence hemicellulose fractions in lignocellulosic biomass. Lignin peroxidase (LiP) can execute the H_2O_2-dependent $C\alpha$-$C\beta$ cleavage of lignin in biomass [239]. H_2O_2 pretreatment undergoes the oxidative delignification by detaching and solubilizing lignin in biomass [240, 241]. Ozonolysis pretreatment can be used for delignification by breaking aromatic rings structures of lignin in bagasse, cotton straw, and wheat straw [88, 242].

Wet oxidation is a simple pretreatment process using air/oxygen along with H_2O or H_2O_2 to fractionate lignocellulosic materials at ≥120 °C [243–246]. The crystalline structure of cellulose in biomass can be opened by wet oxidation [247]. High delignification (65%) is achieved with wheat straw [248]. Alkaline peroxide-assisted wet air oxidation can solubilize 67% of hemicellulose and 88% of lignin in rice husk [249]. Wet oxidation combined with alkali generates the limited formation of fermentation inhibitors (e.g. furan aldehydes and phenolaldehydes), and the main degradation products found from hemicellulose and lignin are carboxylic acids, CO_2, and H_2O. In the wet oxidation, addition of Na_2CO_3 can decrease performance temperature and enhance the removal of hemicellulose, which can avoid the formation of potential inhibitors (e.g., furfurals and HMF) [249]. Hydrolysates could be used to produce microbial lipids from herbaceous lignocellulosic biomass utilizing alkaline hydrogen peroxide pretreatment with NaOH and H_2O_2, which were composed of xylose and glucose as well as acetate and phenolic monomers that could be used as renewable carbon to produce microbial lipids [250].

8.3.3.5 Steam Explosion

Steam explosion is a physico-chemical pretreatment process for the breakdown the lignocellulosic structural components by hot steam (160–260 °C) under the pressure (0.7–4.8 MPa) on the large-scale [75, 98, 101, 102]. Subsequently, the rapid reduction of performance pressure can generate strong shear force in an explosive decompression event, which facilitates the hydrolysis of beta-glycosidic bonds and hydrogen-bonds between the glucose chains, resulting in the degradation of hemicellulose and depolymerization of lignin thereby increasing the cellulose accessibility to cellulases [95, 102]. The advantages of steam explosion include limited use of hazardous chemicals, low energy consumption, and low environmental impact. However, the generation of degradation products from lignin and biomass-derived sugars is unavoidable [75, 251]. Steam explosion pretreatment of microalgae gave the highest lipid extraction yields. The experimental results demonstrate the efficacy and feasibility of the acid catalyzed steam explosion pretreatment, followed by n-hexane lipid extraction. High sugar yields (up to 96%) were obtained with 1.7% H_2SO_4 at 150 °C during steam explosion, and high lipid extraction of exploded microalgae was achieved using n-hexane [252].

8.3.4 Biological Pretreatment (BP)

Lignocellulosic materials are composed of carbohydrate polymers (hemicelluloses and cellulose), coexisting in complex matrices with high carbon content and high aromaticity of highly aromatic biopolymer lignin [38]. Lignin, which is known as the second most abundant terrestrial biopolymers on earth, constitutes ~15–30% of lignocellulosic biomass. Lignin can be underutilized as renewable feedstock for value-added chemicals. The lignin polymer is highly recalcitrant toward chemical and biological degradation due to its molecular architecture. Biomass and its components (e.g., hemicelluloses, lignin, and lignin-derived polyphenols) can be uti-

Table 8.2 Summary of some pretreatment technologies

Pretreatment method	Advantages	Disadvantages
Acid pretreatment	Effective removal of hemicellulose and lignin	High cost, harmful by-products, equipment corrosion requirement of intensive energy
Alkalic pretreatment	Effective removal of lignin and increase of accessible surface areas	Long residence times required, high cost, harmful by-products, requirement of intensive energy
Ammonia fiber explosion (AFEX)	Removal of lignin and hemicellulose to an extent; no generation of inhibitors	Low efficiency for biomass with high lignin content
Biological pretreatment (BP)	Degradation of hemicellulose and lignin to an extent; low energy consumption	Long pretreatment time
CO_2 explosion	Cost-effective; no generation of inhibitors	Low efficiency for removing hemicellulose and lignin; requirement of high cost of high temperature-high pressure equipment and system
Deep eutectic solvent (DES)	Environmentally friendly performance with DES	Lack of economical solutions to DES recycling
Ionic liquid (IL)	Pretreatment by IL with high thermostability, inflammability, low volatility and recyclability; high delignification	Lack of economical solutions to IL recycling
Liquid hot water (LHW)	Requirement of low cost of solvent; generation of minimum inhibitors at low performance temperature	Requirement of a large amount of water; high energy consumption in downstream process
Milling	Environmentally friendly performance without addition of chemicals	High energy consumption, low delignification
Organic solvent	Effective removal of hemicellulose and lignin	Low biomass recovery; solvents need to be drained from the reactor, evaporated, condensed, and recycled; high operation cost
N-methyl-morpholine-*N*-oxide pretreatment (NMMO)	Environmentally friendly performance under below 100 °C	Lack of economical solutions to NMMO recycling
Microwave irradiation	High-heating capacity in a short time, low-energy consumption, easy to operation, and minimum formation of inhibitors	High cost of pretreatment; lack of large-scale equipment
Ozonolysis	Effective removal of hemicellulose and lignin at ambient pressure and temperature	A large amount of ozone required
Pyrolysis	High efficient in the presence of O_2 at lower temperature	High energy consumption

(continued)

Table 8.2 (continued)

Pretreatment method	Advantages	Disadvantages
Steam explosion	Removal of hemicellulose and lignin to an extent, limited use of hazardous chemicals, low energy consumption, and low environmental impact	Generation of degradation products from lignin and biomass-derived sugars at high temperature
Sulfite pretreatment (SPORL)	Removal of hemicellulose and lignin with high efficiency	Degradation of biomass–derived sugar; requirement of large amount of water for post-pretreatment washing of biomass; high cost for recovering pretreatment chemicals
Ultrasonic irradiation	Effective change of the biomass morphology and rupture of carbohydrate fractions in biomass	Lack of large-scale equipment
Wet oxidation	High delignification	High pretreatment temperature

lized via the microbial action by highly efficient bacteria or/and fungi with low-energy consumption, high substrate-specificity, and no generation of undesirable toxic compounds [106, 107, 253–258]. Bacteria and fungi can consume lignin breakdown products and utilize them as carbon sources [16, 33, 35, 259–264], potentiating fuel and chemical production via lignin-consolidated bioprocessing.

Biological pretreatments (BPs) by fungi (e.g., white-rot and brown-rot fungi) have been widely employed as environmentally-friendly approached for pretreating biomass for production of biobased chemicals and biofuels [104, 106, 107, 108, 109, 110, 265–268]. White-rot fungi (e.g., *Trametes versicolor*, *Phlebia radiata*, *Phanerochaete chrysosporium*, *Dichomitus squalen*) are the most effective for delignification in nature [109]. Laccase, manganese peroxidase (MnP), and lignin peroxidase (LiP) are the main extracellular lignin-degrading enzymes of white-rot fungi [255, 269, 270, 271]. LiP catalyzes the homolytic C_a-C_{β} cleavage of lignin and depolymerizes methylated lignin in vitro. MnP has the ability to catalyze the oxidation of Mn^{2+} to Mn^{3+} with H_2O_2 on phenolic (or non-phenolic) lignin units. Laccase is a copper-containing enzyme, part of the group of so-called blue oxidase, that catalyzes the one-electron oxidation of aromatic compounds (e.g., phenolics and amines) by oxygen. Brown-rot fungi, on the other hand, slightly oxidize lignin in wood, which preferentially degrades the polysaccharide components [258, 272]. Although pretreatment with fungi can be carried out with simple protocols, low downstream processing costs, low energy-consumption, and no generation of inhibitors to biofuel fermentation, it has several disadvantages, including slow delignification rates, substantial holocellulose loss, and long pretreatment time.

Compared to fungi pretreatment, BP with bacteria harboring oxidases involving lignin depolymerization are more promising candidates for delignification because of their environmental adaptability and immense biochemical versatility [32, 259, 261, 264, 269, 273, 274]. For bacteria pretreatments, a series of enzymes (demethylase, MnP, LiP, catalase, peroxidase, phenol oxidase, etc.) have been isolated and identified [38, 39, 45, 95, 261, 264]. These enzymes in bacteria can catalyze the demethylation, alkyl-aryl cleavage, cross-linking, and Cα–Cβ bonds cleavage, and value-added lignin derivatives, such as protocatechuic acid, vanillic acid, guaiacol, vanillin, and 4-ethoxy-3-methoxybenzaldehyde, can form under the ambient condition [95, 275]. *R. opacus* PD630 metabolized aromatics, gluconate, alkanes, and acetate, to produce microbial lipids [32, 35, 260, 276], *Rhodococcus jostii* RHA1 degraded lignin to a series of phenolics [38, 263]. Degradation of lignin (39.6%, dry weight) was achieved by performing cofermentation with wild *R. opacus* PD630 and *R. jostii* RHA1 VanA$^-$. Fatty acids (C13–C24), especially palmitic acid (C16:0; 35.8%) and oleic acid (C18:1; 47.9%), were accumulated in cells [41]. Co-culture of *R. jostii* RHA1, *R. jostii* RHA1 VanA$^-$, and *R. opacus* PD630 to produce extracellular peroxidases and oxidases for degrading 33.6% of low-molecular weight lignin derived from dilute acid-pretreated poplar wood, and the lipid content in cells was 0.017 g lipid/g DCW [38].

One-step BP is known as a slow pretreatment process that requires careful control (e.g., growth and equipment conditions) [67]. Combination pretreatments including *Sphingobacterium* sp. LD-1 with NaOH/urea [261], *Pleurotus ostreatus* with 2 wt% H_2O_2 [238], and *Echinodontium taxodii* with 0.25 wt% H_2SO_4 [277] were found to have higher pretreatment efficiency and saccharification than single BP, indicating that combination of BP and other pretreatments has high application for improving enzymatic hydrolysis of biomass and biofuels production [67, 261].

8.4 Conclusion and Future Recommendations

In response to growing concerns about environmental sustainability, energy security, and societal sustainability, various renewable lignocellulosic biomasses have been used as inexpensive feedstock for producing biofuels (e.g., microbial lipids) in recent years. The most applicable pretreatment technologies on lignocellulosic materials such as physical, chemical, physico-chemical, biological, or their combinations for biofuel production have been developed [79, 88, 99, 110, 120, 278–285]. The disadvantages and advantages of these common pretreatment technologies for treating lignocellulosic materials are illustrated in Table 8.2. The trend of future research should be directed to address some issues including the increase in the commercialization on large-scale by analysis of economic aspects and application of a suitable method based on the different biomass structures. Thus, the design of suitable research in order to find an efficient combination of the existing methods is recommended [286–288]. In future, the development of cost-effective pretreatment technologies for improving the enzymatic digestion and microbial lipid production deserve in-depth exploration.

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