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Pretreatments for Enhanced Enzymatic Hydrolysis of Pinewood: a Review

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Abstract Pinewood is an abundant source of lignocellulosic biomass that has potential to be used as renewable feedstock in biorefineries for conversion into advanced biofuels and other value-added chemicals. However, its structural recalcitrance, due to the compact packing of its major components, viz. cellulose, hemicellulose and lignin, high lignin content, and high cellulose crystallinity, is a major bottleneck in its widespread use as a biorefinery feedstock. Typical chemical, thermal, and biological pretreatment technologies are aimed at removing lignin and hemicellulose fractions for improving enzyme accessibility and digestibility of cellulose. This review highlights common pine pretreatment procedures, associated key parameters and resulting enzymatic hydrolysis yields. The challenges and limitations are also discussed as well as potential strategies to overcome them, providing an essential source of information to realize pine as a compelling biorefinery biomass source.

Keywords Pinewood · Pretreatment · Cellulose · Enzymatic hydrolysis · Saccharification yield · Biorefinery

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Introduction

Lignocellulosic biomass is the most abundant source of carbon on earth, but its composition varies, depending on plant species, season of harvest, and geographical location [1]. Pine, a softwood, is one such renewable source of biomass that can be used for the production of advanced biofuels, energy, and value-added chemicals, most of which are currently derived from non-renewable fossil based sources [2]. Softwoods are common in many forests in temperate and subtropical areas of the world. Several countries have well-established systems for the sustainable management of coniferous forests and wood obtained from these forests is majorly used as a raw material in the pulp and paper industry and/or processed into timber for furniture and building purposes; however, a considerable portion of waste is left behind from harvesting activities that could be utilized for novel applications [3]. The USA has 521 million acres of timberland, 40% of which is in the southern region alone [4]. The predominant native species planted in the south is yellow pine [4]; its abundance and an existing year-round supply chain make it a compelling choice of substrate for production of cellulosic biofuels. Several pine species, for example loblolly pine (*Pinus taeda*), lodgepole pine (*Pinus contorta*), scots pine (*Pinus sylvestris*), insignis pine (*Pinus radiata*), and maritime pine (*Pinus pinaster*) could be used as feedstocks in future integrated biorefinery operations.

The three major polysaccharide fractions of lignocellulosic biomass, i.e., lignin, cellulose, and hemicellulose, are closely associated in the cell wall. To maximally utilize biomass carbohydrates, employing effective pretreatment strategies is a necessity as it is key in overcoming the recalcitrance induced by lignin and the crystalline structure of cellulose [5–7]. Pine is typically more resistant to biocatalytic conversion processes compared to most other lignocellulosic substrates due to its higher lignin content [8]. Nevertheless, its high cellulose content makes

investigation of pine saccharification yields from emerging technologies critical, for its use as a biorefinery feedstock [3]. The effects of pretreatment have been described as a disruption of the cell wall matrix, including the linkages of lignin and hemicellulose with cellulose, as well as a decrease in the degree of polymerization and crystallinity of cellulose [1–3, 5–7]. These effects make carbohydrate fractions more accessible to enzyme cocktails, which are key in hydrolyzing cellulose and hemicellulose into fermentable sugars, including glucose. Each fraction of pine, including cellulose, hemicellulose, lignin, and extractives, can be used for the production of value-added products that have wide ranging applications in various industries, making pine a valuable resource for building a bio-based economy. For example, majority of cellulose obtained from Kraft pulping of softwood biomass is currently used in the pulp, paper, and fiber industry; however, it can also be used to produce advanced biofuels such as ethanol [9]. In addition, cellulose crystals and fibers of micro and nanoscale dimensions can be derived from pure cellulose for use in food, cosmetic, polymer, and biomedical industries [10]. Furthermore, the hemicellulose fraction can be extracted using different chemical and enzymatic procedures to obtain prebiotic galactoglucomannan oligosaccharides [11, 12] and reducing sugars [13]. Lignin can be extracted from organosolv [14] and ionic liquid [15] pretreatments and can be used to produce lignosulfonates [16], lignin-coated cellulose nanocrystals (CNC) and cellulose nanofibrils (CNF) [17], and lignin-based phenol-formaldehyde resins [18]. Pine has been shown to be a rich source of phenolic compounds [19, 20], such as flavonoids [21] that have applications in food and pharmaceutical industries due to their antioxidant properties.

This review, however, specifically focuses on different pretreatment strategies that enhance enzymatic hydrolysis of cellulose for conversion into a glucose stream that could be used to produce advanced biofuels. Based on the method that is used to reduce the recalcitrance of lignocellulosic biomass, pretreatments can be divided into four main categories, i.e., physical, chemical, physicochemical, and biological. Mechanical forces in grinding and milling can be used to reduce feedstock particle size and increase surface area; chemical agents, hot water or steam and fungal microorganisms, can be used to remove and/or degrade lignin and hemicellulose. Ultimately, the main purpose of all these pretreatments is to make the cellulose fraction more accessible to enzymes [22, 23]. Most chemical and thermochemical pretreatments require the use of expensive corrosion-resistant reactors and catalysts and generate large volumes of waste streams that, often, need further treatment before being released or reused [5–7]. Moreover, extensive washing of the pretreated material is often required, to remove sugar degradation by-products generated in the process [24]. Applying a suitable pretreatment that requires low capital and operational costs, improves cellulose digestibility without causing significant sugar and lignin degradation, and generates valuable co-products still remains a major technological challenge in commercialization of cellulosic biofuel processes [5–7]. Various methods, such as dilute acid, alkaline, organosolv, steam explosion (SE), CO_2 explosion, wet oxidation, ozonolysis, liquid hot water (LHW), ammonia fiber explosion (AFEX), and ionic liquid (IL) have been reviewed for their capacity to enhance digestibility of lignocellulosic biomass [8, 22, 23]. However, the effectiveness of each pretreatment depends on the type of biomass in use. For example, while AFEX pretreatment is generally not considered suitable for high lignin feedstocks such as pine [22, 23], sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) is a technology that is considered effective for woody biomass [25–27] but is usually not discussed in most review papers. This review specifically examines common chemical, physicochemical, and biological pretreatment procedures that are pertinent to pine biomass and the resulting saccharification yields. Saccharification or hydrolysis yield, sometimes also termed as enzymatic digestibility yield, is calculated as per the formula given in Eq. 1 [28]. It is defined as the percentage of glucan in the pretreated biomass that is converted to glucose during enzymatic saccharification.

$$Yield\ (\%) = \frac{Glucose\ released\ during\ enzyme\ saccharification\ (g)^{*}\ 0.9}{Glucan\ or\ cellulose\ content\ in\ dry\ wt.\ of\ pretreated\ material\ (g)} \times 100 \quad (1)$$

Pinewood Composition

Pinewood is typically composed of 40–50% cellulose, 15–20% hemicellulose, 25–30% lignin, 5–10% extractives, and less than 1% ash [2, 3, 29, 30]; detailed chemical composition analysis of various species is listed in [29, 30]. Under optimal conditions, a fast-growing pine can produce 13.7 g cellulose per day, which corresponds to 8.2 g lignin, 6.5 g polyoses, and 0.3 g extractives, resulting in a total of 27.7 g or 56 cm^3 of woody biomass being produced by one tree per day [31].

Cellulose, the main structural component of cell walls, is a linear high molecular weight polymer of D-glucose units linked through β-(1,4) glycosidic bonds and the degree of polymerization of wood cellulose can be as high as 10,000 [30]. It is characterized by extensive intra and intermolecular hydrogen bonding between adjacent glucose residues in a single chain and between adjacent chains, respectively, resulting in a tightly packed, highly ordered, and partially crystalline structure that is insoluble in most solvents [30]. Crystallinity of pine cellulose has been found to increase with thermal treatments [32] and decrease with physical treatments such as ball milling [33] and alkaline treatments such as mercerization, a process that also causes polymorphic transformation of cellulose I into cellulose II [34]. Hemicelluloses (also called polyoses) are an amorphous mixture of polysaccharides closely associated with cellulose in the cell wall; these are composed of neutral sugars, i.e., pentoses (xylose and arabinose) and hexoses (glucose, galactose, and mannose), and hexuronic acids (4-*O*-glucuronic and galacturonic acids) [29]. Compared to cellulose, the molecular chains of hemicellulose are shorter and more branched due to side groups attached to the backbone. The xylan backbone of softwoods is very different from that of hardwoods; it does not have acetyl groups and it has a higher proportion of arabinofuranose and 4-*O*-methylglucuronic acid side chains [29]; in addition, softwoods also contain higher quantities of chains with a mannan/glucan backbone and galactose side chains, which is why softwood hemicellulose is often called galactoglucomannan and/or arabinoglucuronoxylan whereas that of hardwoods is called *O*-acetyl-4-*O*-methylglucuronoxylan [30]. Softwoods react to strain forces by forming compression wood, which is characterized by higher lignin and galactan content, as opposed to hardwoods that form tension wood under similar conditions [29, 30].

Lignin is a complex aromatic polymer made up of phenolic compounds, typically referred to as *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) phenylpropanoid units. Softwoods are mainly composed of G and H type lignins and minute quantities of S type [35–38]. The precursors of lignin biosynthesis are *p*-coumaryl alcohol (I), coniferyl alcohol (II), and sinapyl alcohol (III); II is the predominant and I is a minor precursor of softwood lignin [30]. Lignin biosynthesis was captured in the differentiating xylem of pine wood using radioactive labeling techniques, giving evidence of the heterogeneous process of ligninification and the stages of incorporation of the different types of lignin in different parts of the cellular matrix [39, 40]. Extractives are a very small percentage of the total wood mass; these are low molecular weight substances that can have great influence on the properties and processing quality of woods [29]. Extractives are a variety of organic compounds including terpenes, aliphatic acids, alcohols, phenolics, alkaloids, fats, waxes, proteins, simple sugars, starches, pectins, mucilages, gums, resins, glycosides, saponins, flavonoids, and essential oils, with solubility in different solvents such as hot water, 1% NaOH, ether, ethanol, and benzene or toluene [30]. Many of these function as intermediates in tree metabolism, as energy reserves, or as part of the tree's defense mechanism against microbial attack and contribute to wood properties such as color, odor, and decay resistance [30]. Ash is the inorganic residue that remains after burning wood at high temperature. Softwoods typically contain less extractives and ash compared to hardwoods [29, 30].

At the anatomical level, softwoods contain 90–95% tracheids, long and slender cells that evolve from conducting function in earlywood to mechanical function in latewood due to formation of thicker cell walls; this change is visible in the form of growth or annual rings [29]. The remaining cells are parenchyma and epithelial cells that perform storing and secreting functions, respectively. The conduction and distribution of aqueous solutions and exchange of cell contents is possible due to presence of openings in cell walls called open and bordered pits. Bordered pits of softwoods usually have smaller apertures [29]; this could be another reason why softwood is more resistant to chemical action compared to other lignocellulosic materials. The structural and functional evolution, densities, dimensions, and percentages of these different cell types are different in softwoods and hardwoods [29]. The cell wall consists of a primary (P) wall, three layers of secondary walls (S1, S2, and S3), and a tertiary (T) wall, where S2 occupies the main portion (90%) of the cell wall. Between the individual cells, there is a thin layer called the middle lamella (ML) which glues the cells together. The transition from the ML to the adjacent primary wall is usually not clear and is termed as compound ML. The arrangement and morphological distribution of cellulose, hemicellulose, and lignin in the different layers of the cell wall is different for softwoods and hardwoods [30]. In pine, the carbohydrate fraction is distributed as 2:10:78:10 in ML + P/S1/S2/S3 [41]. Lignin is located in the secondary wall, compound ML, and cell corners; it is the last component to be incorporated into the cell wall, where it interpenetrates the cellulose fibrils and strengthens the cell walls [29].

Chemical Pretreatments

A key concept for harmonizing pretreatment discussion is that of severity, which is referred to widely in dilute acid, organosolv, and liquid hot water pretreatment literature. As such, combined severity (CS) of chemical pretreatments is a function of time, temperature, and pH [42, 43], as shown in Eq. 2. However, CS and resulting sugar yields also depend on the type of reactor used and the scale of process. Pretreatment studies conducted in different reactor configurations are often difficult to compare even when carried out at similar CS.

$$Combined\ severity = log(R_0) = log\left(t^* exp\left(\frac{T-T_{ref}}{14.75}\right)\right) - pH \quad (2)$$

where T_{ref} is 100 °C and t is in min.

Organosolv

In organosolv pretreatment, pine biomass is treated with organic solvents, such as aqueous ethanol, acetone, acetic acid, methanol, or butanol, at high temperatures, which can vary from 150 to 200 °C, for durations typically ranging from 30 to 60 min [7, 44–53]. The most commonly used solvent is ethanol, concentration ranging between 60 and 75% *v/v*; typical solids loading is 10% *w/v* but it can go up to 25% and an additional catalyst, in the form of dilute mineral acid such as H_2SO_4 and HCl (0.2–1% *w/w*), is often used to accelerate the process and reduce pretreatment times [52–57]. Organosolv is a single-step wood solubilization process that improves enzymatic digestibility of pine biomass by substantially removing lignin [44, 47, 53, 57], preferentially degrading hemicellulose into sugars and furfural [52], and reducing cellulose crystallinity [46]. The simultaneous delignification and degradation reactions have been explained using pseudohomogeneous first-order kinetics in acetic acid pulping of maritime pine [52, 53]. Advantages of organosolv pretreatment include high pulp yield and low cellulose losses, increasing solely with pretreatment severity, and generation of relatively unaltered and highly pure low molecular weight lignin that can easily be recovered as ethanol organosolv lignin (EOL) and used as a precursor for various value-added products [14, 45–47]. It was found that acid-catalyzed cleavage of β-*O*-4 linkages and ester bonds, and hydrolysis of α-aryl ether bonds, were the major mechanisms of lignin degradation during ethanol and acetic acid organosolv treatments of loblolly and maritime pine, respectively, suggesting some structural breakdown does occur [14, 53]. Additional features of this pretreatment are high recovery of fermentable sugars, low energy consumption, and reduced inhibitor formation [45, 52]; however, recovery and recycling of solvents and acid is a limitation [23] and hardwoods and agricultural residues have been found to respond better to this pretreatment compared to softwoods [49, 50, 58]. Results from various organosolv studies on pinewood are discussed below and summarized in Table 1.

It was reported that there is a strong negative correlation between residual lignin content and final hydrolysis yield of organosolv-pretreated substrates [7, 49, 50]. However, besides the total lignin content in the pretreated biomass, the properties of residual lignin, such as its hydrophilicity, nitrogen content, ratio of acid-insoluble lignin with respect to acid soluble lignin after pretreatment, have also been found to influence hydrolysis yields [50]. Organosolv pretreatment of pine was optimized for production of acetone, butanol, and ethanol (ABE), where ABE was obtained from microbial fermentation of sugars produced by enzymatic hydrolysis of pretreated pine. Maximum ABE yield of 87.9 g/kg biomass was obtained from pine treated at 150 °C for 30 min; on the other hand, higher sugar yields were obtained from the enzymatic hydrolysis of pine treated at 180 °C for 60 min, increasing from 17.3 to 20.9% of the theoretical yields as solids loading increased from 5 to 8% [45].

Substrate generated from organosolv pretreatment of lodgepole pine killed by mountain pine beetle (*Dendroctonus ponderosae*) was readily digestible as digestibilities of 93 and 97% were obtained within 24 and 48 h of hydrolysis, respectively [54]. Another study from the same group reported that mountain pine beetle-killed lodgepole pine displayed higher enzymatic hydrolysis potential than healthy lodgepole pine at mild pretreatment conditions; these differences were attributed to lower residual lignin, lower cellulose degree of polymerization and crystallinity, and smaller fiber size, most likely caused by the fungi associated with the beetles [47]. In addition, it was observed that both healthy and mountain pine beetle-killed lodgepole pine were completely hydrolyzed within 12 h as severity of the pretreatment was increased [47].

It was reported that compared to steam pretreatment, organosolv pretreatment of lodgepole pine resulted in 1.4-fold cellulose-to-glucose conversion, when pretreated material was hydrolyzed under similar enzyme loading conditions [50]. Optimizing the organosolv process for insignis pine wood chips, using acetone as the organic solvent, resulted in hydrolysis of 38–72%, depending on process severity [48]. Three different types of catalysts, acidic (1% H_2SO_4), neutral (1% $MgCl_2$), and basic (1–2% NaOH), were evaluated for their enzymatic hydrolysis efficiency of organosolv pretreated pitch pine and it was concluded that sulfuric acid showed the best efficiency even at low temperature [55]. Cellulose-to-glucose conversion yield of mountain pine beetle-killed lodgepole pine reached 82% within 12 h of hydrolysis for butanol/SO_2-pretreated pine and 100% conversion was obtained for both ethanol/H_2SO_4 and butanol/SO_2 pretreated pine after 72 h of hydrolysis under similar enzyme loading [56].

Dilute Acid

Dilute acid pretreatment makes use of dilute mineral acids, with concentrations ranging from 0.1 to 2.5%, at temperatures between 120 to 200 °C, residence times and solids loading varying from 30 min to 1 h and 5 to 15% *w/v*, respectively [59–63]. Sulfuric acid is the most extensively studied acid, but other mineral acids, such as HCl, HNO_3, and H_3PO_4, and organic acids, like maleic, oxalic and acetic acids, have also been investigated [6, 61]. It is highly effective in solubilizing hemicellulose [23, 45, 64, 65]; however, it is not effective in removing lignin, which makes dilute acid pretreatment more suitable for biomass with low lignin content [51]. In general,

Table 1 Literature review of enzymatic studies on pine from organosolv pretreatment

Substrate	Pretreatment conditions	Enzyme loading	Saccharification yield (% cellulose-to-glucose conversion)	Reference
Pinewood	75% *v/v* ethanol, 1% *w/w* H_2SO_4, L:S = 8, 180 °C, 60 min, CS 2.5	25 FPU cellulase and 40 IU β-glucosidase per g pretreated biomass	10.1 and 16.8 g/L total sugar (glc, xyl, man, gal) yield at 5 and 8% loading resp. after 72 h of EH	[45]
Pinus taeda	65% *v/v* ethanol, 1.1% *w/w* H_2SO_4, L:S = 7, 170 °C, 60 min	8 FPU cellulase and 16 IU β-glucosidase per g cellulose	70% cellulose-to-glucose conversion after 80 h	[46]
Pinus taeda	65% *v/v* ethanol, 1.1% *w/w* H_2SO_4, L:S = 7, 170 °C, 60 min	20 FPU cellulase per g glucan	81% after 72 h	[49]
Pinus radiata	50% *v/v* acetone, 0.9% *w/w* H_2SO_4, L:S = 7, 195 °C, 40 min, H factor 6182	20 FPU cellulase and 40 IU β-glucosidase per g pretreated biomass	72% conversion after 72 h	[48]
Pinus contorta	65% *v/v* ethanol, 1.1% *w/w* H_2SO_4, L:S = 4, 187 °C, 60 min	20 FPU cellulase and 40 IU β-glucosidase per g cellulose	100% conversion within 12 h	[47]
Pinus contorta	65% *v/v* ethanol, 1.1% *w*/w H_2SO_4, L:S = 4, 170 °C, 60 min	20 FPU cellulase and 40 IU β-glucosidase per g cellulose	93% and 97% within 24 and 48 h	[54]
Softwood mixture (pine, spruce and Douglas fir)	40–60% *w/w* ethanol, 185–198 °C, 30–60 min, L:S = 7–10, pH 2.0–3.4 (acid catalyst)	20 FPU cellulase per g cellulose	98% conversion within 24 h for the most severe treatment, reaching 100% in 48 h	[7]
Pinus contorta	60% *v/v* ethanol, 1% H_2SO_4, 170 °C, 50 min	10 FPU cellulase and 10 IU per g β-glucosidase per g cellulose	53.1% after 72 h	[50]
Pinus rigida	50% *v/v* ethanol, 1% *w/v* H_2SO_4, L:S = 10, conditions tested: 160–180 °C for 0–20 min or H factor 174–480	Enzyme loading not mentioned but activities for cellulase and β-glucosidase were 700 EGU/g and 250 CBU/g, resp.	53–57% after 72 h	[55]
Pinus contorta	65% ethanol, 1.1% H_2SO_4, 170 °C, 60 min, or 65% butanol, 1.1% SO_2, 170 °C, 60 min	10 FPU per g cellulose	100% cellulose conversion for both conditions after 72 h but 82% for butanol within 12 h	[56]

L:S liquid/solid ratio, *CS* combined severity, *EH* enzymatic hydrolysis, *FPU* filter paper activity, *EGU* endoglucanase unit (typical units for cellulases that break down cellulose into lower DP sugars or oligosaccharides), *IU* international unit, *CBU* cellobiase unit (typical units for glucosidases and cellobiases or that break down oligosaccharides into glucose), *Glc* glucose, *xyl* xylose, *man* mannose, *gal* galactose

dilute acid is a widely studied pretreatment technology for lignocellulosic biomass; however, very few studies have been published w.r.t. pinewood. The results discussed below clearly indicate that dilute acid cannot be used as a stand-alone pretreatment to improve enzymatic saccharification of pine biomass; it has to be combined with a delignification pretreatment to make the cellulose fraction easily accessible to enzymes. The major advantage of using dilute acid at the front end of a sequential pretreatment process for pine is that the easily hydrolyzable hemicellulose fraction and the amorphous fraction of cellulose are obtained in the liquid stream in the form of fermentable sugars, which can be extracted and purified for further use [61, 62, 65]. On the other hand, a major disadvantage is the conversion of sugars into degradation products, such as furans, including furfural and hydroxymethylfurfural (HMF), and carboxylic acids, including acetic acid, formic acid, and levunic acid [66], which are toxic and act as inhibitors in the downstream hydrolysis [24] and fermentation [67] procedures. Use of costly corrosion-resistant equipment and expensive acid recovery and recycling procedures is a limitation [51, 64]. Inhibition mechanisms and removal strategies of these by-products are discussed in detail in [22]. Sugar yields and saccharification efficiencies obtained from dilute acid pretreatment of pinewood are discussed below and results are also summarized in Table 2.

Mixture of pinewood and potato tubers treated with 1% acid resulted in minimal conversion to inhibitors and a yield of 76.2% after 72 h of enzymatic hydrolysis [59]. Recovery of total fermentable sugars in the combined acid and enzyme hydrolysates was lower in softwood pine and hardwood eucalyptus (~50%) as compared to that obtained with agricultural residues, such as wheat straw, sorghum straw, and sugarcane bagasse (80–90%) [60]. Susceptibility to dilute acid pretreatment and enzymatic saccharification was found to be similar for all fractions of scots pine chips, which were stemming from juvenile heartwood, mature heartwood, juvenile sapwood, mature sapwood, and knotwood; on the other hand, the bark fraction was more susceptible to enzymatic saccharification even without pretreatment [3]. The use of maleic acid was reported to yield 1.5-fold higher hydrolysate fermentable sugars than that of sulfuric acid [61]. Dilute acid hydrolysis of pinewood, followed by autoclaving at 121 °C for 1 h, resulted in increased hydrolysate sugar recovery, from 74.3 to 82.4% [62]. Pressurized dilute acid pretreatment of loblolly pine, resulted in increased crystallinity of cellulose in pretreated biomass due to removal of amorphous cellulose and hemicellulose, where up to 70.4% of the latter was recovered in the hydrolysate [65]. Deacetylation prior to dilute acid pretreatment of sorghum, performed at NaOH concentrations of 0.2–0.4% *w/w* and temperatures of 60–80 °C for 30–180 min, resulted in improved sugar yields [68]. This strategy is likely to be effective in less recalcitrant feedstocks such as corn, sorghum, and even hardwoods as these biomass sources have an abundance of acetyl groups; however, it might not be applicable

Table 2 Literature review of enzymatic studies on pine from dilute acid pretreatment

Substrate	Pretreatment conditions	Enzyme loading	Saccharification yield (% cellulose-to-glucose conversion)	Reference
Mixture of pinewood and potato tubers	1% *v/v* acid, 75 g/L solids loading, 130 °C, 30 min	30 FPU cellulase and 60 IU β-glucosidase per g dry pretreated biomass	76.2% after 72 h of EH	[59]
Pinus elliotii	2% *v/v* H_2SO_4, 10% *w/v* solids loading, 134 °C, 60 min	2% cellulase and 4% β-glucosidase	49% total sugar (glc, xyl, ara) recovery after 22 h	[60]
Pinus rigida and *Pinus densiflora*	Maleic acid, 170 °C, 30 min, L:S = 4, CS = 2.5; Sulfuric acid: 170 °C, 90 min, L:S = 4, CS = 2.5	20 FPU cellulase and 80pNPGU β-glucosidase per g pretreated biomass	61 and 49% after 72 h and fermentable sugar (glc, xyl, man) yield of 35 and 23 g/L for maleic and sulfuric acids resp.	[61]
Pine sawdust	1% *w/w* acid, 100 °C, 60 min, L:S = 10 followed by autoclaving for 1 h at 121 °C	–	82.4% sugar recovery	[62]
Pinus taeda	0.5% *w/w* acid, 10% solids loading, 170 °C, 32 min	–	15.3 g/L sugar yield (xmg or xyl man gal)	[65]

L:S liquid/solid ratio, *EH* enzymatic hydrolysis, *CS* combined severity, *FPU* filter paper activity, *IU* international unit, *pNPGU p*-nitrophenyl-β-glucopyranoside unit (typical units for glucosidases and cellobiases or that break down oligosaccharides into glucose), *Glc* glucose, *xyl* xylose, *man* mannose, *gal* galactose

to pine as it is devoid of acetyl groups in the xylan backbone of hemicellulose. But since this process was found to solubilize extraneous components such as proteins, ash, and non-structural carbohydrates [68], it could be evaluated for its effectiveness in enriching xylan and glucan fractions in pinewood for enhanced sugar yields in the subsequent dilute acid pretreatment. A high-temperature two-stage dilute acid pretreatment (180–200 °C, 2–10 min, 0.5–1% *w*/*w* H_2SO_4) resulted in changing the structures of cellulose and lignin in loblolly pine [69]. Crystallinity of cellulose was found to increase due to preferential removal of amorphous regions, decrease in paracrystalline cellulose content and increase in the relative proportion of the more stable cellulose allomorph (I_β); lignin depolymerization by fragmentation of β-*O*-4 linkages was followed by increased degree of condensation [69]. For enhanced conversion of pine biomass into ethanol, there is a need to optimize dilute acid pretreatment such that it results in increased amounts of reactive cellulose, while minimally degrading sugars and re-condensing lignin, both of which are associated with the inhibition of cellulase during enzymatic hydrolysis.

Alkaline

Alkaline pretreatment of pine is performed using bases, such as NaOH, KOH, $Ca(OH)_2$, and NH_4OH, with sodium sulfite (Na_2SO_3) and/or hydrogen peroxide (H_2O_2) supplementation. While NaOH is more effective on low lignin substrates, such as hardwoods, herbaceous crops, and agricultural residues, lime or $Ca(OH)_2$, in addition to being less expensive, is more effective on high lignin substrates, such as softwoods [64]. Alkaline pretreatment efficiency is highly dependent on the temperature and duration of the pretreatment, which can range anywhere between room temperature to as high as 150–250 °C and last from 30 min to weeks, respectively [9, 23]. Compared to dilute acid pretreatment, alkaline pretreatment is less severe and results in reduced degradation of sugars into inhibitory compounds. Alkaline pretreatment is highly selective for delignification, enhancing cellulose digestibility by causing swelling and increase in internal biomass surface area, decreasing the degree of polymerization and crystallinity of cellulose, and disrupting lignin structures and their linkages with carbohydrates [23, 51, 64, 70, 71]. Conventional alkaline processes, known as "kraft pulping," are used to manufacture pulp and paper from wood chips; however, modified processes, such as liquid hot water, dilute acid, or steam pretreatments used prior to kraft pulping, have also been investigated. Integration of such processes into existing pulping mills was proposed and results showed that pulp fiber with improved yield and quality was obtained [28, 72–75]. A techno-economic study on alkaline fractionation of pinewood demonstrated that a typical mill producing 1000 tons of pulp per day could produce 140,000 m^3 and 25,000–63,000 tons of ethanol and lignin per year, respectively [76]. Results from various alkaline studies on pinewood are discussed below and summarized in Table 3.

The alkaline sulfite/anthraquinone pretreatment of insignis pine wood chips followed by disk refining was shown to be an effective pretreatment in producing delignified and highly fibrillated pulp that allowed 70% cellulose-to-glucose and 90% glucose-to-ethanol conversion efficiencies during separate hydrolysis and simultaneous saccharification/fermentation experiments [77]. It was reported that combination of kraft delignification with steam explosion was more effective than its combination with dilute acid pretreatment pertaining to the extent of hemicellulose solubilization, delignification, and enzymatic hydrolysis yield [78]. While examining the synergistic effect of successive pretreatments, it was observed that enzymatic saccharification ratio almost doubled, from 40 to 78%, when steam exploded loblolly pine chips were given alkaline-hydrogen peroxide pretreatment [79]. Alkaline pretreatment of insignis pine resulted in 72% holocellulose, i.e., cellulose + hemicellulose, yield and subsequent 40% conversion of holocellulose to glucose and xylose using a combination of acid hydrolysis and microwave irradiation [80]. Among various pretreatments tested, combination of dilute acid and alkaline pretreatments resulted in the highest sugar yield of 77% from pine sawdust [81].

Sulfite Pretreatment to Overcome Recalcitrance of lignocellulose

Sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) is a novel technology that was specially designed to efficiently overcome recalcitrance of woody biomass [25–27]. Typically, wood chips are directly treated in an aqueous sulfite solution, followed by mechanical size reduction of pretreated biomass using disk milling or refining [25–27], as opposed to most other pretreatment technologies where the energy intensive operation of mechanical size reduction is usually carried out before chemical pretreatment. Due to its low technological and environmental barriers for commercialization, SPORL is a pretreatment that can easily be integrated into the existing infrastructure of the paper and pulp industry. The main features of SPORL pretreatment are that it removes hemicellulose while producing low amounts of fermentation inhibitors, such as HMF and furfural; it also leads to production of readily hydrolysable biomass for cellulosic biofuel applications and sulfonated lignin as a by-product [25, 63]. Results from various SPORL studies on pinewood are discussed below and summarized in Table 3.

SPORL was reported to be superior than dilute acid pretreatment, resulting in higher cellulose-to-glucose conversion rates, 62.1 vs. 18.3%, and better lignin removal and fiber separation [63]. Nearly 100% cellulose-to glucose conversion of SPORL pretreated red pine (*Pinus resinosa*) was achieved within 48 h of hydrolysis [25]. A study comparing various

Table 3 Literature review of enzymatic studies on pine from alkaline, SPORL, and steam explosion pretreatments

Substrate	Pretreatment conditions	Enzyme loading	Saccharification yield (% cellulose-to-glucose conversion)	References
(1): *Pinus radiata* (2): *Pinus caribaea*	7.5% *w*/*w* NaOH, 17.5% *w*/*w* Na_2SO_3, 170 °C, 45 min	(1): 8.8 FPU cellulase and 40 IU β-glucosidase per g pulp (2): 20 FPU and 40 IU per g pulp	(1): 70% after 72 h, 20 g/L ethanol after 24 h of SSF, 260 L/ton wood (2): 72% after 72 h, 16 g/L ethanol after 24 h of SSF, 140 L/ton wood	[77]
Pinus radiata	Steam explosion—190 °C, 28 min, SF = 4.09; dilute acid—170 °C, 25 min, CS = 1.67; kraft—18% active alkali, 30% sulfidity, 1200 H factor	20 FPU cellulase and 20 CBU β-glucosidase per g pretreated material	75.1% with SE and 44.2% with DA after 96 h	[78]
Pinus taeda	2% (*w*/*v*) H_2O_2 and 2 N NaOH; 232 °C for 4 min	–	78% saccharification ratio	[79]
Pinus radiata cones	0.125 M NaOH for 1 h at 70 °C 3 M HCl/5 min	–	40% holocellulose conversion to glc and xyl sugars	[80]
Pine sawdust	6% HNO_3 and 1% NaOH	25 FPU cellulase per g pretreated material	70% sugar yield after 72 h	[81]
Pinus resinosa	8% Na_2SO_3 at H_2SO_4 charge of 3.68% *w*/w, L:S = 3, 180 °C, 30 min	14.6 FPU cellulase and 22.5 CBU β-glucosidase per g substrate	~100% after 48 h	[25]
Pinewood	8% $NaHSO_3$, 2.2% H_2SO_4, 180 °C, 30 min, L:S = 3	7.5 FPU and 11.2 CBU per g cellulose	62.1% after 48 h	[63]
Pinus contorta	8% $NaHSO_3$, low pH—1.9 (2.21% *w*/*w* H_2SO_4), high pH—4.2 (no acid) 180 °C, 30 min	15 FPU cellulase and 22.5 CBU β-glucosidase per g pretreated substrate	Low pH—92.2% High pH—84.1% after 48 h	[26]
Pinus contorta	2.21% *w*/*w* H_2SO_4, 8% *w*/*w* $NaHSO_3$, 180 °C, 25 min, L:S = 3	15 FPU cellulase and 22.5 CBU β-glucosidase per g pretreated substrate	>90% digestibility after 48 h	[27]
Pinus radiata	3.5 MPa, 5 min, 9% SO_2	25 FPU cellulase per g of sawdust	67.1% sugar yield after 48 h	[82]
Pinus contorta	Medium severity—200 °C, 5 min, 4% SO_2	20 FPU cellulase and 10 CBU β-glucosidase per g cellulose	75% after 72 h	[83]
Pinus roxburghii	121 °C for 8 min	1.18 mL of cellulase, 0.31 mL of xylanase, and 0.01 mL of laccase	334 mg per g of dry foliage after 24 h	[13]

L:*S* liquid/solid ratio, *EH* enzymatic hydrolysis, *SE* steam explosion, *DA* dilute acid, *FPU* filter paper activity, *IU* international unit, *CBU* cellobiase unit, *SF* severity factor, *CS* combined severity, *SSF* simultaneous saccharification and fermentation, *glc* glucose, *xyl* xylose

pretreatments for lodgepole pine reported that both low and high pH SPORL systems resulted in highest enzymatic hydrolysis yields of 92.2 and 84.1%, respectively, within 48 h of enzymatic hydrolysis. In addition, it was also found to be more effective in decreasing energy consumption during subsequent size reduction or disk milling stages [26]. Another study concluded that SPORL pretreatment of lodgepole pine using 2.2% acid charge and 8% bisulfite charge resulted in 90% digestibility of glucan in pretreated biomass and 276 L ethanol production per metric ton of wood (or 72% of theoretical yield) after simultaneous enzymatic saccharification and fermentation for 72 h [27]. A study concluded that saccharification efficiency of lodgepole pine, pretreated with dilute acid, alkaline, or kraft pulping and SPORL processes, could be improved by increasing pH from 4.8–5.0 to 5.5–6.0 and adding lignosulfonate during enzymatic hydrolysis [16, 84, 85]. Elevated pH increased the surface charge of lignin, which resulted in greater hydrophilicity and reduction in its nonspecific binding to cellulase [84, 85], while lignosulfonate, a hydrophilic surfactant, directly reduced the nonproductive binding of cellulase with the inherently hydrophobic lignin present in lignocellulosic biomass [16].

Ionic Liquids

Ionic liquids (ILs) are salts composed of organic cations and either organic or inorganic anions. In recent years, ILs have emerged as novel solvents capable of dissolving and enhancing the enzymatic digestibility of lignocellulosic materials, including softwoods, hardwoods, and agricultural wastes [86–91]. Use of ILs has been shown to facilitate the production of fermentable sugars [89, 90, 92–94], furans [95], and lignin as valuable by-products [15, 96]. Wood dissolution and delignification using ILs is dependent on various factors such as wood type, particle size, solvent system, water content, and temperature at which the reaction is conducted [86–96]. IL pretreatment effects can be accelerated by using microwave pulses and ultrasound irradiation [88, 96]. ILs are potential alternatives to traditional pretreatment solvents and chemicals as these can be used for dissolution of lignin and biomass fractionation without any catalyst addition; however, the major bottlenecks for this technology to be commercially viable are as follows: (1) high cost of ILs, (2) long processing times of pretreatment at low temperatures, and (3) lack of effective IL recycling strategies [89].

A study investigated the effect of lignin dissolving switchable ILs and cellulose dissolving conventional ILs; it was observed that DBU-MEA-SO_2 (1,8-diazabicycloundec-7-ene-monoethanolamine-sulfurdioxide) was the most efficient solvent for softwood substrates, such as Scots pine stem wood and bark, due to its high lignin removing capacity [89]. The use of DBU-MEA-SO_2 resulted in high glucose production rates, hemicellulose recovery, and approximately 93% glucan-to-glucose conversion efficiency during enzymatic hydrolysis [89]. Compared to hardwoods and agricultural wastes, microwave-assisted pretreatment of pinewood with dimethyl sulfoxide/1-allyl-3-methylimidazolium chloride (AmimCl) resulted in highest cellulose-to-glucose conversion ratio of 85.4% [88]. The IL pretreatment of lodgepole pine, in both flour and pellet forms, using 1-ethyl-3-methylimidazolium acetate, demonstrated maximum reduction in cellulose crystallinity and roughly 95–100% digestibility within 48 h of hydrolysis, compared to aqueous ammonia and dilute acid pretreatments that only resulted in 40–60 and 15–20% yields, respectively [90]. Isopentenol yields from simultaneous saccharification and fermentation of pretreated substrates followed the same order: 1000, 700, and 500 mg/L for IL, ammonia, and dilute acid pretreated pine, respectively [90]. Superiority of IL pretreatment over dilute acid was reported [87, 89], in addition to its better tolerance to feedstock variability [87]. Acid-catalyzed IL treatment of loblolly pine with 1-butyl-3-methylimidazolium chloride (BMImCl) at 120 °C resulted in complete depolymerization of the carbohydrate fraction into water soluble products including sugars and furans, while lignin was recovered as a solid residue without major chemical modifications [92]. In another study, it was shown that the same solvent can be used to recover pure cellulose from hardwoods as well as softwoods including pine, providing evidence that ILs can be used as greener alternatives over the traditional environmentally detrimental Kraft pulping procedures; however, use of less toxic ILs, minimal use of co-solvents such as DMSO that are solely employed to reduce viscosity and energy requirements of the process need to be evaluated [97]. A techno-economic study of IL pretreatment explained that by reducing IL cost and loading, increasing its recycling and recovering lignin as a revenue stream, ethanol production using IL pretreatment could be economically feasible at large scale [98].

Hydrothermal/Physico-chemical Pretreatments

Liquid Hot Water

Liquid hot water pretreatment is a hydrothermal pretreatment that uses water at elevated temperatures, typically between 150 and 180 °C, at a liquid/solid ratio ranging between 3 to 6 for times that can last from 30 to 120 min [72, 74, 75, 99–101]. Most LHW research is focused on its use as a supplemental treatment to kraft pulping for improved pulp yield and quality. In a biorefinery context, integration of LHW into existing pulp mills is the first step in the sequential and incremental deconstruction of woody biomass [72, 74, 75, 102–104]. Its major feature is pre-extraction of hemicelluloses, generating an additional product stream that can be hydrolyzed to fermentable sugars that have applications in

food, pharmaceutical, and chemical industries [99, 102]. However, there are very few studies investigating the impact of LHW, as a pretreatment for pine biomass, for its conversion into advanced biofuels and chemicals [100], which can be attributed to the results of these studies that are indicative of LHW not being as competitive as dilute acid, SPORL, IL, and hydrotropic pretreatments.

In LHW pretreatment, wood is heated above lignin's glass transition temperature, which can vary from 80 to 140 °C or higher, depending on moisture content, resulting in lignin depolymerization and its migration from cell wall and middle lamella to the surface. The migrated lignin is then deposited as lignin liquid intermediates, and the amount and properties of these structures vary with the severity of pretreatment conditions, thus impacting enzymatic hydrolysis [101]. LHW is a cost-effective pretreatment, as it does not require any catalyst and has low equipment corrosion potential. Compared to steam explosion and most chemical pretreatments, formation of degradation products or inhibitors, such as HMF and furfural, is low in LHW. However, due to its high water and energy requirements, LHW pretreatment technology has not been developed at commercial scale yet [23].

Steam Explosion

Steam explosion, also known as autohydrolysis, is a widely used, cost-effective pretreatment that has low energy requirements and is environmentally friendly, as addition of external catalyst and its recycling are not necessary. In a nutshell, steam explosion pretreatment, can be characterized as a hydrothermal treatment that uses high-pressure saturated steam, in the range of 0.7–4.8 MPa or 160–260 °C, for short time periods, 10 s–2 min, before releasing the material to atmospheric pressure. This sudden pressure release exposes the feedstock to decompression and results in physically opening its internal structure and increasing enzyme accessibility. The key operational parameters of steam explosion pretreatment are temperature, time, particle size, and moisture content with strong interaction between temperature and time effects [5, 8, 22, 64, 71, 105]. During exposure of wood to high-temperature steam, acetic acid and other acids are released from hemicellulose, catalyzing its hydrolysis, depolymerizing and chemically modifying lignin, and disrupting lignin and cellulose bonds thereby improving cellulose digestibility [5, 8, 64]. Higher sugar yields can be achieved by using catalysts, such as H_2SO_4, SO_2, or CO_2, which also can result in decreasing the time and the reaction temperatures [71, 82, 83, 106, 107]; however, this can also lead to increased degradation of carbohydrates into toxic by-products, such as furfural and HMF, that inhibit hydrolysis and fermentation reactions, making detoxification necessary [5, 8, 64]. Steam explosion has been shown to be more effective for hardwoods and agricultural residues than for softwoods, which can most likely be attributed to the low content of acetyl groups in softwood hemicellulose. Of all the pretreatments available, SO_2-catalyzed steam explosion is one of the most effective for softwoods [23]. A review paper evaluated two-step dilute acid and steam pretreatments as promising technologies for softwoods; however, it was concluded that the material cost of enzymes was a major contributor to the overall production cost and that economic feasibility could be achieved by integrating the ethanol production process with a pulp and paper mill or a heat and power plant [108]. A techno-economic study comparing three different pretreatments using various combinations of steam, HCl and SO_2, concluded that none of the processes was less economical than the others based on ethanol production costs and capital costs [109]. Another techno-economic study published back in 2005 concluded that using dilute acid pretreatment could make cellulosic ethanol competitive with starch ethanol within a short-term period of 5 years; however, further research and development would be required to improve conversion efficiencies and reduce capital investments costs of steam explosion and LHW pretreatments to commercialize these technologies for cellulosic ethanol production in middle-term (10–15 years) and long-term periods (>20 years) [110]. Results from various steam explosion studies on pinewood are discussed below and summarized in Table 3.

Acid-catalyzed steam explosion of insignis pine sawdust at 3.5 MPa (~243 °C) for 60 s, using 0.4% (*w*/*w*) H_2SO_4, resulted in a 5–6-fold improvement in its enzymatic digestibility. Increase in digestibility was also observed when pretreatment severity was augmented [106]. Insignis pine chips pretreated with steam at 220 °C for less than 2 min resulted in sugar recovery of 3.5 g/L, which increased to 5.8 g/L with the addition of dilute acid, using acetic acid as a mild catalyst [107]. Steam pretreatment of insignis pine sawdust with SO_2 impregnation was reported to be very effective, as it resulted in extracting 89% of the reducing sugars present in the pretreated substrate during enzymatic hydrolysis [82]. The severity of steam explosion pretreatment was optimized for lodgepole pine to obtain high cellulose-to-glucose conversion yields (~75%) and high ethanol yields (~77% of the theoretical maximum) [83].

The increasing presence of aromatic lignin derived substances was noticed as temperature increased from 190 to 210 °C, while acid-insoluble or Klason lignin content increased by 15% with reduction in chip size, indicating the likelihood of condensation reactions taking place between the degradation products of hemicellulose and lignin and their dependence on chip size and severity

[78], validating the previously reported interdependence of particle size and pretreatment severity [111]. A study demonstrated the use of sequential aqueous ethanol, alkali and steam pretreatments for fractionation of softwood and production of 7.1 kg ethanol from 100 kg of pine; the individual hemicellulose and cellulose fractions could be converted into ethanol with 51 and 75% efficiencies, respectively [42].

Biological/Fungal Pretreatment

Fungi are highly efficient degraders of woody biomass and have an essential role in the global carbon cycle and ecology [1]. The low-cost and environmentally friendly approach of fungal pretreatment of wood species was first investigated in the 1990s. Combining fungal pretreatment with traditional mechanical and chemical pulping operations that are inherent to the pulp and paper industry was found to result in significantly reduced energy costs and toxicity of pulping waste, while producing pulp with improved yield and quality [112–115]. However, fungal pretreatment has received renewed attention as a pretreatment method for enhancing enzymatic digestibility of lignocellulosic biomass for advanced biofuel applications [1, 2, 116–119]. The two main types of fungi that have potential for biomass pretreatment are categorized as white-rot and brown-rot. White-rot fungi are the most effective lignin-degrading microorganisms in nature [116], a property that can be attributed to their extracellular ligninolytic enzyme system, consisting of lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase [120–125]. Brown-rot fungi, on the other hand, preferentially degrade wood carbohydrates and partially oxidize lignin without supporting its removal [2, 118, 119, 123]. The advantages of this technology over thermochemical pretreatments include simple protocols, low energy requirements, no or reduced output of waste streams, lower downstream processing costs, and decreased generation of inhibitors to ethanol fermentation [120–125]. On the other hand, fungal pretreatment is plagued with certain drawbacks, such as substantial holocellulose loss, slow delignification rates that result in pretreatment times that can last from weeks to months, scale-up challenges related with reactor design to ensure uniformity and reproducibility, and likelihood of contamination with unwanted fungi [112–114, 116, 120–125]. Results from various biological pretreatment studies on pinewood are discussed below and summarized in Table 4.

A study evaluating the effect of brown-rot fungi on softwoods and hardwoods revealed that biotreatment of insignis pine chips with *Gloeophyllum trabeum* resulted in maximum hemicellulose loss, with minimal glucan losses and essentially no lignin degradation after 8 weeks of biodegradation [2]. Increase in cellulose-to-glucose conversion from 9% in

Table 4 Literature review of enzymatic studies on pine from biological and combined pretreatments

Substrate	Pretreatment conditions	Enzyme loading	Saccharification yield (% cellulose-to-glucose conversion)	References
Pinus radiata BRF *Gloeophyllum trabeum*	8 weeks	20 FPU cellulase and 40 IU of β-glucosidase per g of pretreated material	14% after 96 h	[2]
Pinus palustris BRF *Gloephyllum trabeum* or *Fomitopsis pinicola*	8 weeks DA: 0.5% H_2SO_4 at 170 °C for 2 h	164.5 CMCU endoglucanase and 6.6 pNPGU β-glucosidase per g cellulose	Fungal sugar yield 2.5 times that of DA sugar yield after 5 days of EH	[118]
Pinus sylvestris BRF *Coniophora puteana*	15 days	60 FPU cellulase and 64 pNPGU β-glucosidase	70% after 168 h	[119]
Pinus densiflora WRF *Polyporus brumalis*	45 days	80 EGU cellulase and 72 IU β-glucosidase per g pretreated biomass	345 mg/L after 24 h	[116]
Pinus radiata BRF *Gloeophyllum trabeum*	4 weeks of fungal pretreatment followed by organosolv pulping (60% *v/v* ethanol, 200 °C, 60 min, L:S = 6)	20 FPU cellulase and 40 IU β-glucosidase per g pretreated biomass	55–70% after 24 h	[126]
Pinus radiata WRF *Trametes versicolor*	6 weeks	20 FPU cellulase and 25 IU β-glucosidase per g pretreated biomass	Fungal + steam glucose yield 25% higher than that obtained with steam alone after 24 h of EH	[127]

BRF brown-rot fungi, *WRF* white-rot fungi, *L:S* liquid/solid ratio, *DA* dilute acid, *EH* enzymatic hydrolysis, *FPU* filter paper activity, *EGU* endoglucanase unit, *CMCU* 5-carboxymethyl cellulase unit (typical units for cellulases that break down cellulose into lower DP sugars or oligosaccharides), *IU* international unit, *pNPGU p*-nitrophenyl-β-glucopyranoside unit (typical units for glucosidases and cellobiases or that break down oligosaccharides into glucose)

controls to 14% in biotreated samples was attributed to decreases in cellulose degree of polymerization and crystallinity index [2]. Cellulose-to-glucose conversion yield of southern yellow pine blocks, treated with two brown-rot fungi, *G. trabeum* or *Fomitopsis pinicola*, for 8 weeks in separate experiments was approximately 2.5-fold that of pine treated with dilute acid [118]; and that of Scots pine blocks treated with brown-rot fungus, *Coniophora puteana*, for 15 days was 70%. In both experiments, the blocks were oven dried and milled to an average particle size of 500 μm prior to enzymatic hydrolysis, indicating the importance of size reduction [119].

A study evaluating several white-rot fungi found that, compared to softwood white pine (*Pinus strobus*), hardwood tulip (*Liriodendron tulipifera*) was more amenable to fungal treatment with *Trametes versicolor*, as it resulted in higher glucose recovery [117]. It was reported that pretreatment of red pine (*Pinus densiflora*) with a recombinant strain of the white-rot fungus *Polyporus brumalis*, which contained an overexpressed laccase gene, resulted in 1.4-fold higher lignin degradation than that obtained using the wild strain; this was followed by 1.7-fold higher sugar yield during enzymatic hydrolysis [116].

Combination of Chemical/Physico-chemical and Fungal Pretreatments

A few studies have also evaluated the synergistic effect of biological and chemical pretreatment on enzymatic hydrolysis of pine [126–128]. Fungal pretreatment of insignis pine wood chips with brown-rot fungus, *G. trabeum*, for a period of 3 weeks resulted in reducing the severity of the following chemical pretreatment. As an example, the process conditions of organosolv pretreatment could be reduced from 200 °C for 32 min for untreated pine chips to 185 °C for 18 min for biotreated chips to obtain similar ethanol yield [128]. In another study, fungal pretreatment of insignis pine chips with brown-rot fungus, *G. trabeum*, was followed by delignification with either alkaline or organosolv pulping. It was observed that alkaline pulping of fungal pretreated wood did not increase hydrolysis yields, but organosolv pulps of fungal pretreated wood had higher glucan-to-glucose conversion yields (55–70%) than that of control organosolv pulps (30–40%), owing to low residual lignin and high glucan retention in organosolv biopulps. The combination of fungal and organosolv processes also resulted in a calculated production of 210 mL ethanol/kg wood, which corresponded to 72% of the maximum theoretically possible [126]. Insignis pine wood biotreated with white-rot fungus *T. versicolor* for 6 weeks, resulted in 50–60% higher cellulose-to-glucose conversion yield compared to non-fungal treated control. Moreover, roughly 25% higher conversion yield was obtained for fungal pretreated and steam exploded pine biomass, compared to non-fungal treated steam exploded control, suggesting that biological and thermochemical pretreatments can work in a synergistic fashion [127].

These results indicate that the combination of biological and chemical pretreatments could prove useful for reduction in chemical pretreatment severity, abatement of energy consumption and environmental footprint of these processes, and improving the overall efficiency of glucose and ethanol production from pinewood. However, there is still a lack of in-depth understanding of the economic implications of these pretreatment systems. Moreover, there is a gap in knowledge about operating parameters, such as particle size, fungal species, fungal incubation periods, pretreatment temperatures, times and severity, and rinsing water requirements, with respect to combined pretreatments. There is a need for studies that will determine these important operating parameters, resulting in the adoption of combined biological and chemical processing protocols that could eventually lead to improved pine saccharification efficiencies.

Conclusions

This paper reviewed the most common chemical, physico-chemical, and biological pretreatment technologies used for pine. The main modes of action of the reviewed pretreatments consisted of lignin and hemicellulose removal, reduction in the crystallinity, and degree of polymerization of cellulose, resulting in improved enzymatic accessibility and digestibility of pretreated biomass. Each pretreatment has its own features and benefits in terms of scale-up potential, cost-effectiveness, environmental impact, reproducibility of operational parameters, and resulting glucose yields. The effects of different pretreatment technologies on the chemical composition and structure of pretreated biomass, as well as a summary of their advantages and disadvantages are outlined in Tables 5 and 6. This review highlights the importance of using combinations of different pretreatments in order to maximally utilize the economic value of each fraction of pinewood. For example, dilute acid, liquid hot water, and steam pretreatments are ideal for extraction of hemicellulose at the front end of the process; these can be combined with organosolv or kraft pulping procedures that are most effective for delignification. On the other hand, SPORL is an energy-efficient pretreatment that should be investigated more as it significantly removes hemicellulose and improves saccharification efficiency of cellulose, despite the presence of the majority of original lignin in pretreated biomass. Finally, the choice of pretreatment is dictated by the end use of each product. Since pine is high in lignin content, recovering pure and/or modified lignin to generate additional revenue to offset other process costs is a strategy that is largely agreed upon. It was also

Table 5 Effect of different pretreatment methods on the chemical composition and physical/chemical structure of lignocellulosic biomass

	Increases accessible surface area	Decrystallizes cellulose	Solubilizes hemicellulose	Removes lignin	Generation of toxic compounds (HMF/furfural)	Alters lignin structure
Organosolv	H	H	H	H	H	ND
Dilute acid	H	L	H	L	M	L
Alkaline	H	H	L	H	L	H
SPORL	H	H	H	H	L	H
ILs	H	H	L	H	H	ND
LHW	L	L	M	ND	L	M
Steam Explosion	H	H	H	M	H	H
Biological	L	L	M	L	ND	M

H high effect, *M* moderate effect, *L* low effect, *ND* not determined

noted that, as applied to pine, most fungal pretreatment studies were focused on changes in composition and structure; unfortunately, few studies reported on saccharification and ethanol yields, and more importantly, there is a need of more studies that elaborate the use fungal pretreatment as a supplement to chemical pretreatments. Overall, factors, such as enzyme loading and particle size of pretreated biomass during hydrolysis, varied widely from one study to another, rendering direct comparison of saccharification yields problematic. New pretreatment technologies, such as wet explosion, ILs, hydrotropic liquid, and alkaline/anthraquinone treatments, have emerged in recent years as promising alternatives; however, they may prove to be costly. Techno-economic analysis of conventional, as well as newly developed pretreatments and ensuing combinations, needs to be done more rigorously by the research community, such that sound decisions on their real-world implementation can be made within the context of a pine based biorefinery. Although the current review is focused on pretreatment techniques that improve enzymatic saccharification efficiency of pinewood, it would be worth noting that some of these techniques that target hemicellulose and lignin removal are also suitable for extraction of pure cellulosic pulp from pine, for further conversion into novel materials such as cellulose nanocrystals and nanofibrils.

Table 6 Summary of advantages and disadvantages of different pretreatment methods

	Advantages	Disadvantages
Organosolv	- Lignin and hemicellulose hydrolysis - Unaltered lignin as by-product	- Recovery and recycling of solvents and acid - Generates toxic by-products
Dilute acid	- Hemicellulose solubilization	- Not effective in lignin removal not effective - Generates degradation products - Causes corrosion of equipment - Acid recovery
Alkaline	- Effective lignin removal - Reduced generation of toxic by-products	- Toxic waste stream that needs treatment
SPORL	- Reduced generations of inhibitors	- Energy intensive
ILs	- Effective fractionation of lignin and polysaccharide fractions - Unaltered lignin as by-product	- High cost of ILs - Recycling of ILs
LHW	- Hemicellulose removal - Less generation of toxic by-products	- Pretreated material needs more processing to remove lignin and improve cellulose digestibility
Steam explosion	- Cost-effective and fast - Solubilizes hemicellulose and disrupts/transforms lignin	- Degrades hemicellulose and lignin into toxic by-products - Acid recovery
Biological	- Low energy requirements - Reduced waste streams - Less generation of inhibitors	- Long pretreatment times - Delignification and/or increase in cellulose digestibility not significant

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