



Toward a Fundamental Understanding of the Role of Lignin in the Biorefinery Process

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As one of the main components in biomass, lignin plays a vital role in the biorefinery industry. Its unique structural feature increases the dose of cellulases during enzymatic deconstruction and is an attractive resource for many high valued products. The inhibition of lignin on cellulases is proposed to occur in several ways, with the most studied being nonproductive enzyme binding, which is attributed to hydrogen bonding, hydrophobic and/or electrostatic interactions. This review provides a comprehensive review of how lignin is transformed during various pretreatment methods as well as how these changes impact the cellulases inhibition. Future pretreatment directions for decreased cellulases inhibition are also proposed.

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1 INTRODUCTION

Due to the depletion of fossil fuels, environmental and energy security concerns, alternative renewable energy from biomass has attracted intensive attention worldwide (Ragauskas et al., 2014; Cole-Hamilton, 2020). Cellulose, hemicellulose, and lignin are the three main components in biomass (Wörmeyer et al., 2011). Cellulose and hemicellulose can be hydrolyzed to glucose or xylose which can be subsequently fermented by yeast or bacteria to yield various types of liquid fuel (e.g., ethanol and butanol). Lignin, a polymer comprised of cross-linked phenylpropane units, contributes to several biomass features, including hydrophobicity, structural rigidity, and microbial resistance to plant cell walls (Saini et al., 2016). Typically, biofuels production from lignocellulosics mainly consists of three key steps: namely biomass pretreatment, enzymatic hydrolysis/saccharification, and fermentation.

However, it has been found that lignin adversely impacts enzymatic hydrolysis, resulting in an increased dose of cellulases, which accounts for almost half of bioethanol cost (Luterbacher et al., 2014). It was suggested that ideal pretreatments should maximize lignin removal and minimize polysaccharide modification (Ding et al., 2012). Therefore, how to decrease the inhibitory effect of lignin on cellulases becomes one of the major challenges in biofuels production for assorted bioresources. To mitigate the adverse effects of lignin and fundamentally decrease the overall cost of biofuel, how lignin impacts cellulases performance during enzymatic hydrolysis needs to be understood and has been extensively explored.

TABLE 1 | Typical biomass constituents for select plant resources (Ragauskas et al., 2014).

Plant resource	Cellulose %	Hemicellulose %	Lignin %
Miscanthus	45–52	24–33	9–13
Switchgrass	37–42	26–33	17–18
Corn stover	37	31	18
Poplar	42–48	16–22	21–27
Eucalyptus	39–46	24–28	29–32
Pine	46	23	28

1.1 Structural Features of Lignin

Lignin is a heterogeneous macromolecular polymer composed of guaiacyl (G), syringyl (S), and/or *p*-hydroxyphenyl (H), which are connected by carbon-carbon and aryl ether interunit linkages. Depending on the plant species, the contents of lignin and its G, S, and H units vary significantly. As shown in **Table 1**, softwoods, such as pine, contain ~28% lignin, which mainly consists of G units. Lignin from hardwoods (i.e., poplar and eucalyptus) comprises both of G and S units and accounts for 21–32% of the plant cell wall. Besides G and S units, lignin from herbaceous species (i.e., miscanthus, switchgrass, and corn stover) also contains small amounts of H units. This latter bioresource contains the least amount of lignin, typically less than 20%.

The common interunit linkages in lignin include β -O-4, β -5, β - β' , β -1, and 5-5'/4-O- β' , as depicted in **Figure 1**. As shown by earlier researchers, the β -O-4 linkage is the dominant interunit linkage in lignin and its presence in “natural levels” indicates the intactness of native lignin. The remaining key interunit linkages include phenylcoumaran (β -5), resinols (β - β'), and dibenzodioxocin (5-5'/4-O- β') and spirodienone (β -1).

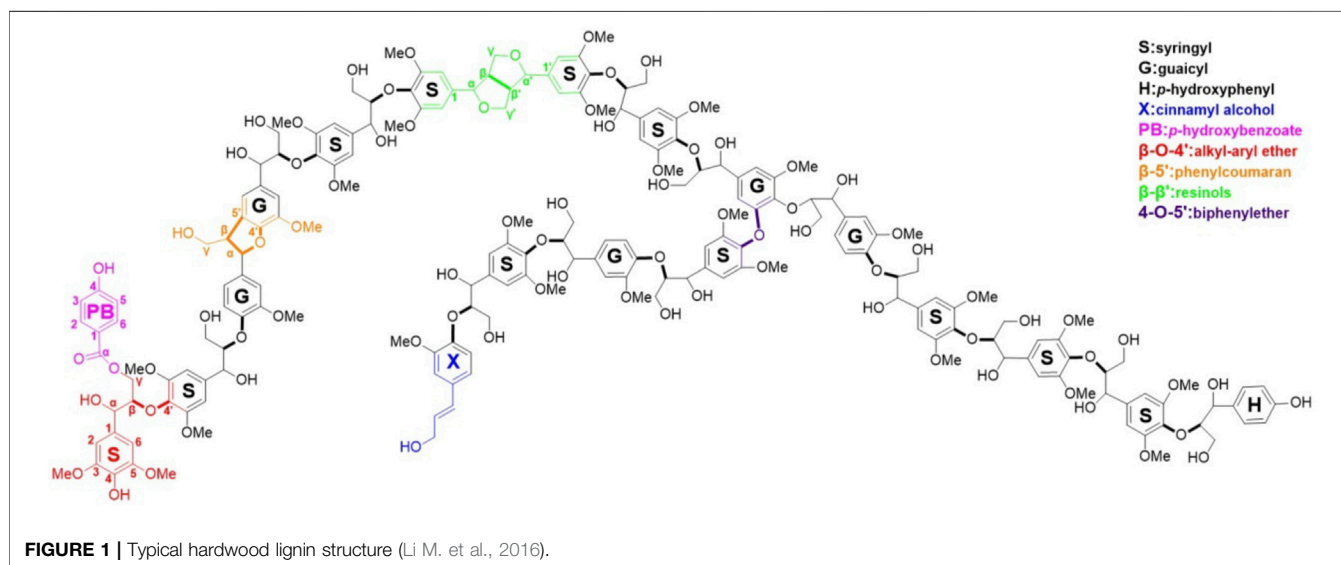
1.2 Cellulases-Lignin Interactions During Enzymatic Hydrolysis Process

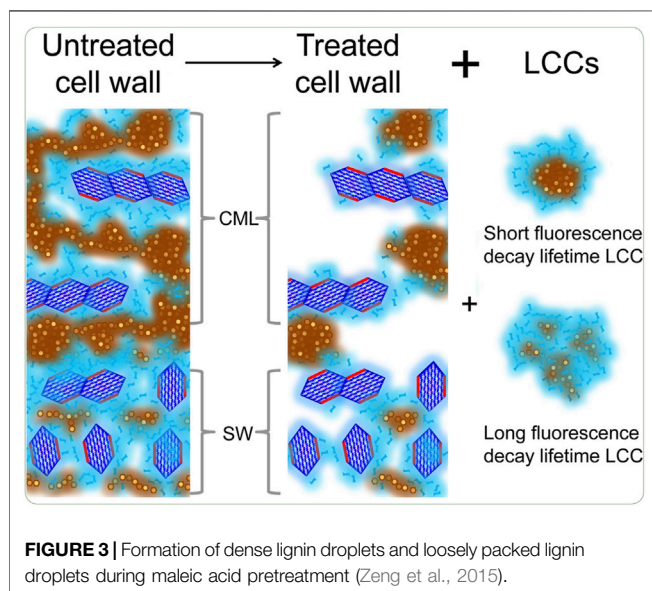
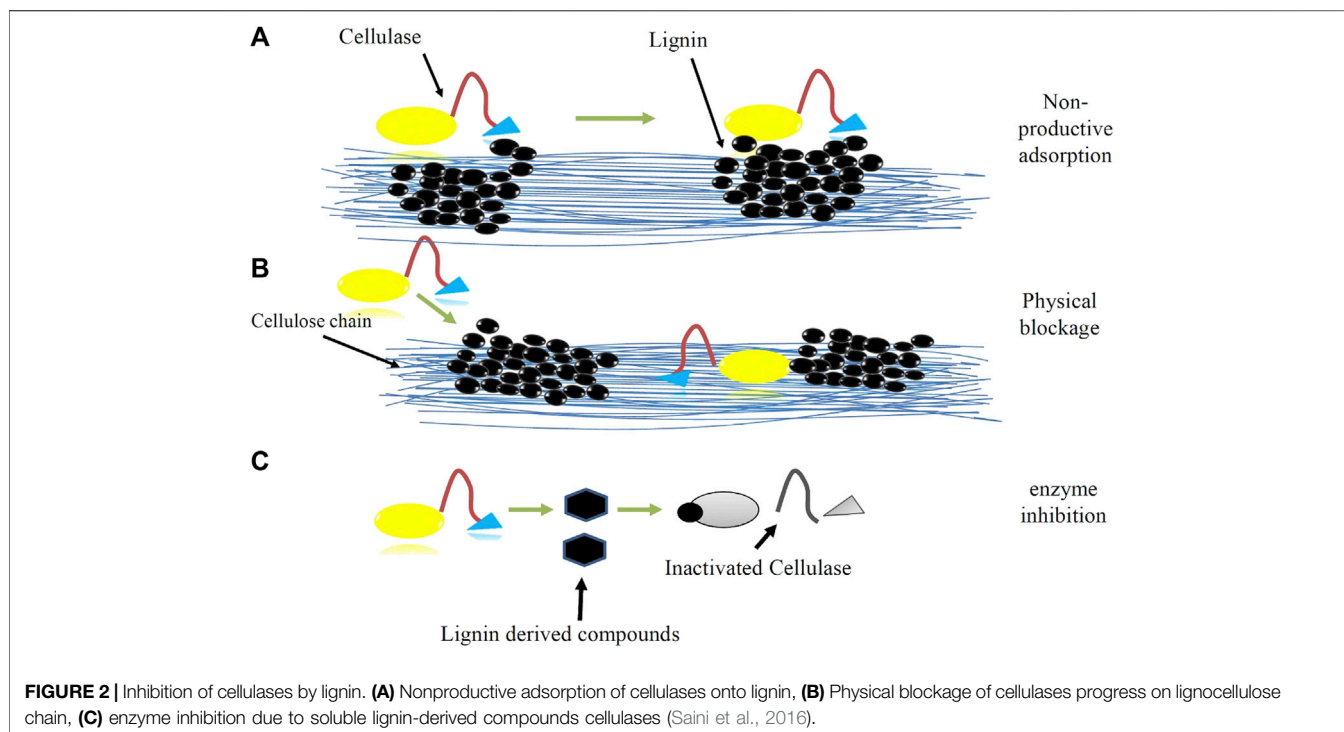
The interaction between lignin and cellulases is very complicated. The most focused research area is lignin-derived inhibition,

which has been proposed to occur in three ways: (I) physical barrier, (II) nonproductive binding, and (III) deactivation of enzymes by lignin fragments of small molecular weight, as shown in **Figure 2**.

1.2.1 Lignin's Physical Barrier Properties

Lignin can block the access of enzymes to cellulose as a physical barrier, which is one of the main reasons for the low glucose yield from the enzymatic hydrolysis of native biomass. Earlier studies supported the hypothesis that lignin extraction and deposition during hydrothermal pretreatment might decrease the enzymatic hydrolysis rate over time (Selig et al., 2007; Donohoe et al., 2008; Hansen et al., 2011). In a series of studies, Lai et al. demonstrated that these solvent extractable lignin droplets from ethanol organosolv pretreatment could also have positive effect on enzymatic hydrolysis (Lai et al., 2018b). A micro-spectroscopic approach combining stimulated Raman scattering microscopy and fluorescence lifetime imaging microscopy was employed to understand the roles lignin play in biomass recalcitrance after maleic acid pretreatment (Zeng et al., 2015). Results showed that both dense lignin droplets and loosely packed lignin droplets were formed (**Figure 3**). Li and his coworkers found that lignin droplets relocated onto the Avicel surface after hydrothermal pretreatment of a mixture of Avicel cellulose and poplar wood as a lignin source, significantly inhibited cellulose hydrolysis (Li et al., 2014). Similarly, researchers also found that after liquid hot water pretreatment, spherical droplets appeared on the surface and in the corners of the disrupted cell walls, which might act as a physical barrier inhibiting the access of enzymes to the inner region of cell walls (Donohoe et al., 2008; Ko et al., 2015b; Wang et al., 2015b). The removal of droplets by 1, 4-dioxane extraction was shown to increase the glucose yield from cellulases treatment from 16.3 to 23.0% (He et al., 2020a). Recently, THF-water co-solvent system with dilute sulfuric acid was applied to solubilize redeposited lignin and overcome the limitations of traditional dilute sulfuric acid pretreatment. It was suggested that this co-solvent could





prevent lignin redeposition onto the biomass surface and prolong cellulases activity (Patri et al., 2021).

Microscopic analysis showed that after autohydrolysis pretreatment, lignin from *Eucalyptus globulus* migrates out of the cell wall and redeposits in certain regions of the fibers to form droplet-like structures. The authors reported that the regions of the cell wall adjacent to the coalesced lignin appear to open and improved the accessibility of cellulose to enzymes (Araya et al., 2015). Another study using a lime pretreatment showed that tiny

droplets were formed on the sweet sorghum bagasse surface, which could increase cellulose conversion rate and at the same time reduce carbohydrates loss by forming a complex with lignin and calcium ions (Yan et al., 2015). The inconsistency of the obtained results indicated that the fundamentals behind the impact of lignin droplets on cellulose accessibility are still not fully understood, and more studies are needed to clarify the mechanisms.

1.2.2 Nonproductive Binding of Lignin to Cellulases

The nonproductive binding of cellulases to lignin has attracted much attention recently. This binding effect has been attributed to hydrophobic interactions, electrostatic forces, and/or hydrogen bonding. Hydrophobic interactions were reported to be the dominant driving force in cellulases binding to lignin, which was revealed by atomic force microscopy between specialized tips with hydrophobic, -OH, and -COOH groups, and immobilized cellulases (Qin et al., 2014). Lignin is more hydrophobic than any other major component in biomass (Hodgson and Berg, 1988). It was found that proteins could be adsorbed at the hydrophobic surfaces of lignin (Saini et al., 2016). A study on cellulases adsorption to different lignin preparations correlated carboxylic acid groups in lignin and cellulases adsorption. They suggested that the carboxylic acid content could impact the hydrophilicity of the lignin, which in turn affects the nonproductive binding of the cellulases to lignin (Pareek et al., 2013). By investigating the association of aspen lignins after hydrothermal pretreatment at various pretreatment severities with cellulases binding, Sun and his coworkers found that the condensed aromatic rings enhanced the hydrophobic interactions between lignin and cellulases, which are mainly responsible for

the inhibitory effect of lignin on cellulases (Sun et al., 2016). Other studies also obtained similar results (Yu et al., 2014; Ko et al., 2015a; Yao et al., 2018a).

Previous studies found that hydrogen bonding was another major driving force for cellulases adsorption onto lignin. These studies indicated that hydroxyl groups in lignin, especially phenolic OH, played an essential role in cellulases-lignin interaction (Pan, 2008). Many other researchers later confirmed this hypothesis (Yu et al., 2014; Sun et al., 2016; Yao et al., 2017). It was found that hydroxypropylation of phenolic OH could reduce the negative inhibitory effect of lignin on cellulases (Yang and Pan, 2016). Similarly, pretreated biomass with decreased phenolic OH contributed to an increased digestibility of biomass (Mou and Wu, 2017). All these results support the hypothesis that hydrogen bonding plays a vital role in the nonproductive binding of cellulases to lignin.

The isoelectric point (pI) is a unique feature to each enzyme which is defined as the pH at which the overall net charge is zero. When the solution pH is above the pI, the overall charge of the enzyme is negative, and vice versa. Cellulases and lignin will bind together if they possess opposite charges by electrostatic interactions. At pH 4.8, which is the typical optimum pH of cellulases, lignin showed a negative zeta potential, and cellulases (such as Cel6A and Cel5A) were positively charged (Saini et al., 2016; Yang et al., 2020) while others (such as Cel7A and Cel7B) were negatively charged (Lai et al., 2018b). Thus, depends on the pH and the types of enzymes, there could be either attractive or repulsive forces between cellulase and lignin substrates. A study on the *Trichoderma reesei* cellulases cocktail and lignin showed that electrostatic interactions contributed to cellulases adsorption, and their effect was most pronounced for β -glucosidase from *T. reesei* (Ko et al., 2015a). An elevated pH in the cellulases hydrolysis process could significantly reduce the nonspecific binding of cellulases to lignin residues after various pretreatment (Lou et al., 2013). However, some studies in the literature report that electrostatic interactions were not significantly involved in the cellulases lignin interactions (Lou et al., 2013; Yang et al., 2020) which suggests further investigations are needed on this aspect.

1.2.3 Deactivation of Cellulases by Lignin-Derived Phenolic Compounds

As pretreatment is a necessary step to reduce the recalcitrance of biomass, lignin-derived phenolic compounds formed during pretreatment are nearly universal in the hydrolysate of pretreated lignocellulosic biomass. These soluble lignin derivatives could influence the performances of cellulases enzymes and fermentation yeast. The impacts of phenolics on yeast include damage to internal proteins structures, a decrease in cell growth, and changes in cell morphology (Fitzgerald et al., 2004). Studies showed that the properties of generated phenolics were significantly affected by the applied pretreatment technologies.

It has been confirmed that the phenols could inhibit cellulose hydrolysis. The rate of cellulose hydrolysis reduces by 50% by

adding 8 mg vanillin per FPU (Ximenes et al., 2010). Toxic and inhibitory compounds vary with applied pretreatment. Phenolics were identified as the most inhibitory components, which decreased the rate and extent of cellulose hydrolysis by half due to both inhibition and precipitation of the enzymes (Kim et al., 2011). The different influence of various phenolic compounds on cellulases and yeast might be due to the incorporation of amide group in phenolics which are formed during AFEX pretreatment process (Chen et al., 2020). Inhibition of different phenolics compounds on cellulases has been studied by Qin and his coauthors (Qin et al., 2016). It was found that phenolics compounds with aldehyde and ketone group exhibited a more inhibitory effect on cellulases than those with phenol and carboxyl group. In addition, the carbonyl group and methoxy group in phenolics also exhibited inhibition toward enzyme activity, as shown in **Figure 4** (Qin et al., 2016). The effects of three phenolic acids on the structure of cellulases were also investigated. It was indicated that the addition of the phenolic acids (i.e., ferulic acid, *p*-coumaric acid, and salicylic acid) significantly changed the secondary structure of cellulases by decreasing α -helix content, increasing β -sheet and random coil contents (Tian et al., 2013), which accounted for the decreased enzyme activity. Although lignin-derived phenolic compounds play negative effect on cellulases, as the hydrolysates have been separated from pretreated substrates, it may not affect the enzymatic hydrolysis process in some cases.

Most research studies have focused on the three lignin-enzyme interactions mentioned above, but not all three interactions were included in each enzyme adsorption mechanism study, as summarized in **Table 2**. In recent years, intriguing studies have shown that pretreated lignin from certain plant cell wall locations or with certain physicochemical properties could have a positive effect on the enzymatic hydrolysis process. For example, hardwood organosolv lignin was reported to enhance the enzymatic hydrolysis, while softwood organosolv lignin played a traditional negative role (Lai et al., 2014). Similarly, solvent extractable lignin and the residual bulk lignin also demonstrated distinct roles (Lai et al., 2015). Enhanced sugar release during hydrolysis was also reported by incorporating sulfonate groups (Wang et al., 2013a; Wang et al., 2013b; Zhou et al., 2013) and acid groups (Wu et al., 2011) onto the lignin during the biomass pretreatment step. Finally, Huang et al. concluded that the exact effect of lignin on enzymatic hydrolysis is a function of both inhibitive hydrophobic interactions and the stimulative electrostatic repulsions, which are controlled by lignin hydrophobicity and surface charges, respectively (Huang et al., 2017).

Although years of research on lignin-enzyme adsorption study has focused on using lignin model compound or modifying lignin structure in order to build the “structure-function” relationship, the relative contribution of each individual factors on lignin-enzyme interaction is still unknown. This is mainly due to the difficulty of differentiating each driving forces and the lack of analytical techniques that could be used to directly measure these factors (Li and Zheng, 2017). For example, phenolic OH group from lignin has been proposed to contribute significantly to the hydrophobic interactions; however, these OH groups also affect

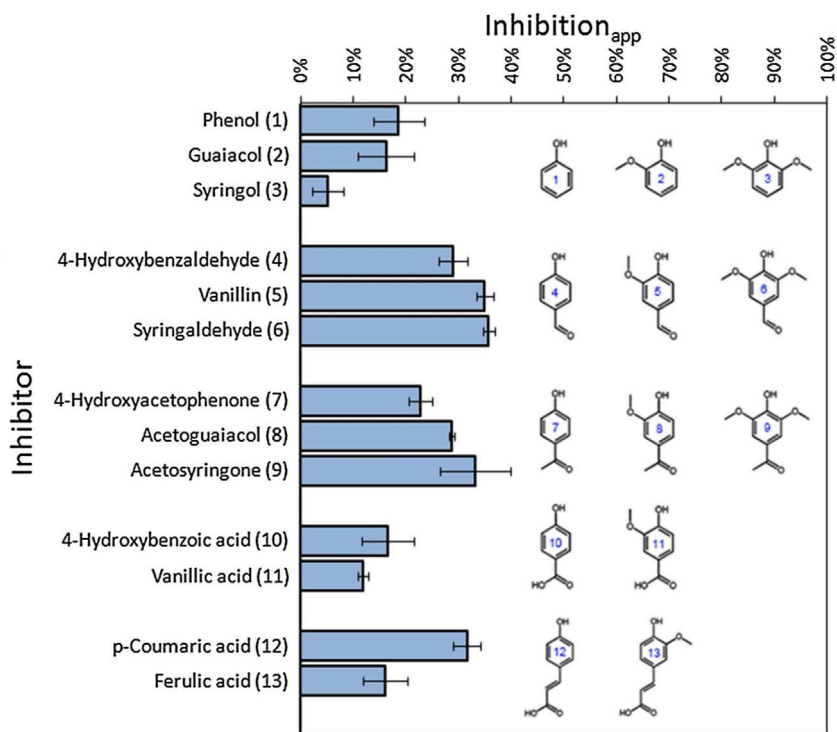


FIGURE 4 | Apparent inhibitions of different phenolic compounds on cellulases (Qin et al., 2016) Note: names and structures with the same number refer to the same phenolic compounds.

TABLE 2 | Mechanisms of nonproductive cellulases adsorption to lignin.

Cellulases	Lignin	Adsorption conditions	Concluded mechanism	References
Celluclast 1.5 L, Novozyme 188	alkali lignin, hydrolytic lignin, organosolv lignin, lignosulphonate acid sodium salt	pH 4.8, at temperatures of 4°C and 45°C	hydrophobic interactions	Pareek et al. (2013)
Cellulase C2730	Isolated from hot water hydrothermal pretreated aspen	2% (w/w) lignin at 4°C and 150 rpm for 2 h	Hydrophobic interactions, hydrogen bonding	Sun et al. (2016)
<i>T. reesei</i> β -glucosidase	liquid hot water pretreated mixed hardwood chips	pH 4.8, at 25°C for 1.5 h	Electrostatic interactions	Ko et al. (2015a)
Cellobiohydrolase I, endocellulase E1, eno-1,4-xylanase	Extracted from switchgrass pretreated by methyl isobutyl ketone, ethanol, water (16/34/50 w/w/w), 0.1 M sulfuric acid at 160°C	1 mg/ml of lignin, flowing enzyme solution at 0.1 ml/min	Hydrophobic interactions, electrostatic interactions	Sammond et al. (2014)
<i>Trichoderma reesei</i> , ATCC 26921	water-soluble, low sulfonate kraft lignin (MW = 10,000)	0.10 mg/ml Celluclast at room temperature in pH 4.8	hydrophobic interaction	Qin et al. (2014)
Cellic CTec2, CellulaseC2730	Ethanol organosolv lignin isolated from organosolv pretreatment of cottonwood, black willow, aspen, eucalyptus, and loblolly pine	2% (w/v) EOL lignins in 0.05 M citrate buffer (pH 4.8), at 4°C and 150 rpm for 3 h	Hydrophobic interactions and electrostatic interactions	Huang et al. (2017)
<i>Trichoderma longibrachiatum</i> cellobiohydrolase	lignin isolated from dilute acid pretreated <i>Broussonetia papyrifera</i>	lignins (2%, w/v) in acetate buffer (50 mM, pH 4.8) at 50°C for 4 h	hydrogen bonding	Yao et al. (2017)
<i>Trichoderma longibrachiatum</i> cellobiohydrolase	lignin isolated from dilute acid pretreated poplar	lignin samples (2%, w/v), pH 4.8 at 50°C	hydrophobic interactions	Yao et al. (2018a)
Celluclast 1.5 L and β -glucosidase	enzymatic residual lignin treated with dilute sulfuric acid at different severities	lignin (1%, w/v), at 4°C for 1 h	hydrophobic interactions	Wang et al. (2020a)
Celluclast 1.5 L and β -glucosidase	enzymatic residual lignin treated with sodium hydroxide at different severities	lignin (1%, w/v) at pH 5.0 and 50°C	Hydrophobic interactions and electrostatic interactions	Wang et al. (2020b)

(Continued on following page)

TABLE 2 | (Continued) Mechanisms of nonproductive cellulases adsorption to lignin.

Cellulases	Lignin	Adsorption conditions	Concluded mechanism	References
CBH and BGL from <i>Penicillium oxalicum</i> JU-A10-T	Milled wood lignin from untreated and LHW pretreated corn stover	50 mM acetate buffer (pH 4.8) at 50°C for 48 h under 50 rpm	Hydrophobic interactions	Lu et al. (2016)
EG and xylanase from <i>Penicillium oxalicum</i> JU-A10-T	Milled wood lignin from untreated and LHW pretreated corn stover	50 mM acetate buffer (pH 4.8) at 50°C for 48 h under 50 rpm	electrostatic interactions	Lu et al. (2016)
Cellic CTec2	lignin extracted from corn stover	at room temperature for 60 min at pH 4.8	hydrophobic and electrostatic interactions	Yarbrough et al. (2015)

lignin's hydrogen bonding ability which is difficult to measure directly. Additional work is much needed to resolve these challenges.

2 FACTORS INFLUENCING LIGNIN-CELLULASES INTERACTIONS FROM A LIGNIN PERSPECTIVE

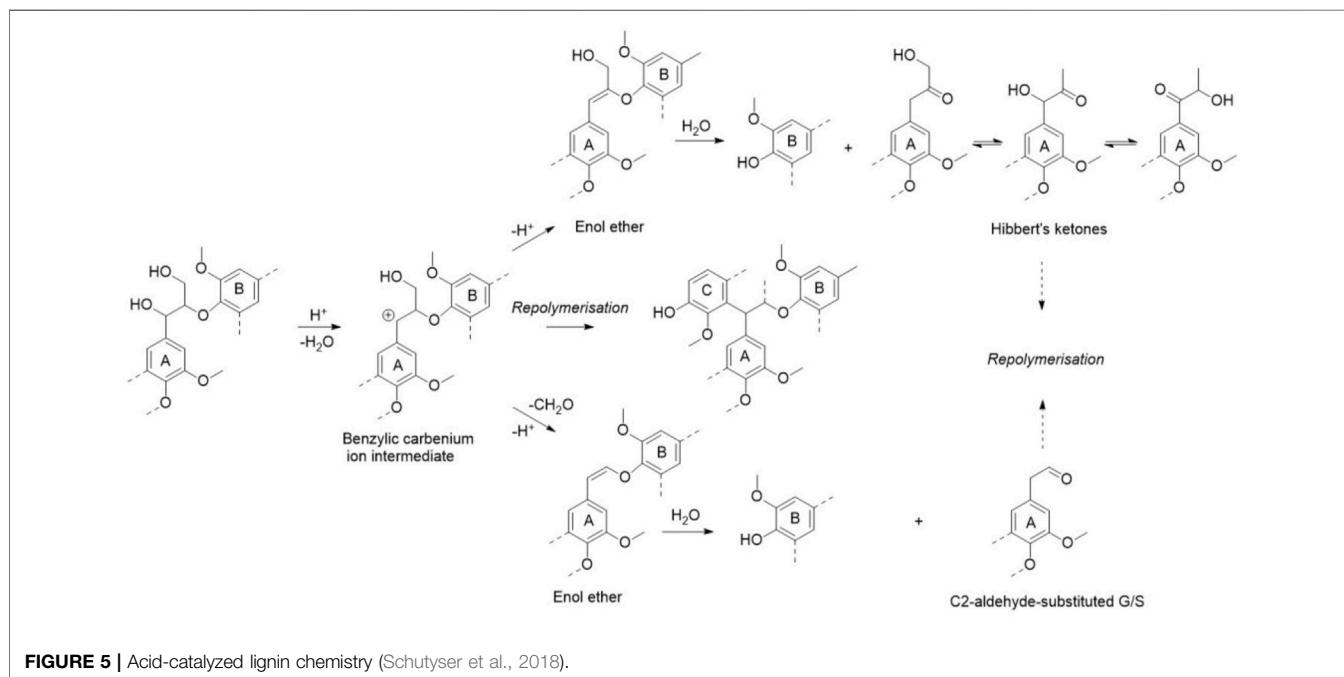
2.1 Lignin Transformation During Pretreatment and Its Effect on Cellulases Interaction

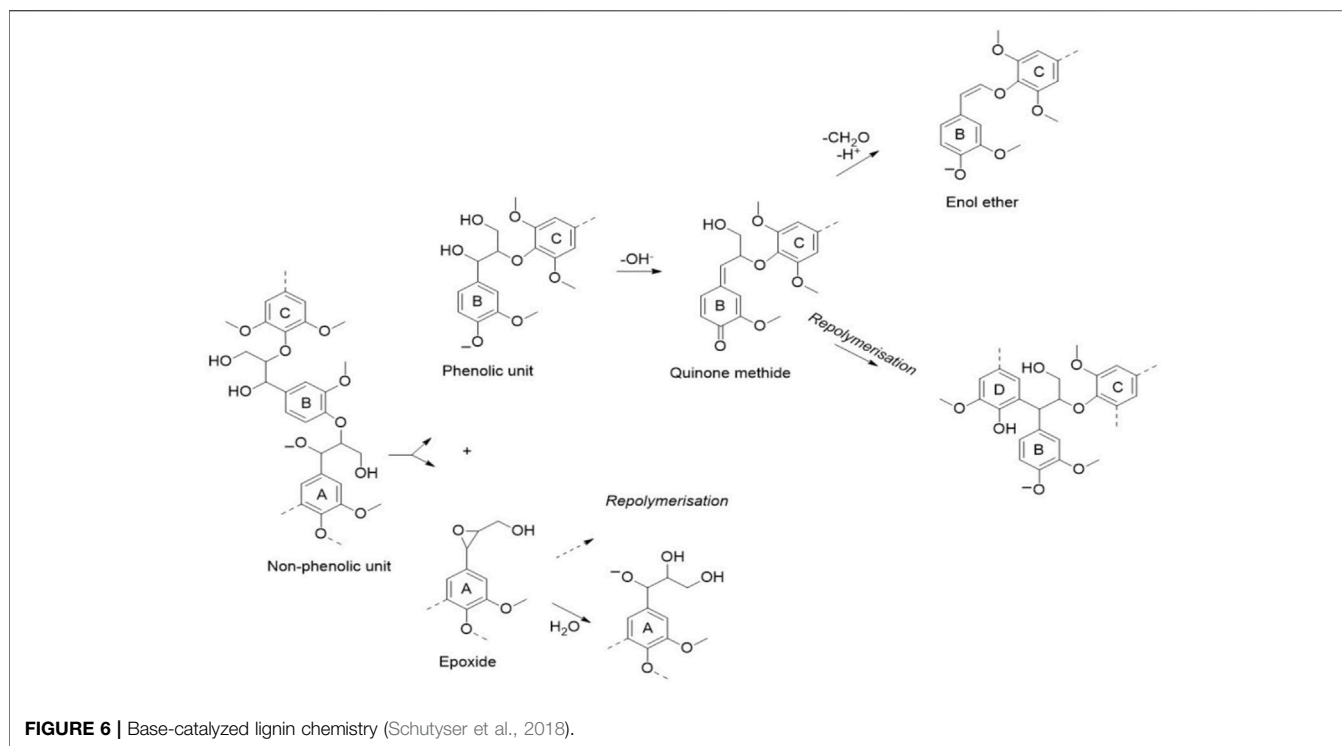
2.1.1 Dilute Acid Pretreatment

Dilute acid pretreatment is a widely studied pretreatment method which typically exhibits limited delignification. Studies showed that the inhibitory effect of lignin from DA pretreated biomass was more significant than the untreated one (Xu et al., 2020). It is well known that both polymerization and depolymerization can happen during acid-catalyzed pretreatment (Schutyser et al., 2018; Yang et al., 2020). During DA pretreatment, lignin has been reported to be

significantly depolymerized by acid catalyzed hydrolysis of β -O-4 linkages as reported in several earlier studies (Lundquist et al., 1972; Karlsson et al., 1988). Usually, the molecular weight of lignin after DA pretreatment was decreased (Yang et al., 2020), and the lower molecular weight favored CBH (Cellobiohydrolase I) adsorption to lignin (Yao et al., 2018a). Poplar lignin was only partially degraded after DA pretreatment at 170°C for 8 min using 0.5% H_2SO_4 as catalyst (Hu et al., 2013). Structural analysis of milled wood lignin from switchgrass after DA pretreatment showed that the β -O-4, β - β , β -5 linkages, and syringyl units were all decreased (Samuel et al., 2010). By comparing four different pretreatment methods (including dilute sulfuric acid, sodium hydroxide, ethanol, hot liquid water) on the structural features of residual lignin and their impact on cellulases, the results indicated that dilute sulfuric acid pretreated lignin showed the maximum adsorption capacity to cellulases (Xu et al., 2020). The reason might be due to the lowest negative zeta potential of residual lignin after dilute sulfuric acid pretreatment.

The condensation reaction is another important lignin transformation during DA pretreatment (Samuel et al., 2010; Imai et al., 2011; Cao et al., 2012; Moxley et al., 2012; Pu et al.,





2013; Deuss et al., 2015; Sun et al., 2015; Ko et al., 2015b), which could cause increased cellulases adsorption to lignin (Yao et al., 2018a). The formation of condensed lignin process is depicted in **Figure 5**. Depending on the applied acid, the formation of a benzylic carbonium ion could be transformed into two enol ether structures. Subsequent hydrolysis products of the acid-labile enol ethers could participate in a complex network of repolymerization reactions, resulting in a condensed lignin polymer. It was found that the addition of formaldehyde during pretreatment can partly prevent lignin condensation. The resulting stable 1,3-dioxane structure with the 1,3-diols on lignin side-chains could enhance the amount of uncondensed lignin by blocking the formation of benzylic cations after pretreatment (Shuai et al., 2016a), which was later verified by other researchers (Zhang et al., 2021). Furthermore, 2-naphthol and 2-naphthol-7-sulfonate additives were employed during DA pretreatment to block lignin bridging reactions and consequently, improved cellulases hydrolysis yield by 47.8% (Lai et al., 2018a).

Another impact of DA pretreatment on cellulases was caused by the formation of pseudo-lignin (Wan et al., 2019), which was shown to be more detrimental to enzymatic hydrolysis of cellulose than residual lignin (Hu et al., 2012; He et al., 2018; Wang and Jönsson, 2018). Several studies demonstrated that the pseudo-lignin was a polyaromatic (i.e., lignin-like) structural substances derived from carbohydrate degradation products (Sannigrahi et al., 2011) which increased the Klason lignin content after DA (Shinde et al., 2018; Wang and Jönsson, 2018). A recent study revealed that soluble lignin model compounds could participate in pseudo-lignin's formation during acid pretreatment by assembling a platform of spherical droplets (He et al., 2020b). As pseudo-lignin is

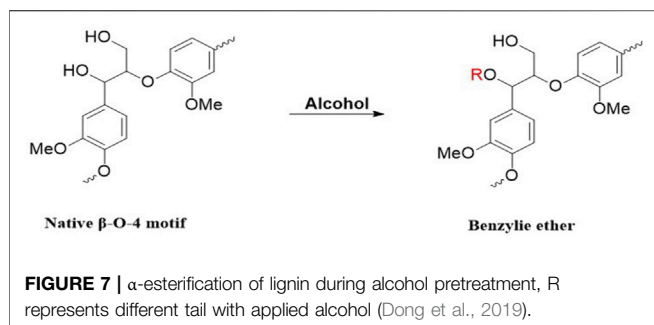
typically formed at high pretreatment severity, thus, severe conditions should be avoided during DA pretreatment.

2.1.2 Alkaline Pretreatment

A review of the alkaline (AL) pretreatment literature indicates that different bases (e.g., NaOH and lime) could be used in AL pretreatment for lignin solubilization (Yang and Pan, 2016). These kinds of AL based pretreatments are usually carried out at lower temperature and pressure when compared with hydrothermal pretreatments (Fang et al., 2018). NaOH-based pretreatment could reduce biomass recalcitrance by rupturing the linkages between lignin and polysaccharides (mainly hemicellulose), degrading β -O-4 linkages, cleaving ester bonds, as well as disrupting the lignin structure (Xiao et al., 2014; Yan et al., 2015; Yang et al., 2016). Base-catalyzed cleavage of the β -O-4 bonds in non-phenolic units has been postulated to produce an epoxide intermediate and a phenolic unit that could be transformed into quinone methides (**Figure 6**). The quinone methide can repolymerize *via* the formation of a carbon-carbon bond (Schutyser et al., 2018). Alternatively, the removal of the terminal γ -CH₂OH group could form an alkali-stable enol ether motif and formaldehyde.

In addition to NaOH-based techniques, ammonia fiber explosion/expansion (AFEX) is another well-known alkaline pretreatment (Bouxin et al., 2015). Cleavage of LCC and ester linkages during the pretreatment process could result in partial solubilization of lignin, which is mainly composed of oligomeric fragments, and β -O-4 bonds are typically well preserved during the ammonia based pretreatment (Chundawat et al., 2011).

Our previous research indicated that lignin could be easily dissolved during AL pretreatment, and the recovered lignin



termed as alkaline lignin showed the least effect on cellulases performance when compared with milled wood lignin and Klason lignin (Yao et al., 2012). Hydrophilic sulfonated lignin and hydrophobic kraft lignin were compared of their effects on the enzymatic digestibility of various pretreated biomass. The impact of lignin addition on the enzymatic digestibility varied with both added lignin type and the applied pretreatment methods (Wang et al., 2015a). It was implied that the influence of lignin addition on the enzymatic digestibility of pretreated biomass was dependent not only on the properties of introduced lignin, but on the residual lignin in pretreated biomass as well. By comparing alkali lignins with different molecular weights on enzymatic hydrolysis of lignocellulose, it was showed that the addition of alkali lignins improved the cellulases hydrolysis, and it was increased with increasing molecular weights of alkali lignins (Li Y. et al., 2016). Due to the adsorption of cellulases on alkali lignins, the filter paper activity could be enhanced. Another study demonstrated that when kraft pine lignin was precipitated on the surface of cellulose, a detrimental effect on the hydrolysis performance of enzymes was noted (Li W. et al., 2018). Kraft pine lignin with different molecular weights showed that lignin of lower molecular weight might form a complex with cellulases, and lignin of higher molecular weight resulted in steric repulsions by lignin deposition on cellulose (Li W. et al., 2018). By comparing residual lignins in acid pretreated and kraft pulped bamboo, their extents of nonproductive enzyme adsorption were investigated. It was found that the maximum adsorption capacity and the inhibitory effect on enzymatic hydrolysis efficiency was more obvious in acid pretreated lignin than kraft pulped bamboo lignin (Huang et al., 2016), due to more hydrophobicity, phenolic hydroxyl group, and the degree of condensation of residual lignin after dilute acid pretreatment.

2.1.3 Organosolv Pretreatment

Organosolv (OS) pretreatment is a well-known delignification method employing various organic solvents including acetone, methanol, ethanol, diol, THF, and γ -Valerolactone (GVL). Lignin could be substantially removed due to the acid catalyzed cleavage of β -aryl ether bonds (Kangas et al., 2015) resulting in a cellulose-rich solid residual with enhanced reactivity toward enzymes (Zhang et al., 2016; Dong et al., 2019; Meng et al., 2020). The advantage of OS pretreatment includes the enhanced digestibility of pretreated biomass and the high potential valorization ability of the obtained organosolv lignin (García et al., 2017; Sadeghifar et al., 2017; Yao et al., 2020).

Ethanol is the most studied green solvent applied in organosolv pretreatment. Structural analysis of lignin from *Populus* after auto-catalyzed ethanol organosolv pretreatment indicated that part of the β -O-4' linkages were cleaved whereas, β - β' and β -5' units were intact. Stilbene units were also formed during this process (Guo et al., 2015). It was also observed that ethanol organosolv resulted in an increase in the S/G ratio, cleavage of part of β -O-4' and β -5' (Sannigrahi et al., 2009; Hallac et al., 2010; Wen et al., 2013; Zhu et al., 2015; Rinaldi et al., 2016; Yao et al., 2018b). Furthermore, organic solvents such as ethanol and methanol have been reported to quench the benzyl carbonation intermediate by forming ether linkages at the α position of lignin (Mateo et al., 2020), which could hinder lignin repolymerization and condensation (Huang et al., 2015).

A modified organosolv pretreatment employing 1,4-butanediol (1,4-BDO) was developed by Dong and his coworkers (Dong et al., 2019). The results showed that lignin deposition was not formed compared with conventional organosolv pretreatment using ethanol, and a relatively higher amount of β -O-4' interunit linkages was retained in residual lignin by forming α -etherified lignin with hydroxyl tail and protecting the β -O-4 substructure from degradation and condensation, shown in **Figure 7** (Dong et al., 2019). In another study, by applying ethanol (EtOH), THF, and γ -Valerolactone (GVL), it was found that the total content of condensed S and G units was significantly higher in EtOH lignin than that in THF and GVL lignins. Furthermore, more β -O-4 linkage was preserved in GVL lignin, making it suitable for producing mono-aromatic lignin compounds (Shuai et al., 2016b; Meng et al., 2020). Similar to GVL, cyrene, a cellulose-derived aprotic dipolar organic solvent, also has high potential as a green pretreatment solvent in terms of lignin fractionation/recovery and sugar release in the follow-up enzymatic hydrolysis (Meng et al., 2020b).

Various co-solvent pretreatments have demonstrated advantages relative to aqueous-only methods by enhancing lignin removal, among which tetrahydrofuran (THF) has been identified as a highly effective co-solvent for solubilizing and extracting lignin from biomass by adopting extended coil configurations (Smith et al., 2016). Not only enhanced solubilization of cellulose and high yields of fermentable sugars can be achieved by the THF-water co-solvent system (Mostofian et al., 2016), but also facilitates lignin transformation into valuable chemical precursors (Patri et al., 2019).

Lai and her coauthors found positive effects of extractable lignin (EL) on enzymatic hydrolysis of ethanol organosolv-pretreated wood sawdust by blocking nonproductive enzyme binding sites on the milled wood lignin (Lai et al., 2019). Subsequent research found that different organosolv lignin showed disparate effects on cellulases. It was indicated that organosolv lignin with PB units enhanced MCC digestibility (Huang Y. et al., 2020). The reason is because the partial negative charges on the carbonyl groups in PB units result in lower cellulases binding on lignin. Acid-catalyzed glycerol (AG) pretreatment of sugarcane bagasse was investigated and it was found that glycerol had modified the bagasse lignin through α -etherification of β -aryl ethers and γ -esterification of

hydroxycinnamic acids. The obtained lignin was highly hydrophilic, and it did not inhibit the enzymatic hydrolysis of pretreated bagasse (Hassanpour et al., 2020). By comparing liquid-hot-water (LHW) pretreatment with acid-free ethanol-water (EW) pretreatment, it was found that the non-productive adsorption between EW pretreatment-induced lignin and cellulases was significantly weakened due to the advantages of suppressing the deposition of lignin condensates (Shi et al., 2018).

2.1.4 Other Promising Pretreatments

As new and rapid emerging green solvents, deep eutectic solvents (DESs) have gained much attention lately, which are composed of a hydrogen-bond acceptor (HBA) and a hydrogen-bond donor (HBD) component. In addition, DESs are biocompatible, non-toxic, and have been shown to be recycled for at least 5 rounds in pretreatment with the same digestibility improvement (Kim et al., 2018; Li A-L. et al., 2018; Guo et al., 2019; Kim et al., 2019; Kim et al., 2019; Sai and Lee, 2019; Song et al., 2019; Lin et al., 2020; Huang et al., 2021; Ji et al., 2021). Compared with dilute acid and alkaline pretreatment, DES pretreatment led to significantly higher lignin removal (Li A-L. et al., 2018; Li W-X. et al., 2021; Tan et al., 2019; Guo et al., 2018; Hansen et al., 2021; Fernandes et al., 2021). The central lignin transformation during DES pretreatment of wood lignin was the cleavage of β -O-4 linkages, resulting in decreased molecular weight and increased hydroxyl groups (Das et al., 2018; Kim et al., 2019; Wang J. et al., 2020; Wang Y. et al., 2020; Wang Y. et al., 2021). Similar research also proved that acidic DESs could degrade a small portion of C-C bonds in lignin (Li A-L. et al., 2018; Ma et al., 2021). In addition to depolymerization, repolymerization of lignin fractions can also occur during acidic DES pretreatment *via* acid-catalyzed recondensation (Liu et al., 2021). Alkaline DES pretreatment disrupts lignin-carbohydrate compounds and breaks ether linkages in lignin (Ho et al., 2019). Studies indicated that acidic DESs showed higher lignin solubility than alkaline DESs, and lignin from bamboo dissolved in DES was mainly syringyl lignin (Li C. et al., 2021). DES-extracted lignin from willow and corn stover showed an inhibitory effect on cellulases by hydrophobic interactions and hydrogen bonds (Song et al., 2020).

Acid hydrolytic fractionation (AHF) was another promising method developed by Zhu's group to improve lignin valorization by reducing lignin condensation (Chen et al., 2017). AHF refers to fractionate lignocelluloses using a group of acids with hydrolytic properties (Cai et al., 2020). It was suggested that acid hydrolytic could aggregate around extracted lignin to prevent lignin from aggregation (Ji and Lv, 2020). The impact of the hydrolytic on ether and/or ester bonds cleavage can promote the removal of lignin from the plant cell wall. Results indicated that lignin carboxylation during pretreatment could improve glucose yield during the saccharification process by reducing nonproductive cellulases adsorption to lignin (Cai et al., 2020). Furthermore, maleic acid pretreated switchgrass showed improved enzymatic digestibility by carboxylated lignin and cellulose in the fractionated cellulosic water-insoluble solids (Su et al., 2021).

Due to the limitation of each pretreatment method, combined pretreatment technologies have been developed. As early as 2012,

deacetylation with 0.1 M NaOH before acid pretreatment was suggested by Chen and his co-workers to improve the monomeric sugar yield and minimum ethanol selling price (Chen et al., 2012). Two-stage hydrothermal pretreatment was proposed by Min *et al.* to diminish inhibitors and enhance the total sugar recovery at the same time (Min et al., 2015). Then, combinatorial pretreatment with 1% H₂SO₄ for 30 min followed by 1% NaOH for 60 min at 120°C of corn stover were investigated in an effort to synergistically improve carbohydrate conversion and lignin processability into polyhydroxyalkanoate (Liu et al., 2017). Similarly, a highlighted application of combinatorial pretreatments of autohydrolysis followed by dilute alkali extraction was applied by Huang and his coworkers to maximize the carbohydrate output from bamboo (Huang C. et al., 2020). Furthermore, combination pretreatment using dilute sulfuric acid, liquid hot water, sodium hydroxide, and ethanol were developed by the same research group (Liu et al., 2018). Results showed that combinatorial pretreatment was an effective strategy to facilitate lignin valorization. In an effort to efficiently produce sugars and improve lignin processability for the fabrication of lignin nanoparticles, combinatorial organosolv pretreatment (COP) was thus developed (Liu Z-H. et al., 2019).

Sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) is another combined pretreatment technology which consists of sulfite pretreatment with acid catalyst followed by mechanical refining (Zhu et al., 2009). SPORL-treated softwood chips were significantly softened and over 90% of enzymatic cellulose conversion could be obtained (Zhu et al., 2009). It was indicated that hemicellulose removal and lignin sulfonation during SPORL were beneficial for the following cellulose hydrolysis process (Zhu et al., 2009). When applied to a hardwood, near complete cellulose conversion to glucose could be achieved with only about 4% sodium bisulfite charge on aspen at 180°C for 30 min (Wang et al., 2009). Afterwards, by comparing SPORL with dilute acid (DA) pretreatments, it was found that higher enzymatic digestibility and ethanol yield were produced after SPORL than DA pretreated wood chips (Tian et al., 2011). More effective in xylan removal and decreased extent of lignin condensation during SPORL might explain this observation. Furthermore, lower amount of furfural and HMF were formed, which was favorable for the following yeast fermentation (Tian et al., 2011).

Fenton oxidation is an environmental friendly process operated under mild conditions (Zhang et al., 2018). Lignin and hemicellulose could be degraded during the process, which enhances the enzymatic hydrolysis subsequently (Wang S. et al., 2021). To further explore ultrasound-assisted Fenton reaction, it was compared with dilute acid-catalyzed steam explosion. Results showed that ultrasound-assisted Fenton reaction removed more lignin and created slightly more accessible area and pores on the surface of the substrate compared with dilute acid-catalyzed steam explosion (Wang et al., 2016). During Fenton pretreatment, C1 of cellulose was oxidized, which plays an important role in improving enzymatic hydrolysis by making cellulose more accessible (Yang et al., 2019). In addition, lignin structure was transformed after Fenton pretreatment. The S/G and phenolic OH group content were decreased, while the carboxylic content and the negative zeta potential were increased, all of which might be the reason for

decreased unproductive adsorption of cellulases on lignin compared with untreated milled bamboo lignin (Wu et al., 2018).

Furthermore, sequential Fenton oxidation and sulfomethylation pretreatment was applied to increase the enzymatic accessibility by 20% after pretreatment. Fenton oxidative reaction could help introduce sulfomethyl group on aromatic ring more easily by oxidative demethylation, cleave some interunit linkages and increase the hydrophilicity of residual lignin (Ying et al., 2019). Two-pot sequential pretreatment, comprising of ultrasound ethanol and deep eutectic solvent, was developed to efficiently fractionate lignocellulosic biomass into cellulose with high digestibility and high-quality lignin with tailored chemical structures (Ji et al., 2021).

Reductive catalytic fractionation (RCF) has emerged in the past few years to pursue value-added lignin derived products and a cellulose-rich residue that could be further converted to its sugar monomers (Korányi et al., 2020). The key steps include solvolytic extraction, catalytic degradation and upgrading of lignin (Liu et al., 2020). Many different feedstocks (softwood, hardwood and Gramineae), catalysts (Raney-Ni, Zn-Pd/C, Pd/C, Ru/C, Ni/C, Ni₂P/SiO₂, Ni@ZIF-8) and process (solvent, temperature, pressure and time, one-pot method, two-step method) have been investigated to obtain various value-added lignin derived phenolic chemicals up till now (Ferrini and Rinaldi, 2014; Parsell et al., 2015; Schutyser et al., 2015; Luo et al., 2016; Shuai et al., 2016a; Anderson et al., 2017; Cao et al., 2018; Graça et al., 2018; Liu X. et al., 2019). Recently, hydrogenolysis of lignin in birch sawdust with the selectivity towards 4-propyl guaiacol and 4-propyl syringol was achieved with noble metal based catalysts of 5% Ru/C (Liu et al., 2020). A low-cost MoO₂/C catalyst was prepared for the RCF of biomass feedstocks, with high selectivity towards methyl coumarate and methyl ferulate (Gong et al., 2021). Effective removal of the catalyst from the pretreatment residue to enable subsequent enzymatic hydrolysis will be essential for the efficient biofuel production and catalyst recycle at industrial scale.

2.2 Interactions Between Lignin and Cellulases by Model Compounds

Until now, often conflicting conclusion have been reported on the impact and the underlying mechanisms of lignin on cellulases and its hydrolysis properties. It is challenging to investigate the impact of a single lignin feature on cellulases, as any change will result in a concurrent change in other lignin properties. Thus, model compounds are a useful tool in the mechanism study. Homodimers of guaiacyl (LGG) were used as a representative to predict lignin binding sites of β -glucosidases by AutoDock (Lu et al., 2017). Based on the predicted results, the adsorption of the CBM onto lignin was decreased by altering the charge properties of amino acid in CBH, indicating the feasibility of model compounds' application in the mechanism research. Three types of ¹³C-labeled β -O-4 lignin oligomer models were then synthesized and applied in binding sites research of Cellobiohydrolase I (Cel7A) in lignins by Tokunaga and his coworkers (Tokunaga et al., 2020). To explore the effect of lignin composition on cellulases

adsorption, H-DHP, G-DHP, and S-DHP (dehydrogenation polymers) were prepared. The results clearly showed that lignin composition had a significant impact on cellulases performance, and that G-type lignin exhibited the most detrimental effect (Yao et al., 2021). With the development of synthetic chemistry, lignin models with higher molecular weight would simulate further investigations into the lignin-cellulases adsorption process with more conclusive results.

2.3 Decrease Nonproductive Adsorption by Additives

Lignin-blocking additives, such as bovine serum albumin, soybean protein, and surfactants, have been applied in the enzymatic hydrolysis process to improve glucose yields (Kim et al., 1982; Yang and Wyman, 2006; Selig et al., 2008; Mukasekuru et al., 2018; Bhagia et al., 2019; Florencio et al., 2019; Luo et al., 2020). It was reported that these additives could improve glucose release by cellulases treatment by binding to lignin, thus decreased nonproductive cellulases adsorption to lignin (Borjesson et al., 2007; Zheng et al., 2008). Furthermore, biomass washing combined with soybean protein addition was evaluated recently on glucose release during the enzymatic hydrolysis process, which is cost-competitive in a large-scale industrial process (Pinto et al., 2021).

3 PERSPECTIVE FOR FUTURE CHALLENGES OF LIGNIN IN BIOREFINERY PROCESS

Lignin is expected to play an essential role in the future integrated biorefinery process and the development of renewable chemicals and biomaterials. The mechanism underlying cellulases adsorption to lignin has not been fully elucidated so far, mainly due to the complex structure of enzymes, and the heterogeneity of lignin which was also the cause for undesirable lignin performances of particular applications (Yoo et al., 2020).

The unique biosynthesis of lignin causes the initial heterogeneity of this biopolymer. Furthermore, most pretreatments result in an increase the structural heterogeneity of lignin. The depolymerization/repolymerization of lignin during the pretreatment often increase the polydispersity of lignin molecular weight. Future pretreatment methods that decrease the heterogeneity of lignin might favor the following enzymatic hydrolysis process.

The application of recovered lignin from pretreatment is vital to the biorefinery process. It was reported that over-cleavage of β -O-4 linkages to yield more dissolved lignin could damage the lignin's structure for future lignin valorization (Dong et al., 2019). Promising pretreatment should offer a reaction pathway for lignin structural integrity and enhanced cellulose digestibility simultaneously.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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