

Changing the role of lignin in enzymatic hydrolysis for a sustainable and efficient sugar platform

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ABSTRACT

Hydrolyzing lignocellulose, the most abundant biomass raw material in nature, into monosaccharides by enzymes and then transforming the sugars into chemicals and fuels by biological or chemical methods is one of the most important parts of biorefining. In lignocellulosic biomass, cellulose is often tightly wrapped by lignin along with hemicelluloses. Although some pretreatments can partially remove lignin, residual lignin in pretreated substrates still physically blocks the accessibility of cellulose, non-productively adsorbs cellulases, and sterically hinders the enzymatic hydrolysis of cellulose. So, lignin is generally considered to be an inhibitor to the enzymatic hydrolysis of lignocellulosic substrates. However, new research shows that under the right conditions, lignin does not inhibit the enzymatic hydrolysis of lignocellulose, and can even increase the final sugar yield. For example, recently, some novel lignin-target pretreatments have been developed, they can reduce or overcome the inhibitory effect of lignin. And researchers also find that lignin can be functionalized and used as an activator/promoter of enzymatic hydrolysis of lignocellulose, their effectiveness is comparable to common high-efficiency surfactants. What's more, different lignin-based carriers were also synthesized to carry and recycle cellulases during the hydrolysis, they can obviously reduce the load and cost of cellulases. These works indicate that lignin can play a positive role in the enzymatic hydrolysis of lignocellulose. In order for readers to dialectically view lignin and understand its multiplicity, this review is to report the progress in the studies of how researchers tame the "lignin dog" to facilitate enzymatic hydrolysis of lignocellulose.

1. Introduction

Lignocellulose is a very important biomass resource on earth, composed mainly of cellulose, hemicelluloses, and lignin. More than 100 billion tons of lignocellulose are produced through photosynthesis on

earth every year [1]. The lignocellulose can not only sequester carbon to reduce environmental impact but also can be a renewable source of energy, chemicals, and materials for sustainable development [2–5]. The sugar platform in which cellulose in lignocellulose is hydrolyzed into glucose by cellulases (Fig. 1), and then further converted into different target products through chemical and biological means is well

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Nomenclature

Abbreviation

AcL	acetic acid lignin
AFM	atomic force microscope
Alk-SP	alkaline steam pretreatment
AO-SP	alkaline oxygen steam pretreatment
Auto-SP	steam pretreatment without chemicals
CBD	cellulose binding domain
CBHs	exoglucanases
CMC	carboxymethyl cellulose
DA	dilute acid
DHPs	dehydrogenated polymers
EGs	endoglucanases
EHL	enzymatic hydrolysis lignin
G	guaiacyl
H	<i>p</i> -hydroxyphenyl
LCCs	lignin-carbohydrate complexes
Mw	weight-average molecular weight

Oxy-SP	steam pretreatment under oxygen pressure
PEG	polyethylene glycol
pH-LZC	pH-responsive lignin zwitterionic carrier
pH-L-PEG	pH-responsive lignin-based carriers
pl	isoelectric point
<i>p</i> -TsOH	<i>p</i> -toluenesulfonic acid
QCM-D	Dissipative quartz crystal microbalance
QALC	quaternary ammonium lignin carboxylate
QALS	quaternary ammonium lignosulfonate
S	syringyl
SEM	scanning electron microscopy
SL	sodium lignosulfonate
SPORL	sulfite pretreatment to overcome recalcitrance of lignocellulose
β -GLs	β -glucosidases
3H2N	3-hydroxy-2-naphthoic acid
[Ch][Lys]	cholinium lysinate
[C2mim][OAc]	1-ethyl-3-methylimidazolium acetate

recognized as a promising biorefinery strategy [6,7]. For example, we can obtain liquid fuels such as bioethanol and butanol, degradable polymer materials such as polylactic acid and polyethylene glycol, and chemical materials such as 2,5-dimethylfuran and furan-2,5 dicarboxylic acid [6,8,9]. Enzymatic saccharification of lignocellulose has the advantages of mild reaction conditions, high selectivity, and low environmental impact, compared with acid hydrolysis [10,11].

Enzymatic saccharification of cellulose requires the synergistic actions of each enzyme in the cellulase cocktail [12], as shown in Fig. 1. Endoglucanases (EGs) randomly hydrolyze cellulose chain to produce oligosaccharides of different chain lengths with the formation of new ends. Exoglucanases (CBHs) act on the reducing or non-reducing end of cellulose sequentially, breaking the β -1,4-glycosidic bond of cellulose to produce cellobiose. The above two steps can effectively destroy the crystalline structure of cellulose. β -Glucosidases (β -GLs) further catalyze the cellobiose to produce glucose and reduce the feedback inhibition of

cellobiose in the hydrolysis system, promoting the hydrolysis reaction [13,14].

Although it has been almost 100 years since the development of glucose production by enzymatic hydrolysis of cellulose, glucose production from cellulose is still not competitive with production from starch in terms of efficiency and cost. Making the cellulose enzymatic saccharification process economically competitive can not only avoid food shortages, but also greatly expand the source of sugar platforms, which is of great significance for sustainable development. At present, there are still some factors that limit the development of lignocellulose enzymatic saccharification industry, for example, 1) crystalline structure of cellulose [15,16], 2) lower activity of cellulases than amylases [17,18], 3) the low accessibility of cellulose to cellulases, due to the steric hindrance of other components (lignin and hemicelluloses) in lignocellulose [19,20], 4) strong and tough matrix of lignocellulosic substrates [21,22]; and 5) the non-productive adsorption of cellulase on

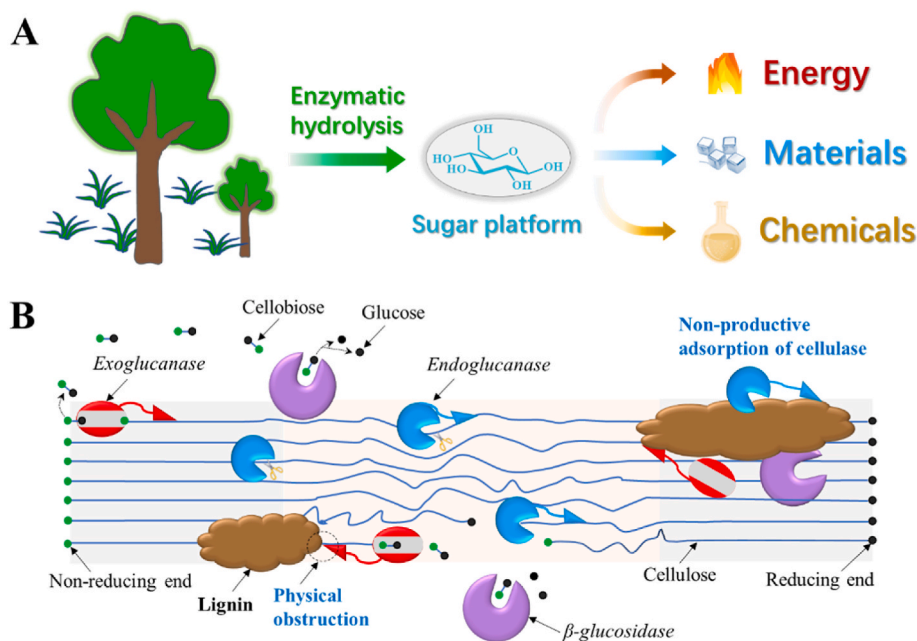


Fig. 1. A: Hydrolyzing lignocellulose into sugars by enzymes and then converted into different products; B: Schematic diagram of enzymatic saccharification of lignocellulose by cellulase.

lignin lead to cellulase not being able to effectively hydrolyze cellulose [23–25]. It has been well recognized cellulose accessibility and non-productive adsorption of cellulase are the two major barriers to efficient and low cost sugar production from lignocellulose, and they are both closely related to lignin in substrates [26].

Effective pretreatment can remove, at least partially, lignin and hemicelluloses in lignocellulosic feedstocks, thus increasing cellulose accessibility to cellulases [27]. However, removing all lignin to obtain pure cellulose substrates for enzymatic hydrolysis is not economically feasible because it consumes a lot of chemical reagents and needs to be performed under harsh conditions [28]. Therefore, the influence of lignin on the enzymatic hydrolysis of cellulose is a problem that must be dealt with, and it has been widely studied in past decades [22,23,29]. Freudenberg, Ralph, Boerjan et al. have done pioneering work for a better understanding of lignin structure, including the natural structure in plants and the structure after different pretreatments [30–34]. Ragauskas, Wyman, Saddler et al. provided many strategies and suggestions for the development of effective pretreatments [35–37], and they also studied the interaction of lignin and cellulases during enzymatic hydrolysis [15,38]. In most studies, lignin has been widely recognized as a negative inhibitor during the enzymatic hydrolysis of cellulose, and it increases the cost of lignocellulosic biomass conversion [25,39].

In recent years, in addition to developing pretreatment strategies to overcome lignin inhibition by removing or modifying lignin, researchers have used modified lignin to promote the enzymatic hydrolysis of lignocellulose. Uraki and Zhu et al. discovered that adding appropriate lignin derivatives can enhance the enzymatic hydrolysis of lignocellulose, which opened the prelude to the use of modified lignin as enzymatic hydrolysis additives [40–42]. Nonaka et al. [43,44]. used the adsorption force between lignin and cellulase to immobilize cellulase on lignin for cellulose hydrolysis and verified the feasibility of recovering cellulase by a lignin-based carrier. As a following up, more lignin-based additives and carriers have been developed to enhance enzymatic hydrolysis and recycle cellulases, thereby improving the hydrolysis efficiency and reducing the amount of cellulase. However, due to the short development time of this area, the related work has not been sorted and summarized, so many researchers have not realized that lignin is not just a “troublemaker”.

Therefore, this review is to provide insights into the different roles of lignin in enzymatic hydrolysis from four perspectives: 1) lignin structure and its inhibition to enzymatic hydrolysis; 2) alleviating lignin

inhibition to cellulose hydrolysis by chemical pretreatment; 3) lignin-based additives to enhance enzymatic hydrolysis; 4) lignin-based carriers for cellulase recovery (Fig. 2). The future research directions of lignin in lignocellulosic enzymatic hydrolysis are also addressed.

2. Lignin structure and its inhibition to enzymatic hydrolysis

2.1. Lignin structure

Lignin in the cell walls of plants plays an important role in increasing plant toughness and resistance to microbial deconstruction of cell walls. It is generally believed that lignin is an amorphous polymer of three phenylpropane structural units, guaiacyl (G), syringyl (S), *p*-hydroxyphenyl (H), through deoxy-radical polymerization [31,34]. The ratio of the three units in lignin varies with plant species [45]. Grass lignin contains all three units, softwood lignin mainly contains G unit, and hardwood lignin mainly contains S and G units (Table 1) [46–48]. Lignin units are connected by C–O and C–C bonds, including β -O-4, α -O-4, β - β , β -5, 5-5, and β -1 bonds (Fig. 3) [49,50]. In addition to the benzene ring, lignin molecules also contain other functional groups, such as methoxy, phenolic hydroxyl, alcoholic hydroxyl, carbonyl, conjugated double bond, etc., which provide great possibilities for its subsequent modification and application [51,52].

Native lignin in plants generally has a large molecular weight (currently impossible to measure accurately), light color, and poor solubility in water. Lignin forms lignin-carbohydrate complexes (LCCs) with hemicelluloses through chemical bonds. The existence of LCCs makes the complete removal of hemicelluloses and/or lignin difficult [54,55]. The molecular weight of lignin is decreased through extraction processes using acid, base, or organic reagents due to depolymerization after breaking chemical bonds [56]. The color of extracted lignin is often brownish due to the formation of chromophores [57]. Lignin isolated from kraft, alkali, sulfite, organosolv pulping, and enzymatic hydrolysis is often called technical or industrial lignin. The lignin isolated using

Table 1

The abundance of the primary lignin precursors in different types of plants [53].

Monolignol	Grass (%)	Softwood (%)	Hardwood (%)
Sinapyl alcohol (S)	20–50	0–1	50–75
Coniferyl alcohol (G)	20–50	90–95	20–50
<i>p</i> -Hydroxyphenyl (H)	10–25	0.5–3.4	Trace

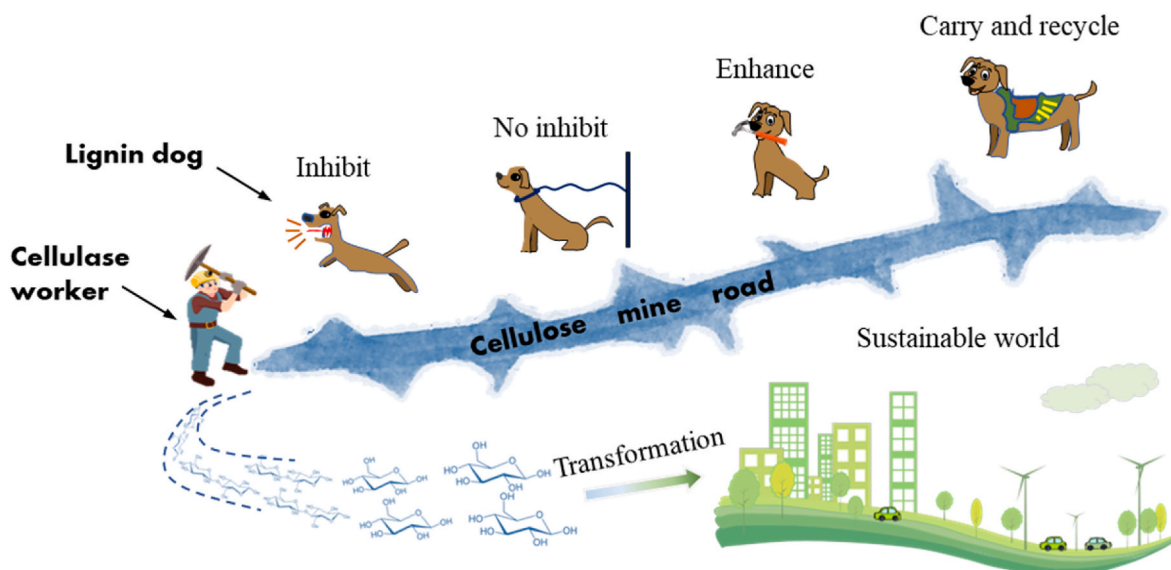


Fig. 2. Taming the “lignin dog” to facilitate enzymatic hydrolysis of lignocellulose for a sustainable and efficient sugar platform.

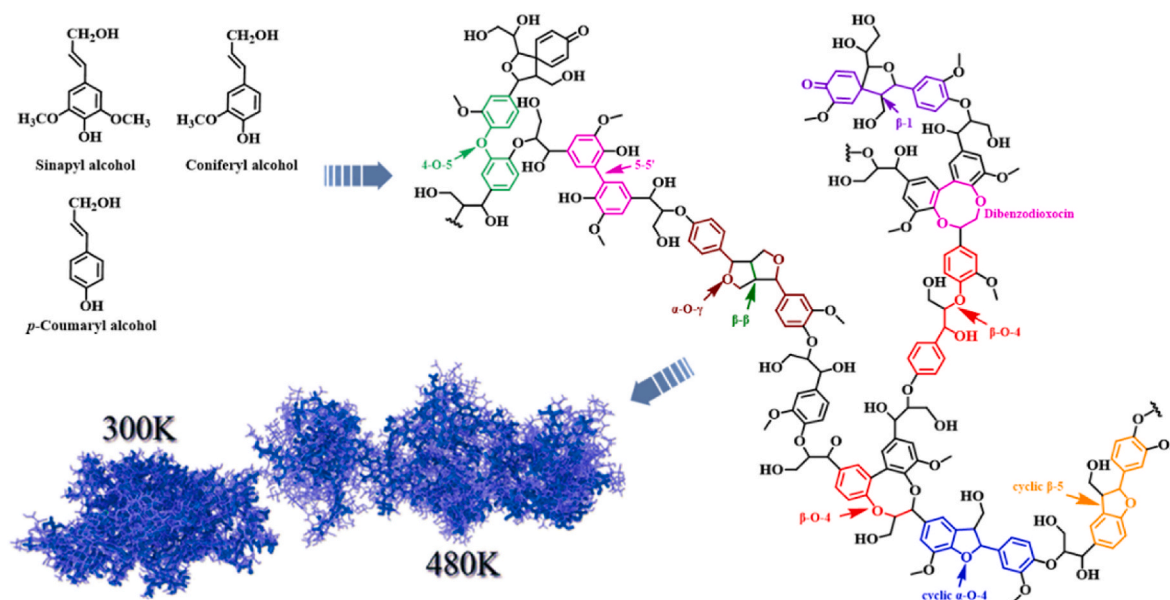


Fig. 3. Lignin units, molecular structure, and molecular simulation morphology [59,60].

different methods varies in terms of purity, molecular weight, structure, and functional groups [34]. For example, organosolv lignin often has a high purity (>90%), while enzymatic hydrolysis lignin (EHL) is often contaminated with carbohydrates and/or proteins (from enzymes or yeasts) and has a large molecular weight [58].

2.2. Steric hindrance of lignin to cellulase

Cellulases need to bind to the surface of cellulose to initiate hydrolysis [22]. In plant cell walls, cellulose is wrapped by hemicelluloses and/or lignin to form a physical barrier, making it difficult for cellulase to access cellulose [61,62]. Pretreatment can remove part of lignin and hemicelluloses and increase the accessibility of cellulose, thus improve the efficiency of enzymatic hydrolysis [63].

However, the residual lignin in substrates after pretreatment can still restrict the accessibility of cellulose depending on how much and where lignin is located [64]. The removed lignin can re-precipitate on the surface of substrates under the pretreatment conditions [65,66]. This is especially true in dilute acid (DA) and hydrothermal pretreatment processes [67,68]. Through scanning electron microscopy (SEM), Ko et al. [69], observed that some spherical aggregates were formed on the

surface of the cell wall during hydrothermal pretreatment of mixed hardwood, and the aggregates were mainly composed of lignin. When the removed lignin from pretreatment was added to the enzymatic hydrolysis system of Avicel, it was adsorbed on the surface of Avicel and inhibited the hydrolysis of Avicel [70].

2.3. Inhibition of lignin degradation products to cellulase activity

Pretreatment of lignocellulose usually leads to lignin degradation, resulting in a variety of phenolic compounds, including monophenols and oligophenols (Fig. 4) [71,72]. During enzymatic hydrolysis, these phenols have different effects on cellulase activity [73]. Many of them inhibit cellulase activity significantly, such as 4-hydroxybenzoic acid, 4-hydroxybenzaldehyde, syringaldehyde, syringic acid, conifer aldehyde, and vanillin [74].

Ximenes et al. [75,76], proved that four phenols, vanillin, syringaldehyde, *trans*-cinnamic acid, and 4-hydroxybenzaldehyde inhibited the activity of different types of cellulases. When vanillin and cellulase (*Spezyme CP*) were added at 4 mg and 1 mg, respectively, the hydrolysis yield of cellulose was reduced by 50%. However, the same concentration of vanillin did not significantly inhibit the activity of β -GL from

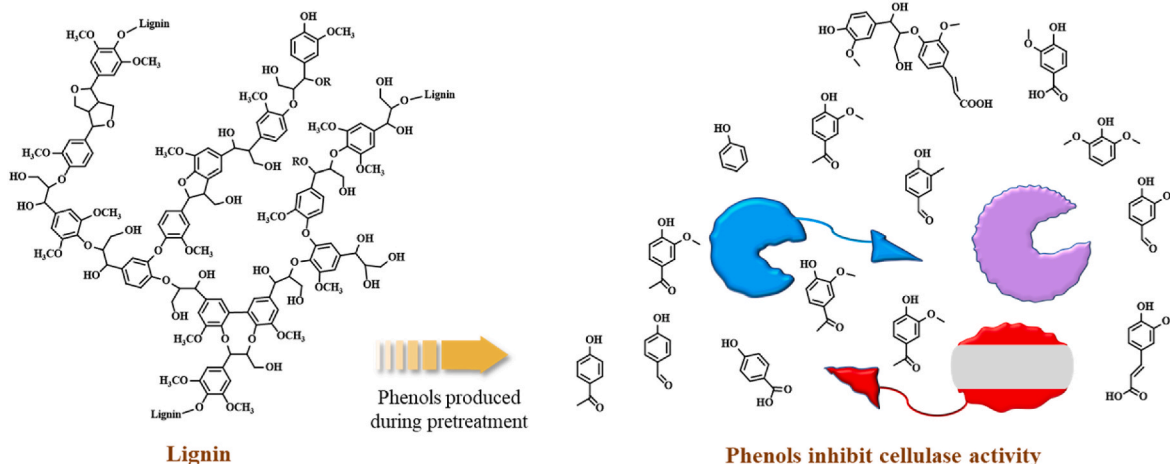


Fig. 4. Lignin is degraded into phenolic compounds during pretreatment and inhibits cellulase activity.

Trichoderma reesei or *Aspergillus niger* [75]. The study of Xu et al. [77] showed that at the same concentration, oligophenols caused more cellulase inactivation than monophenols. They found that the cellulase inactivation by oligophenols was observed at concentrations much lower than those of monophenols. Water-soluble phenolic compounds from different pretreatments affect enzymatic hydrolysis of substrate cellulose very differently [78]. For example, water-soluble phenolic compounds from ammonia fiber expansion pretreatment strongly inhibited enzymatic hydrolysis, while water-soluble phenolic compounds produced by alkaline pretreatment had little inhibition.

2.4. Non-productive adsorption of lignin in substrates to cellulase

Cellulases adsorbed to lignin cannot effectively hydrolyze cellulose due to restricted movement (except for some β -GLs that only act on soluble cellobiose), which is often called non-productive adsorption or binding [79]. The interactions between lignin and cellulase include hydrophobic adsorption, electrostatic attraction/repulsion, and hydrogen bonding [23,80,81].

Hydrophobic adsorption is generally considered to be the predominant interaction between cellulases and lignin [23,82]. The cellulose binding domain (CBD) of cellulase is relatively hydrophobic and is generally considered to be the main part of cellulase adsorbed on lignin [82,83]. When one of the three amino acid residues of CBD of TrCel7A was mutated to a more hydrophobic tryptophan, the binding force between the CBD and lignin was increased [83]. Clarke et al. [84] used modified atomic force microscope (AFM) probes with hydrophobic groups, $-\text{COOH}$, and $-\text{OH}$, and tested their interactions with cellulase from *Trichoderma reesei*. They found that the hydrophobically modified probe had a strong attractive force with the cellulase, which was 13% and 43% higher than that of $-\text{COOH}$ and $-\text{OH}$ modified probes, respectively.

Lou et al. [85] identified that electrostatic interaction can play an important role in the adsorption of cellulase on lignin. By increasing the hydrolysate pH from 4.8 to 5.2–6.2, above the isoelectric point (pI) of common cellulase, cellulase became negatively charged. In the meantime, the elevated pH also enhanced the negative charge of lignin due to the presence of the negatively charged functional groups on lignin surface [85]. These functional groups are either native to wood lignin or introduced by chemical treatments, such as sulfonic groups by the sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) [85,86] or carboxyl groups by maleic acid hydrolytic fractionation [87,88]. As a result, the electrostatic repulsion between cellulase and lignin was greatly enhanced to such a level that significantly reduced non-productive adsorption of cellulase on lignin, thus increasing the enzymatic hydrolysis sugar yield of lignocellulose [85,89]. Similarly, it was found that substituting positively charged lysine for the polar amino acids in Cel7A CBM increased its adsorption on lignin, while substituting negatively charged glutamate reduced its adsorption on lignin [90]. Recently, Timothy et al. [91] used computational design to negatively supercharge the surfaces of *Ruminoclostridium thermocellum* family 5 CelE and a CelE-family 3a carbohydrate-binding module fusion. The resulting designs showed a complete lack of inhibition by lignin because of electrostatic repulsion between the designs and lignin.

Some studies have also shown that hydrogen bonds affect cellulase adsorption on lignin. Pan [92] studied the inhibitory effects of different lignin model compounds on the enzymatic hydrolysis of cellulose and found that the model compounds containing phenolic hydroxyl groups had a more significant inhibitory effect on enzymatic hydrolysis. In this case, the phenolic hydroxyl group was likely to interact with cellulase through hydrogen bonding. For real lignin, the total hydroxyl content has an obvious effect on the adsorption of cellulase, and blocking phenolic hydroxyl groups via hydroxypropylation could reduce the adsorption of cellulase on lignin and thereby improve the final hydrolysis yield [93,94]. Due to the influence of other interactions, it is difficult to isolate the influence of hydrogen bonding.

2.5. Correlation between lignin structure and inhibition to enzymatic hydrolysis of cellulose

Lignin inhibitory effect on cellulases is greatly dependent on its structure. For example, lignin from different plants and different preparation methods showed varying inhibition to enzymatic hydrolysis of cellulose [95,96]. Selectively removing the highly inhibitory fraction of lignin without extensive delignification can dramatically enhance the enzymatic hydrolysis of lignocellulosic substrates [97]. For example, when the steam-exploded Douglas fir substrate was washed with 1% NaOH at room temperature, the lignin content was reduced only by 7.7 points from 43.2% to 35.5%, but the enzymatic hydrolysis yield was increased significantly from 30.4% to 54.2%. This observation suggested that the cold-NaOH extracted lignin be more inhibitory than the residual lignin in the substrate.

Due to the complexity and heterogeneity of lignin structure, it is difficult to attribute lignin inhibitory effect on enzymatic hydrolysis to a specific lignin structural feature or property, because altering functional groups or chemical bonds often leads to altering other properties. Nevertheless, lignin inhibition is correlated to its physicochemical properties. Pan's research group studied the effects of lignin functional groups on the enzymatic hydrolysis of Avicel using a series of lignin model compounds with well-defined structures and found that the phenolic hydroxyl group inhibited cellulose hydrolysis, while the methoxy group did not show obvious inhibitory effect [98]. By studying the effect of two lignin samples from hardwood and softwood with varied physicochemical properties (molecular weight, aliphatic hydroxyl, phenolic hydroxyl, and hydrophobicity) on the enzymatic hydrolysis of Avicel, they found that both the hydrophobicity and the phenolic hydroxyl group are closely related to the lignin inhibition [95]. Similar results were also reported by Park and Yong et al. [93,99]. In general, the formation of C–C bonds (lignin condensation) and the cleavage of $\alpha\text{--O--4}/\beta\text{--O--4}$ bonds lead to high hydrophobicity and more phenolic hydroxyl, respectively, which usually occur under severe pretreatment conditions [32,100].

Other studies explored the effects of alcohol hydroxyl, syringyl/guaiacyl (S/G) ratio, $\beta\text{--}\beta$, $\beta\text{--}5$, etc. on the enzymatic hydrolysis of cellulose, but further investigations are needed to reach solid conclusions [101–104]. It should be noted that the effect of lignin structure on enzymatic hydrolysis is greatly affected by the content and distribution of lignin in substrates [24,26].

3. Alleviating lignin inhibition to cellulose hydrolysis by chemical pretreatment

Chemical pretreatment of lignocellulose is the most efficient means to remove lignin recalcitrance [35,105]. Common chemical pretreatments include dilute acid, alkali, hydrothermal, and organic solvent processes [27]. The severe conditions (strong acid or base, high temperature, and high pressure) required by most pretreatment processes could lead to lignin condensation [32,106–110]. As mentioned earlier, condensed lignin not only deposits on cellulose surface forming physical obstacles but also adsorbs cellulase due to increased hydrophobicity, resulting in cellulase inactivation [32,111]. Furthermore, chemical pretreatment, especially dilute acid pretreatment often enriches lignin content in the pretreated substrates due to the removal of hemicelluloses. In general, the following three strategies can be adopted in pretreatment to reduce lignin inhibition: 1) increase the hydrophilic groups of lignin to promote the removal (dissolution) of lignin and weaken the hydrophobic interaction between cellulase and residual lignin in substrates; 2) prevent the formation and condensation of benzyl carbocations by adding protective agents; 3) decrease pretreatment severity to reduce the rate of the lignin condensation reaction (Fig. 5).

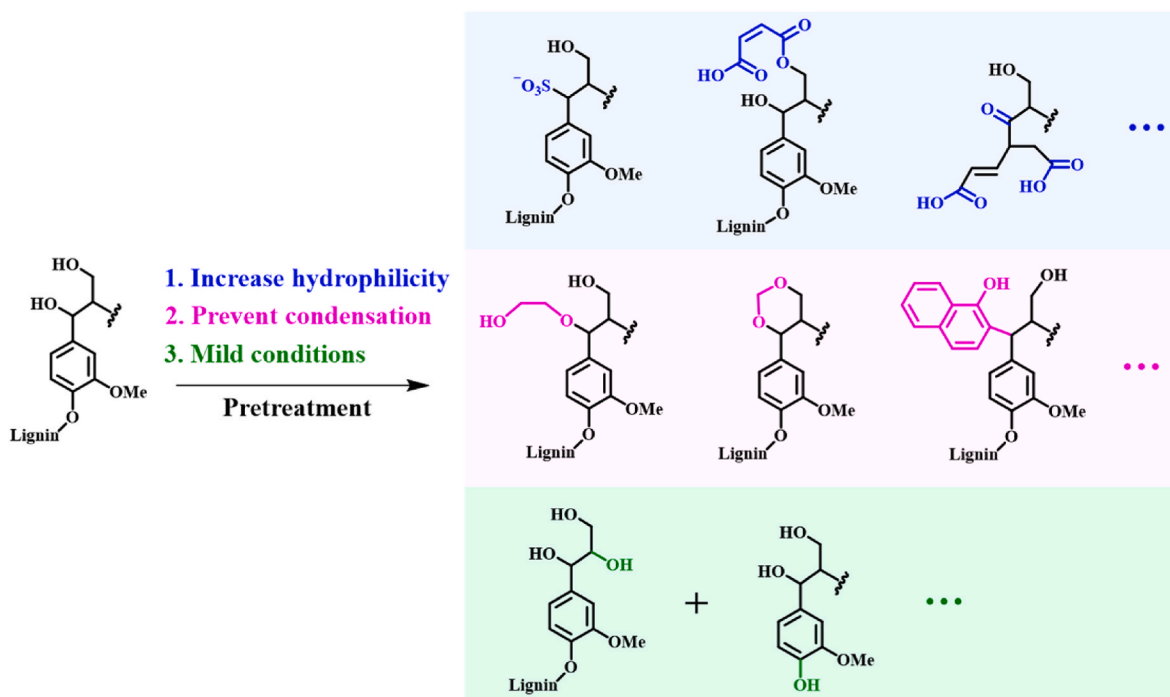


Fig. 5. Three pretreatment strategies to alleviate the inhibitory effect of lignin on cellulose hydrolysis.

3.1. Increasing the hydrophilicity of lignin by pretreatment

3.1.1. SPORL pretreatment

SPORL pretreatment uses sulfite to treat lignocellulose under weakly acidic conditions. The treatment temperature can be varied from 120 to

190 °C [86,112], and the solid-liquid ratio can be as low as 2–3 [86,113].

Zhu and Pan et al. [114–117], systematically explored the SPORL pretreatment of different lignocellulosic feedstocks. During the pretreatment, the α -C of the side chain of the lignin-phenylpropane

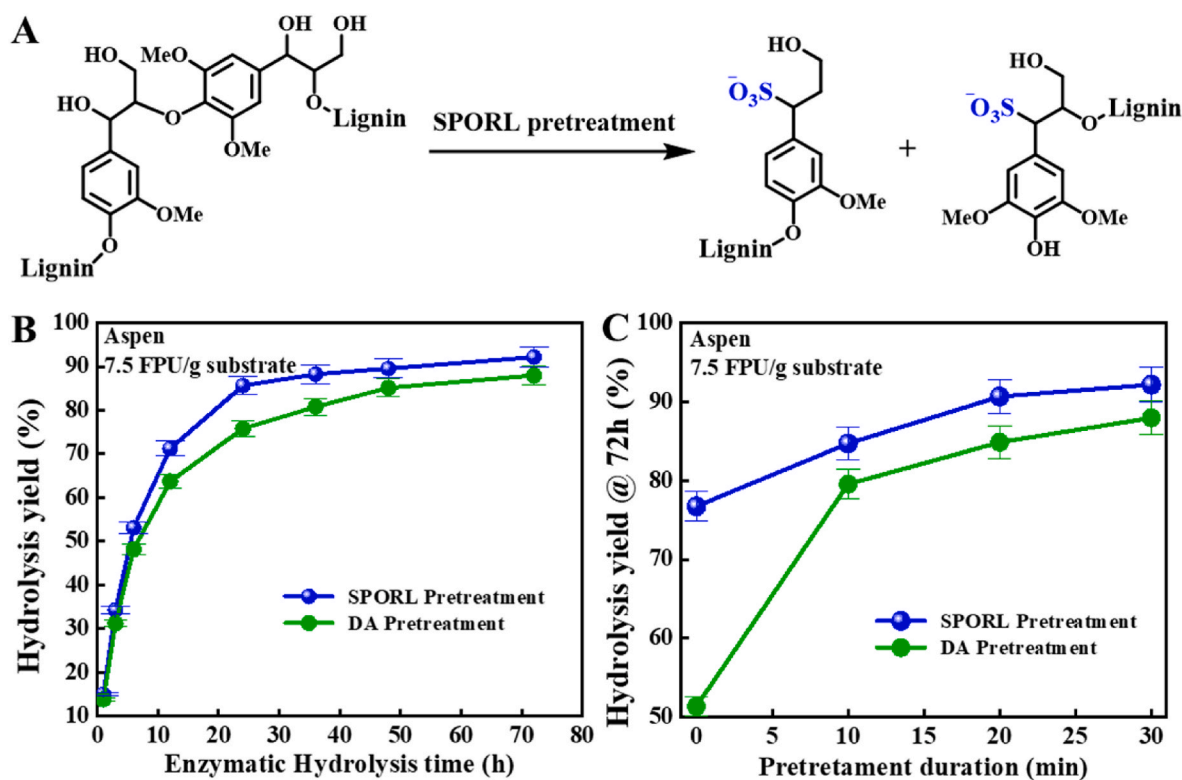


Fig. 6. Schematic diagram of lignin structure changes during SPORL pretreatment (A); changes of hydrolysis yields of DA-pretreated and SPORL-pretreated aspen substrates with different hydrolysis times (B) and different pretreatment durations (C) [125,126]. (DA pretreatment: 1.1 wt% H_2SO_4 ; SPORL pretreatment: 1.1 wt% H_2SO_4 and 3 wt% NaHSO_3 ; chemicals all based on the dry weight of biomass).

structural unit is sulfonated (Fig. 6), and part of lignin with a relatively high degree of sulfonation is dissolved in the pretreatment liquid. Most hemicelluloses are hydrolyzed into monosaccharides, while cellulose is retained as an insoluble solid [118]. The residual lignin in the substrate is also partially sulfonated, resulting in relatively hydrophilic lignin [118,119]. In addition, at a relatively high enzymatic hydrolysis pH (5.5–6.0), lignin sulfonation enhanced its surface charge [85]. Meanwhile, because the pH range is slightly higher than the pI of commonly used cellulases, the negative charge of the cellulases is enhanced. As a result, the electric static repulsive interaction between substrate lignin and cellulases reduces the non-productive adsorption of the cellulases on the lignin [85]. For example, the hydrolysis yield of SPORL pretreated Douglas-fir (softwood) forest residue reached 90% within 48 h at a substrate loading of 21 wt% with an enzyme loading of only 15 FPU/g glucan [120]. SPORL pretreatment also resulted in a low formation of fermentation inhibitors because of its moderate pretreatment temperature and acidity [121]. The separation of pretreatment liquor and the pretreated substrate is not necessary, and the entire slurry can be saccharified and fermented together [112]. The SPORL process has been successfully scaled up to pretreating 60 tons of forest residue and produced 1000 gallons of certified isoparaffinic kerosene for a commercial flight [122].

Lignocellulose pretreated with SO₂ steam explosion and SO₂-ethanol-water system can also achieve a relatively high enzymatic hydrolysis yield [123,124]. Their mechanism is similar to that of SPORL pretreatment.

3.1.2. Alkaline oxygen pretreatment

Alkaline oxygen pretreatment removes lignin by oxidation, which increases the carboxyl and carbonyl content in lignin while opening the benzene ring structure, thereby increasing the hydrophilicity of lignin (Fig. 7) [127,128].

Saddler et al. [129] synthesized dehydrogenated polymers (DHPs) from monolignols (coniferyl alcohol and ferulic acid) and studied their effect on the enzymatic hydrolysis of Avicel. The DHP from ferulic acid

was rich in carboxyl groups, which had little adsorption to cellulases and therefore did not negatively affect the enzymatic hydrolysis of Avicel. However, the DHP from coniferyl alcohol absorbed more cellulases and inhibited the hydrolysis of Avicel. Saddler et al. [128] then studied the effect of alkaline oxygen on the steam explosion pretreatment of eucalyptus. The wood chips were impregnated using Na₂CO₃ and O₂ at 110 and 135 °C before the steam explosion (210 °C, 5 min). It was found that the alkaline oxygen steam pretreatment (AO-SP) delignified the wood by 58.3%, significantly higher than the steam explosion after using.

Oxygen (Oxy-SP) or sodium carbonate (Alk-SP) alone (24.4% and 42.0%, respectively). The content of carboxyl groups in AO-SP lignin were increased to 151 mmol/kg from 33 mmol/kg in the lignin treated only by the steam explosion (Auto-SP). The significantly improved hydrophilicity of AO-SP lignin weakened its hydrophobic interaction with cellulases during enzymatic hydrolysis, and thus the hydrolysis yield of the AO-SP substrate reached 86% within 48 h at a substrate concentration of 10 wt% and an enzyme loading of 10 FPU/g glucan [128].

Recent studies also demonstrated that oxidative pretreatment under alkaline conditions greatly improved the enzymatic hydrolysis yield of substrates, including the pretreatment using hydrogen peroxide as an oxidant under atmospheric pressure [130,131].

3.1.3. Polycarboxylic acid hydrotropic pretreatment

Acid hydrotropes are organic acids that possess the properties of both an acid and a hydrotrope. They not only can catalyze hydrolysis reactions but also dissolve the hydrophobic compounds in water [132]. Zhu et al. [133] pioneered the application of *p*-toluenesulfonic acid (*p*-TsOH) in treating poplar wood and found that more than 90% delignification and more than 85% xylan dissolution was achieved at 80 wt% *p*-TsOH concentration and a temperature of 80 °C. In following-up studies, a series of acid hydrotropic pretreatments were developed based on aromatic sulfonic acids [134–137].

Polycarboxylic acids could also be used as acid hydrotropes to treat lignocellulose [87,132]. When birch chips were treated in 60 wt% maleic acid (MA) aqueous solution at 120 °C under atmospheric pressure,

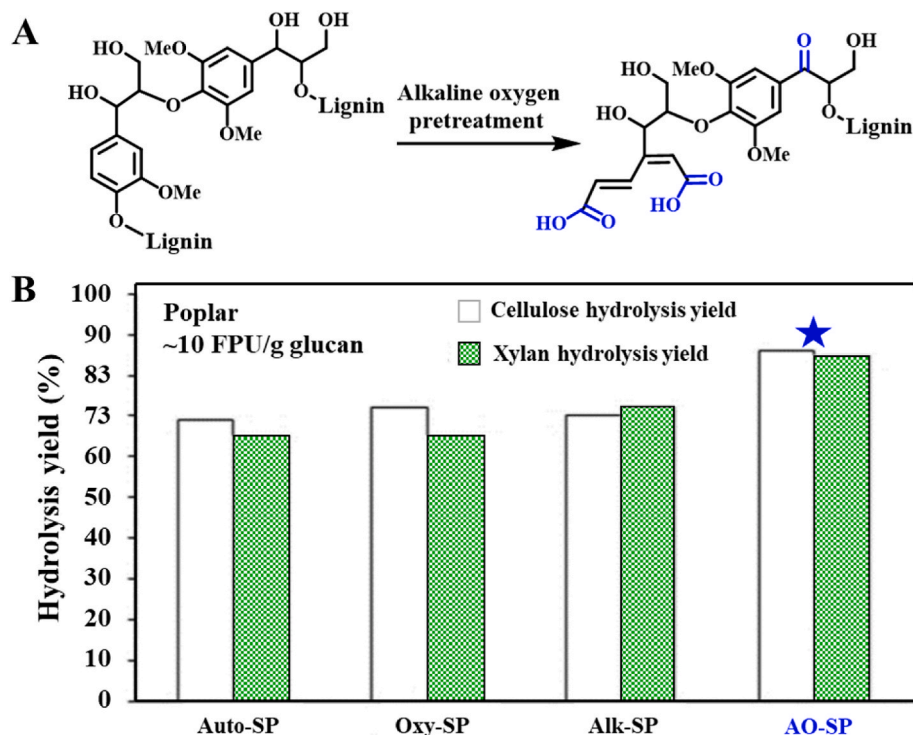


Fig. 7. Schematic diagram of structural changes of lignin during alkaline oxygen pretreatment (A); enzymatic hydrolysis yields of carbohydrates in poplar after different pretreatments (B) [127,128]. (Auto-SP: steam pretreatment without chemical reagents; Oxy-SP: steam pretreatment under oxygen pressure; Alk-SP: steam pretreatment with Na₂CO₃).

60% of lignin was dissolved and 70% of hemicelluloses were hydrolyzed [88]. The enzymatic hydrolysis sugar yield of pretreated solid substrate exceeded 98% at a low enzyme loading of 10 FPU/g glucan. More than 90% MA in the pretreatment liquid could be recovered through concentration and crystallization [88]. It was confirmed by 2D NMR that the γ -OH of the lignin dissolved in the pretreatment liquid was esterified by MA. So, the content of the carboxyl group of the lignin was significantly increased, and the hydrophilicity of the lignin was improved (Fig. 8) [87]. Due to the increase of carboxyl content, the electrostatic repulsion between the lignin in substrates and cellulases during enzymatic hydrolysis was increased at an elevated pH of 5.0–6.5, which substantially increased the hydrolysis yield of the substrate [87,138,139].

Similarly, the substrates treated with citric acid and oxalic acid also showed relatively high hydrolysis yields [140,141]. As polycarboxylic acids are weak organic acids, fewer fermentation inhibitors are produced during the pretreatment, and the pretreatment liquid is suitable for direct fermentation to produce ethanol and succinic acid, etc [142]. The advantages and disadvantages of the above three types of pretreatments are compared in Table 2.

3.2. Preventing the condensation of lignin during pretreatment

During most hydrothermal and acidic pretreatments, the α -OH of lignin is removed to form a benzyl carbocation, which is highly active and can easily couple with a negatively charged carbon on lignin benzene ring and side chain or carbohydrate degradation products to form new C–C bonds, resulting in lignin condensation, thereby affecting the enzymatic hydrolysis of the pretreated substrates [39,107,143]. The condensation also makes the downstream lignin utilization more difficult. Therefore, how to prevent lignin from condensation, such as by adding protective agents to reduce the formation and condensation of benzyl carbocations, has drawn more and more attention [106,144].

Table 2

Advantages and disadvantages of three types of pretreatments by increasing the hydrophilicity of lignin.

Pretreatments	Advantages	Disadvantages
SPORL pretreatment	Low cost, less fermentation inhibitors, by-product sodium lignosulfonate	The use of sulfur compounds can easily cause air pollution
Alkaline oxygen pretreatment	Low cost, high sugar yield, environment-friendly	Partial lignin and hemicellulose degradation, overall economics affected
Polycarboxylic acid hydrotropic pretreatment	High-quality lignin can be obtained, high sugar yield, simultaneous conversion of hemicellulose into furfural	High reagent concentration and high recovery cost

3.2.1. Acidic dioxane pretreatment with aldehydes as protecting agents

Luterbacher et al. [145]. developed a strategy for lignin extraction with acidic dioxane under the protection of formaldehyde, in which the α and γ hydroxyl groups of lignin reacted with formaldehyde to form 1, 3-dioxane structures (Fig. 9). Since lignin was hardly condensed, an enzymatic glucose yield of 90% was achieved after removing formaldehyde (substrates without removing formaldehyde were not suitable for enzymatic hydrolysis) [145]. At the same time, the DA-pretreated substrate under the protection of formaldehyde also reached a hydrolysis yield of about 80%, indicating that the inhibition of enzymatic hydrolysis by lignin was alleviated. Subsequently, they replaced formaldehyde with other aldehydes such as acetaldehyde and propionaldehyde and found that the structure of lignin was also protected, and the enzymatic hydrolysis yields of pretreated substrates were still high [146].

It is worth mentioning that recently, Luterbacher et al. [147]. developed a new process to prepare sustainable polyesters via the direct functionalization of lignocellulosic sugars with glyoxylic acid. In the

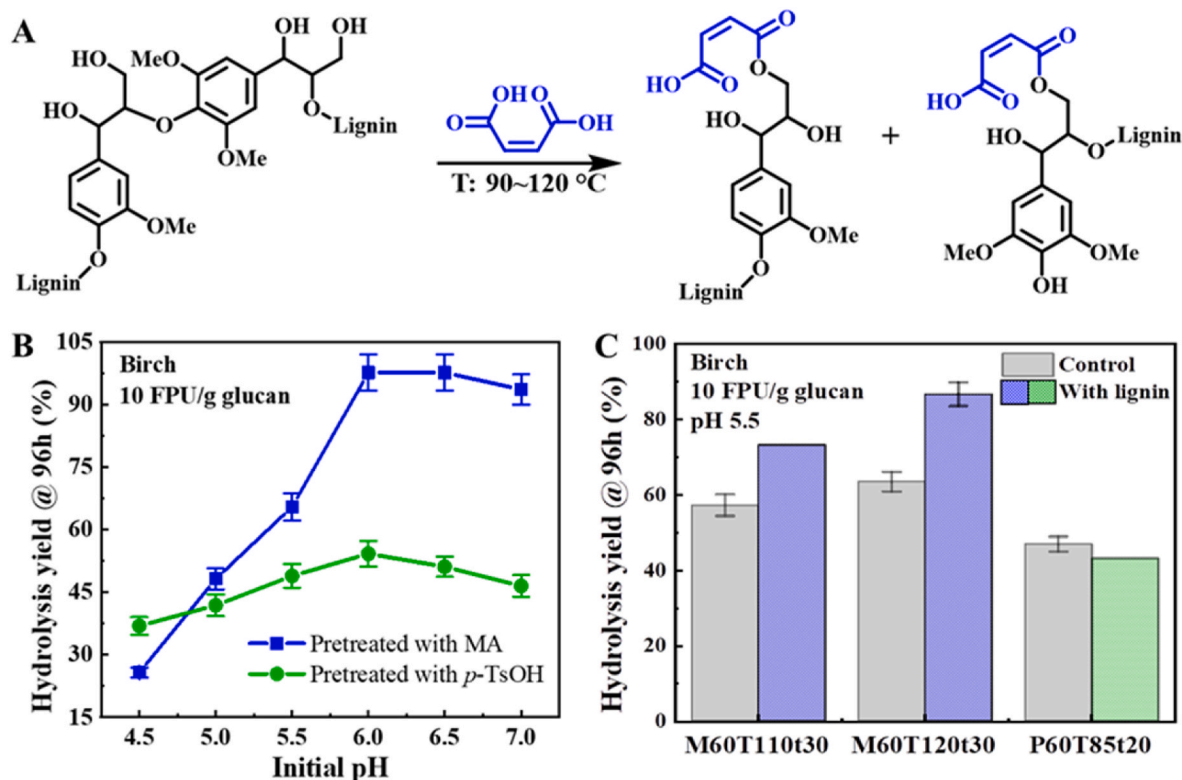


Fig. 8. Schematic diagram of structural changes of lignin during MA pretreatment (A); hydrolysis yields of pretreated birch by MA and *p*-TsOH (with similar lignin and hemicellulose dissolution but without lignin carboxylation) at different pH (B); the influence of adding the corresponding dissolved lignin from pretreatment on the enzymatic hydrolysis of pretreated solids (C) [88].

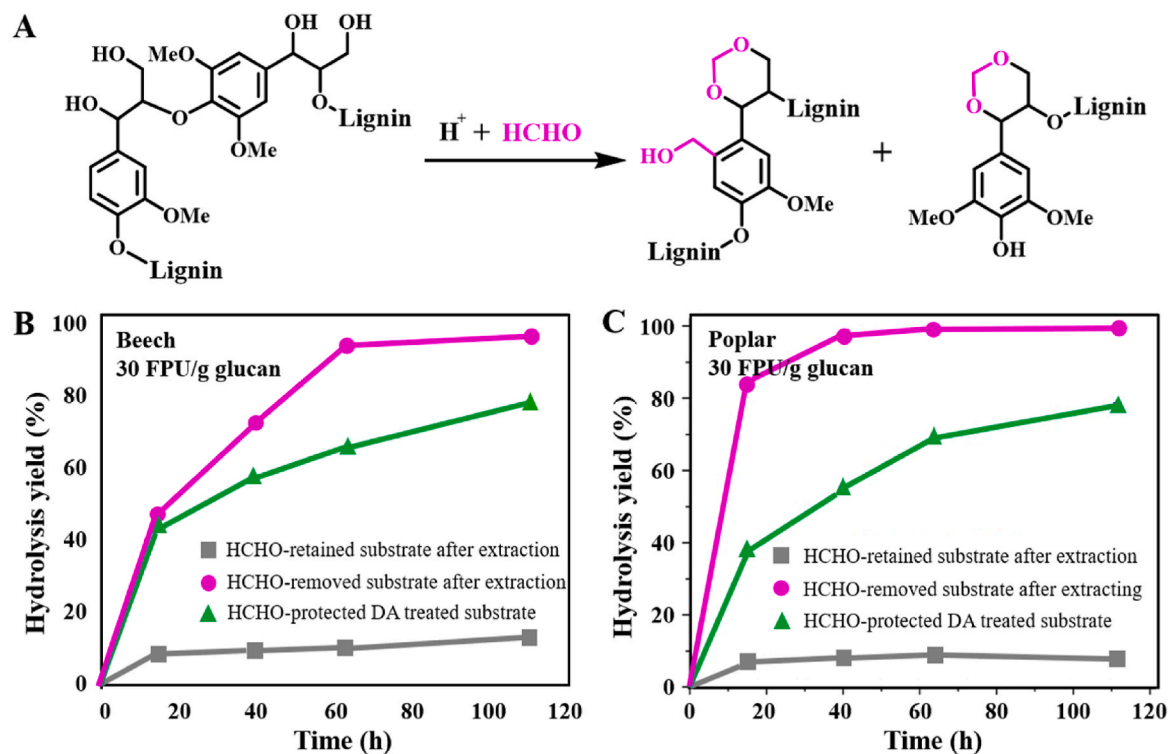


Fig. 9. Schematic diagram of the reaction of lignin and formaldehyde under acidic conditions (A); changes of hydrolysis yields of different substrates (B: beech; C: poplar) after extracting lignin by acidic dioxane or DA pretreatment (both protected by formaldehyde) with time [145].

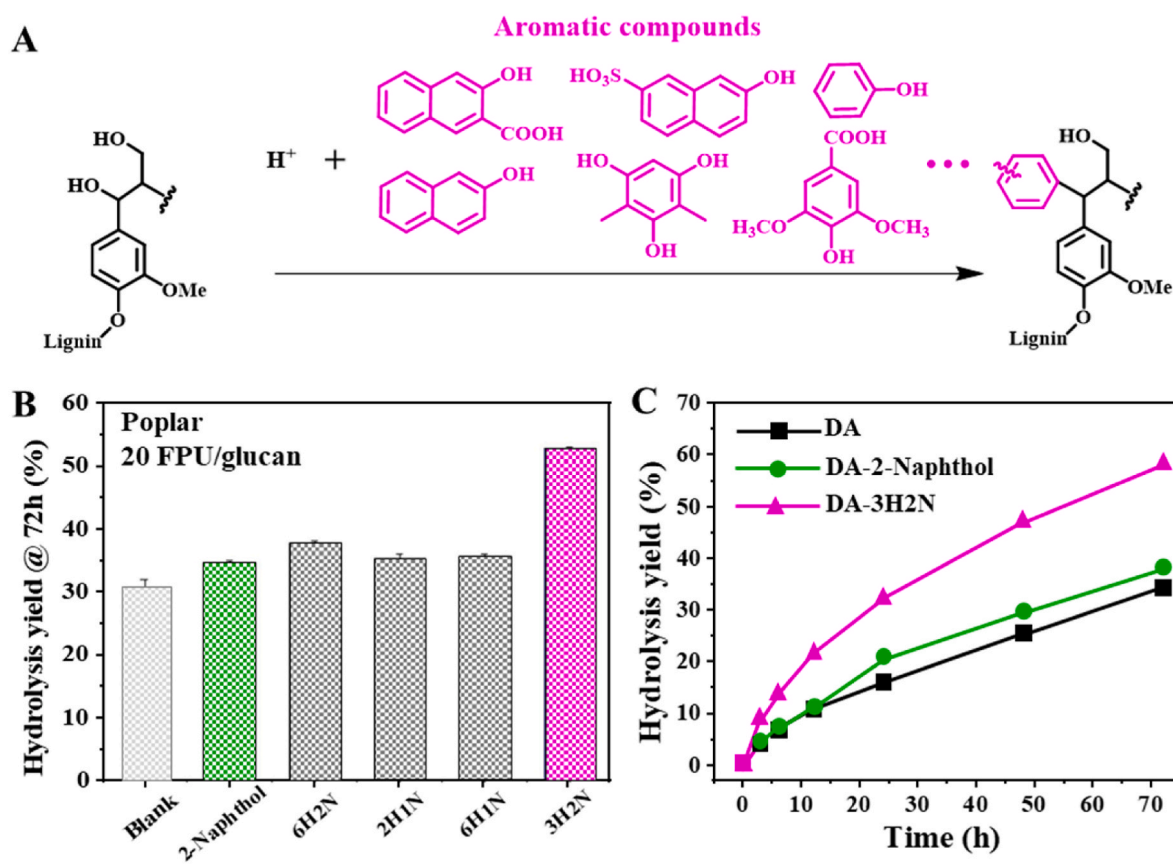


Fig. 10. Aromatic compounds act as protective agents to prevent the condensation of lignin (A); hydrolysis yields at 72 h of pretreated substrates with the addition of 2-naphthol and carboxylic acid derivatives of naphthol (B); changes in hydrolysis yields of pretreated substrates with time (C) [151]. (6H2N: 6-hydroxy-2-naphthoic acid; 2H1N: 2-hydroxy-1-naphthoic acid; 6H1N: 6-hydroxy-1-naphthoic acid; 3H2N: 3-hydroxy-2-naphthoic acid, DA: diluted acid).

process, glyoxylic acid reacted with hemicellulose sugars to form tri-cyclic diester as plastic precursors. Meanwhile, lignin in the substrate also reacted with glyoxylic acid to form 1,3-dioxane structures and introduce carboxyl groups into lignin [148], which not only prevented lignin from condensation but also improved the hydrophilicity, resulting in a nearly 100% enzymatic hydrolysis yield of the substrate [147].

3.2.2. Pretreatment with aromatic compounds as protective agents

In the early studies, 2-naphthol was added to the autohydrolysis process of aspen wood to prevent lignin from repolymerization (Fig. 10), which effectively increased the removal rate of lignin and improved the reactivity of extracted lignin [149]. Thomas et al. [150], systematically studied the effect of different aromatic compounds on the repolymerization of lignin during the autohydrolysis of pine and found that dimethyl phloroglucinol and 2-naphthol were effective to prevent lignin from the repolymerization. However, adding nucleophiles with multiple reactive sites, such as phloroglucinol, caused the cross-linking of lignin and severely inhibited the subsequent enzymatic hydrolysis efficiency.

Recently, syringic acid and naphthol derivatives were used as nucleophiles to block the benzyl carbocation of lignin during pretreatment to prevent lignin from condensation [151,152]. The addition of the nucleophiles effectively weakened the inhibitory effect of the lignin on cellulase and greatly increased the hydrolysis yield of substrates. Adding 4 wt% (based on biomass) 3-hydroxy-2-naphthoic acid (3H2N) during DA pretreatment of poplar increased the hydrolysis yield of the substrate by 72% [151].

3.2.3. Acidic polyol pretreatment

Ragauskas et al. [153], attempted to treat eucalyptus with acidic diols. During the pretreatment, the diols acted as nucleophiles to attack the benzyl carbocation of lignin, turning it into a more stable benzyl ether structure and preventing lignin from further condensation

(Fig. 11). Compared with monoalcohol-pretreated lignin, diol-pretreated lignin contained more aliphatic hydroxyl groups and fewer phenolic hydroxyl groups. The diol-pretreated lignin also had a relatively lower cellulase adsorption affinity, which was beneficial to enzymatic hydrolysis. The hydrolysis yield of the eucalyptus treated by acidic diols reached 90% in 48 h at an enzyme loading of 7.5 FPU/g glucan. Increasing the severity of the diol pretreatment did not affect the hydrolysis yields of substrates, which showed a good linear correlation with the content of residual lignin. However, the hydrolysis yield of the monoalcohol-treated substrates decreased with the pretreatment severity at high severities. These observations were attributed to the limited condensation and lower adsorption affinity to cellulases of diol-pretreated lignin than that of monoalcohol-treated lignin [153, 154].

Recently, mannitol, as a polyol, was also used as a nucleophile to react with the benzyl carbocation of lignin during pretreatment. As a result, the inhibitory effect of lignin on cellulose hydrolysis was alleviated, and the glucose yield of the substrate was increased to 53% from 42% [152], suggesting the polyol as an additive has the potential to prevent lignin from condensation. The advantages and disadvantages of the above three types of pretreatments are compared in Table 3.

3.3. Mild pretreatment

Besides improving the hydrophilicity of lignin and preventing it from condensation, mild pretreatments can partially remove lignin from lignocellulose [155,156]. They are usually carried out at a low temperature and low pressure and avoid the use of strong acids and bases [157–159].

Since the degree of structural changes to lignin in substrates and pretreatment liquids is generally positively correlated during most mild pretreatments [160,161], the dissolved lignin is often characterized to

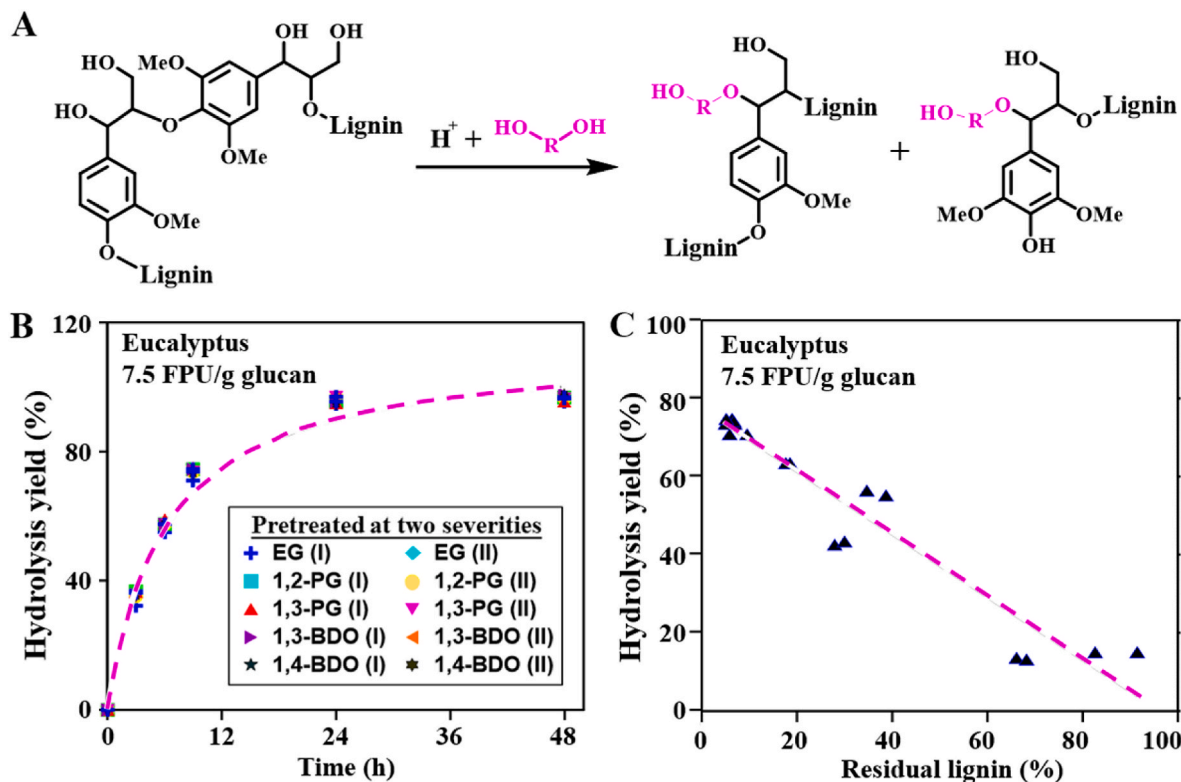


Fig. 11. Schematic diagram of structural changes of lignin under acidic diol pretreatment (A); changes of hydrolysis yields of substrates (eucalyptus treated with diols at two different intensities: 20 mM H₂SO₄, 65% diol concentration; 25 mM H₂SO₄, 85% diol concentration) with time (B); the relationship between the hydrolysis yield and the lignin content in substrates treated by diols under different conditions (C) [153]. (EG: Ethylene glycol; 1,2-PG: 1, 2-propylene glycol; 1,3: 1, 3-propylene glycol; 1,3-BDO: 1,3-butanediol; 1,4-BDO: 1,4-butanediol).

Table 3

Advantages and disadvantages of three types of pretreatments by preventing the condensation of lignin.

Pretreatments	Advantages	Disadvantages
Acidic dioxane pretreatment with aldehydes as protecting agents	High lignin extraction rate, the structure of extracted lignin is well preserved, high sugar yield	Aldehydes need to be removed before enzymatic hydrolysis, requiring the use of a large amount of organic solvents
Pretreatment with aromatic compounds as protective agents	The amount of additives is small, and no need to use organic solvents	The price of additives is high, and the effect of different additives varies greatly
Acidic polyol pretreatment	The extracted lignin has high activity, high sugar yield	Polyols usually have high boiling points, are difficult to recycle, and have limited ability to prevent lignin condensation

reflect the structural changes of the lignin in substrates. Sousa et al. [162]. treated corn stalks with liquid ammonia during which the crystal form of natural cellulose was transformed from type I to type III (conducive to enzymatic hydrolysis), and resulted in 45% of lignin in the stalks being extracted. Due to the low pretreatment temperature (115 °C), the structure of extracted lignin was well maintained, and the hydrolysis yield of the substrate reached 90% at a relatively low enzyme load (7.5 g cellulase protein/g glucan). Luterbacher et al. [163]. used a mild acidic γ -valerolactone/water system (80:20) to treat beech at

120 °C for 45 min. The process dissolved 80% of lignin which retained high β -O-4 content, and the enzymatic hydrolysis yield of the pretreated substrate reached 99% in 48 h, almost complete hydrolysis.

Deep eutectic solvents (DES) and ionic liquids have also been widely used to treat lignocellulose recently [165,166]. Shen et al. [167]. treated eucalyptus sawdust using lignin-derived DES reagents (lactic acid and choline chloride) at 110 °C for 6 h. About 80% of lignin was dissolved with low molecular weight and low condensation degree, and the final glucose yield of the substrate reached 94.3% at 72 h. Dutta et al. [168]. treated switchgrass using ionic liquid cholinium lysinate [Ch][Lys] at 140 °C for 1 h, which dissolved 74% of lignin. The lignin dissolved in [Ch][Lys] and that remained in the substrate did not show obvious condensation, and the enzymatic hydrolysis yield of the substrate exceeded 90%. Sun et al. [164]. carefully studied the structural changes to lignin in ionic liquids, 1-ethyl-3-methylimidazolium acetate ([C2mim][OAc]), and found that under different conditions, lignin underwent the reactions of dehydration, demethylation, depolymerization, and condensation (Fig. 12). The cleavage of β -O-4 bonds and condensation reaction occurred at high temperatures.

4. Lignin-based additives to enhance enzymatic hydrolysis of lignocellulose

Many surfactants have been used to enhance the enzymatic hydrolysis of lignocellulose, of which non-ionic and zwitterionic surfactants perform the best [169,170]. The promotion mechanism of surfactants is

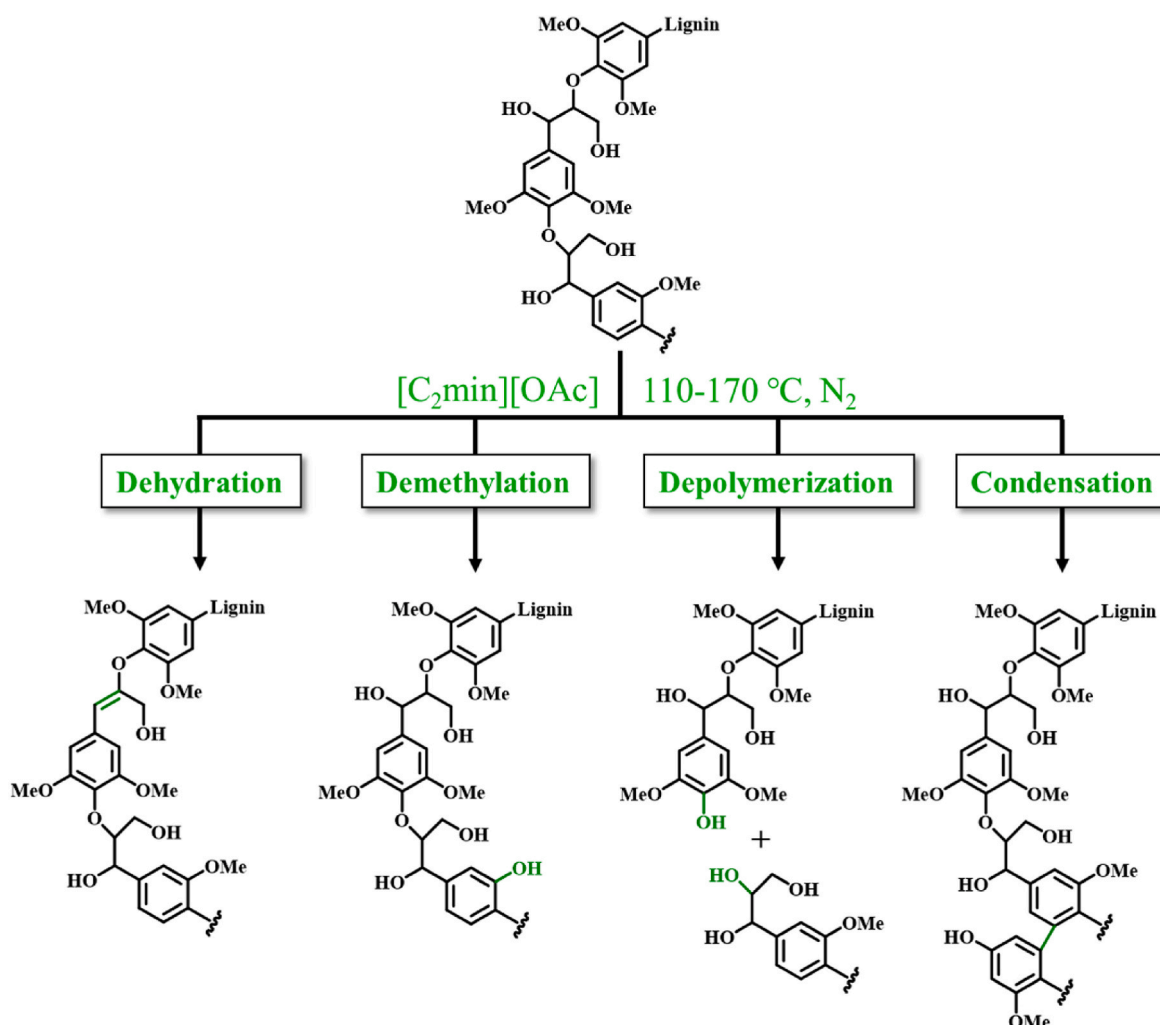


Fig. 12. The proposed structure changes of lignin during [C2mim][OAc] pretreatment [164].

mainly attributed to the surfactant-reduced non-productive adsorption of cellulases on lignin and maintained cellulase activity [171,172]. Since most effective surfactants are expensive and unrecyclable, researchers have tried to modify inexpensive and easily available lignin as additives to enhance enzymatic hydrolysis [40,41].

Isolated lignin without modification generally inhibits or does not significantly affect cellulase activity [173–175]. It was reported that grafting anionic groups, anionic & cationic groups, and hydrophilic segments on lignin can synthesize effective lignin-based additives to enhance the enzymatic hydrolysis of lignocellulose (Fig. 13) [40,176,177].

4.1. Lignin-based anionic additives

4.1.1. Carboxylated lignin

Most isolated lignin is insoluble or non-dispersible in water due to its low content of hydrophilic groups. Grafting carboxyl groups can improve the water solubility of lignin and even make lignin completely water-soluble.

Lou et al. [178]. synthesized lignin carboxylate by reacting enzymatic hydrolysis lignin with sodium 1-chloroacetate. The resultant lignin carboxylate had a carboxyl group content of 2.38 mmol/g and was almost completely soluble in enzymatic hydrolysate at pH 4.8. Adding 2 g/L of lignin carboxylate increased the hydrolysis yield of DA-pretreated eucalyptus and SPORL-pretreated Masson pine from 43% to 29%–48% and 51%, respectively. The lignin carboxylate enhanced the hydrolysis by reducing the adsorption of cellulases on the lignin in the substrates.

Carboxyl groups can also be introduced onto lignin by grafting polycarboxylic acid. Cai et al. [88]. directly modified native lignin by treating birch powder with maleic acid (MA) as an acid hydrotrope. The water dispersibility of MA-modified lignin was significantly improved, but it was not completely soluble in water. Adding 1 g/L of MA-modified lignin enhanced the enzymatic hydrolysis yield of MA-treated birch from 63.4% to 86.6%, because MA-modified lignin could maintain cellulase activity.

4.1.2. Sulfonated lignin

Zhu et al. [40,41]. discovered that sodium lignosulfonate (SL) obtained by grafting sulfonic acid groups onto lignin could effectively enhance the enzymatic hydrolysis of lignocellulose, and the promotion effect was closely related to the molecular weight and sulfonation degree of SL [41]. Further studies showed that SL could increase the negative charge of cellulases and reduce the non-productive adsorption of cellulases on lignin by combining with cellulases during the enzymatic hydrolysis of lignocellulose, thus increasing the hydrolysis yield [40]. Adding SPORL pretreatment liquid containing SL could increase the hydrolysis yields of poplar and lodgepole pine substrates by 25.9% and 31.8%, respectively [40]. Subsequently, Wang et al. [179]. also found the addition of SL increased the hydrolysis yield of Masson pine pretreated with green liquor from 42% to 75%.

In addition, SL can also enhance the enzymatic hydrolysis of pure cellulose [180]. It was proposed that SL with a relatively small weight-average molecular weight (Mw) and a relatively high degree of sulfonation could stabilize the structure of cellulases and facilitate the binding of cellulases to cellulose, thereby enhancing the enzymatic hydrolysis of cellulose. SL with a relatively large Mw and a relatively low degree of sulfonation limited the movement of cellulases and reduced the hydrolysis yield of cellulose (Fig. 14) [180].

Recent studies found that when compounded with other surfactants, SL could more effectively enhance the enzymatic hydrolysis of pure cellulose and lignocellulose because the surfactants could appropriately adjust the interaction between SL and cellulases, as well as the interaction between SL and substrates [181–183].

4.2. Lignin-based zwitterionic additives

Zwitterionic surfactants are formally neutral but contain both anion and cation groups. Unlike anionic and cationic surfactants, they do not significantly affect the steric structure of cellulase proteins [184–187]. Some zwitterionic surfactants can form effective hydrophilic films on the surface of substrates, preventing the adsorption of proteins [188,189]. Previous work has shown that several petroleum-derived zwitterionic surfactants can effectively enhance the enzymatic hydrolysis of lignocellulose [184,190], but only recently have lignin-based zwitterionic additives been explored.

Quaternary ammonium lignosulfonate (QALS) could be synthesized by grafting quaternary ammonium groups on SL (Fig. 15) [176]. Since the quaternary ammonium groups in QALS had an electrostatic attraction with negatively charged lignin in substrates, the adsorption of QALS on lignin was greater than that of SL. The lignin film covered by QALS became more hydrophilic than that covered by SL. So, adding QALS could form a more effective steric hindrance and hydration membrane on lignin in substrates to reduce the non-productive adsorption of cellulases on lignin. With increasing degree of quaternization, the promotion effect of QALS on the enzymatic hydrolysis of lignocellulose gradually increased. Adding 2 g/L of QALS-80 (mass ratio of quaternization reagent to SL was 80%) could increase the enzymatic hydrolysis yields of pretreated eucalyptus and corn cob from 36.7% to 37.8%–84.3% and 90.5%, respectively [176]. Because of its pH responsiveness, QALS-80 could be recycled by adjusting pH.

Zhan et al. [178]. grafted lignin with carboxyl and quaternary ammonium groups to obtain quaternary ammonium lignin carboxylate (QALC). By adjusting the content of carboxyl and quaternary ammonium groups, a series of QALCs with different isoelectric points (pIs) were prepared. The promoting effect of QALCs with different pIs on the enzymatic hydrolysis of lignocellulose was compared, and it was found that the QALC with a pI close to the pH of enzymatic hydrolysate had the best promotion effect on enzymatic hydrolysis. The excess charge on QALC would change the spatial structure of cellulases and affect cellulase activity [183,191]. Adding 2 g/L QALC-40 (pI = 5.0, the mass ratio

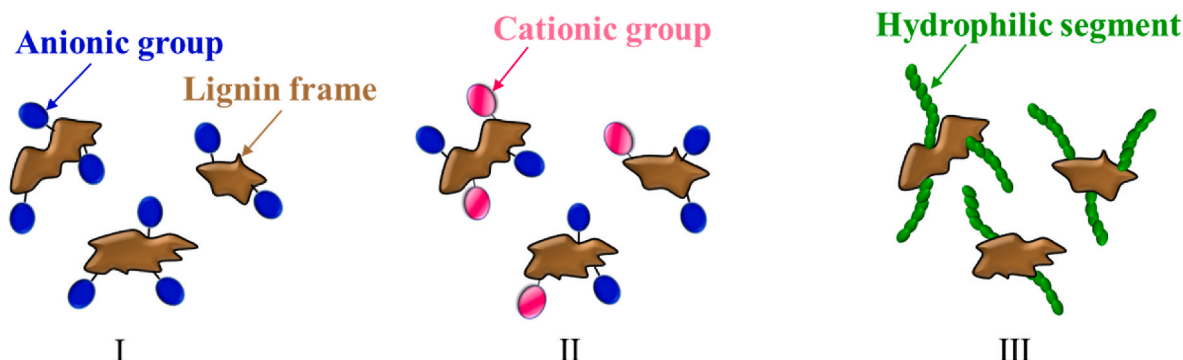


Fig. 13. Three types of lignin-based additives for enhancing the enzymatic hydrolysis of lignocellulose.

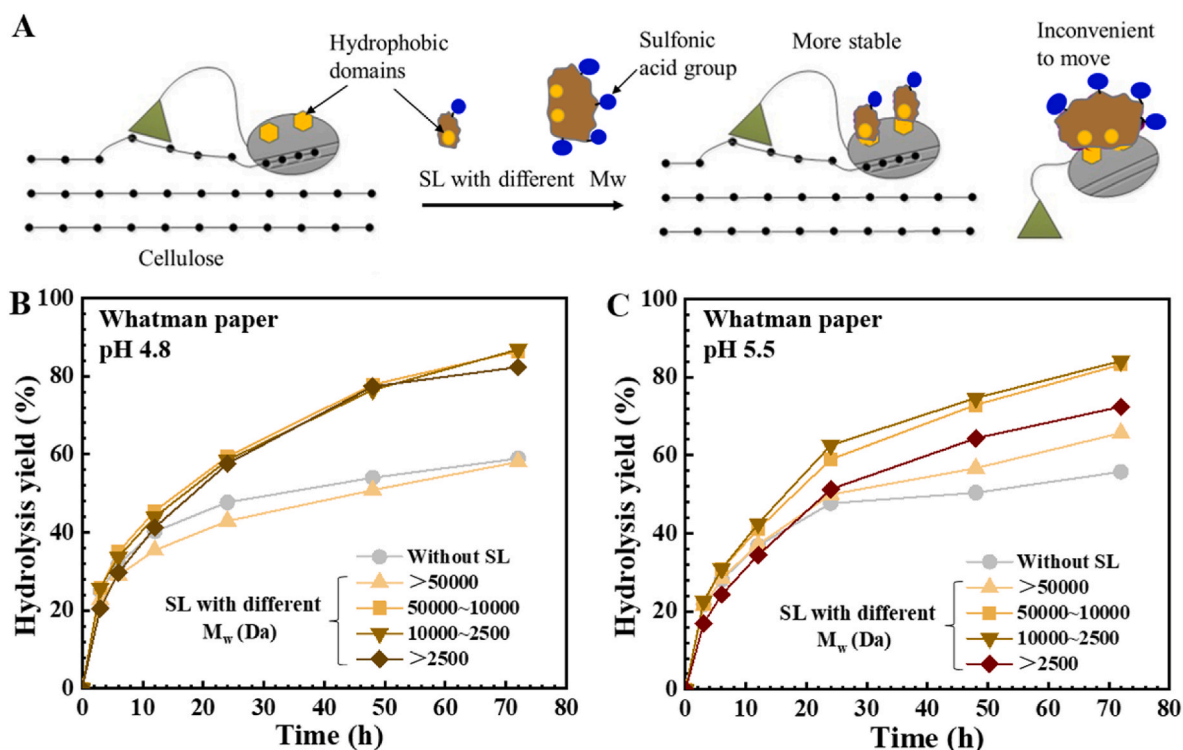


Fig. 14. Mechanism diagram of the effect of SL with different Mw on the enzymatic hydrolysis of pure cellulose (A); Effects of SL with different Mw on the enzymatic hydrolysis of Whatman paper at pH 4.8 (B) and pH 5.5 (C) [180].

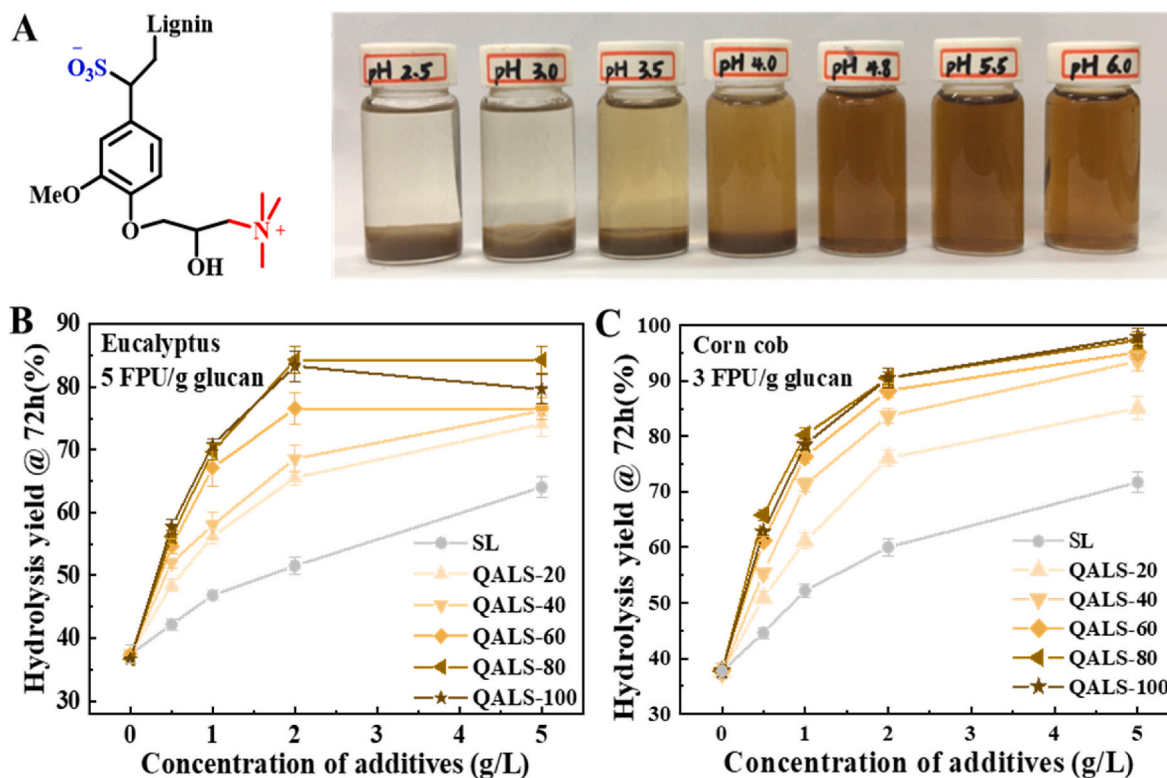


Fig. 15. Structure and pH responsiveness of QALS (A); effects of SL and QALS on the enzymatic hydrolysis of eucalyptus (B) and corn cob (C) [176]. (QALS-x, x was the mass ratio of quaternization reagent to SL).

of quaternization reagent to lignin was 40%) increased the enzymatic hydrolysis yield of pretreated eucalyptus from 35.2% to 95.3% [178].

Recently, molecular docking was used to study the binding between

β -glucosidase (β -GL) and lignin-based surfactants [192]. It was found that QALC had a lower binding free energy to β -GL than hydroxymethylated lignin and the activity of β -GL was increased by QALC but

inhibited by hydroxymethylated lignin. The increased activity might be attributed to the optimized conformation of β -GL by QALC.

4.3. Lignin-based additives grafted with hydrophilic segments

Water-soluble lignin additives can be synthesized by grafting hydrophilic segments. Among them, lignin polyoxyethylene ether (L-PEG) is the most studied, which is prepared by grafting nonionic surfactant polyethylene glycol (PEG) on lignin [177,193,194].

Twenty years ago, Uraki et al. found that AcL-PEG, prepared from acetic acid lignin (AcL) with polyethylene glycol diglycidyl ether as a crosslinker, could preserve the activity of cellulases for a long period [194]. Then, they found that AcL-PEG could also promote enzymatic hydrolysis of lignocellulose [42]. Next, Lin et al. [177,195], synthesized EHL-PEG by grafting PEG onto EHL and meticulously studied its effect on the enzymatic hydrolysis of lignocellulose. Dynamic light scattering revealed that EHL-PEG could disperse cellulase aggregates to form smaller particles, thereby increasing the chance of cellulase contact with the substrate (Fig. 16). Dissipative quartz crystal microbalance (QCM-D) analysis also showed that EHL-PEG reduced the non-productive adsorption of cellulases on lignin by 88%. Adding EHL-PEG could increase the glucose yield of corn stover at 72 h from 16.7% to 70.1%, while adding PEG alone only increased the glucose yield to 52.3%. Similar results were obtained by Yong et al. [196]. The effect of EHL-PEG on the enzymatic hydrolysis kinetics of cellulose membranes was also studied using QCM-D and AFM [197], and it was speculated that cellulase aggregates dispersed by EHL-PEG excavated extensive cavities into the surface of cellulose membrane, making the membrane looser and more exposed. When the hydrolysis rate reached the maximum, the membrane was peeled off layer by layer until it was completely hydrolyzed.

Lin et al. [198], also grafted PEG onto lignosulfonate to obtain LS-PEG and found it effectively enhanced the enzymatic hydrolysis of lignocellulose just like EHL-PEG. The promotion effect was related to the PEG content and molecular weight of LS-PEG. Most recently, Lai et al. [199], in situ modified residual lignin in DA-pretreated Masson pine

with PEG-epoxides and found that the non-productive adsorption of cellulase on lignin was reduced and the fiber swelling of the substrates was enhanced, thus an improved hydrolysis yield. The advantages and disadvantages of three types of lignin-based additives above are shown in Table 4.

5. Lignin-based carriers for cellulase recovery

Recycling cellulases is an effective way to reduce the cost of cellulases, thus immobilizing cellulases on recyclable carriers has been widely studied [200,201]. Cellulases can be immobilized on different carriers, including insoluble carriers and environment-responsive carriers [202–205]. In general, it is difficult to obtain high-performance immobilized cellulases due to the multi-component nature of cellulases and the mass transfer barriers between immobilized enzymes and solid substrates [206]. Therefore, the immobilization of cellulases is usually limited to a single component or tested on soluble substrates [207–209]. The immobilization through chemical grafting often leads to the loss of cellulase activity [210,211].

Due to multiple non-covalent interactions between lignin and cellulases, lignin can be used to immobilize different components of cellulases through adsorption [43,212]. The advantages of cellulase recovery by lignin-based carrier are obvious. On the one hand, lignin is a by-product of lignocellulose enzymatic saccharification, which is cheap and easy to obtain; on the other hand, lignin can adsorb cellulase through non-covalent interaction, avoiding the loss of enzyme activity

Table 4

Advantages and disadvantages of different types of lignin-based additives.

Types	Advantages	Disadvantages
Anionic zwitterionic	Easy to synthesize, low price Highly efficient, can be recycled	Promotion effect is limited Difficult to synthesize, expensive
Non-ionic	Convenient to synthesis, highly efficient	Relatively high price

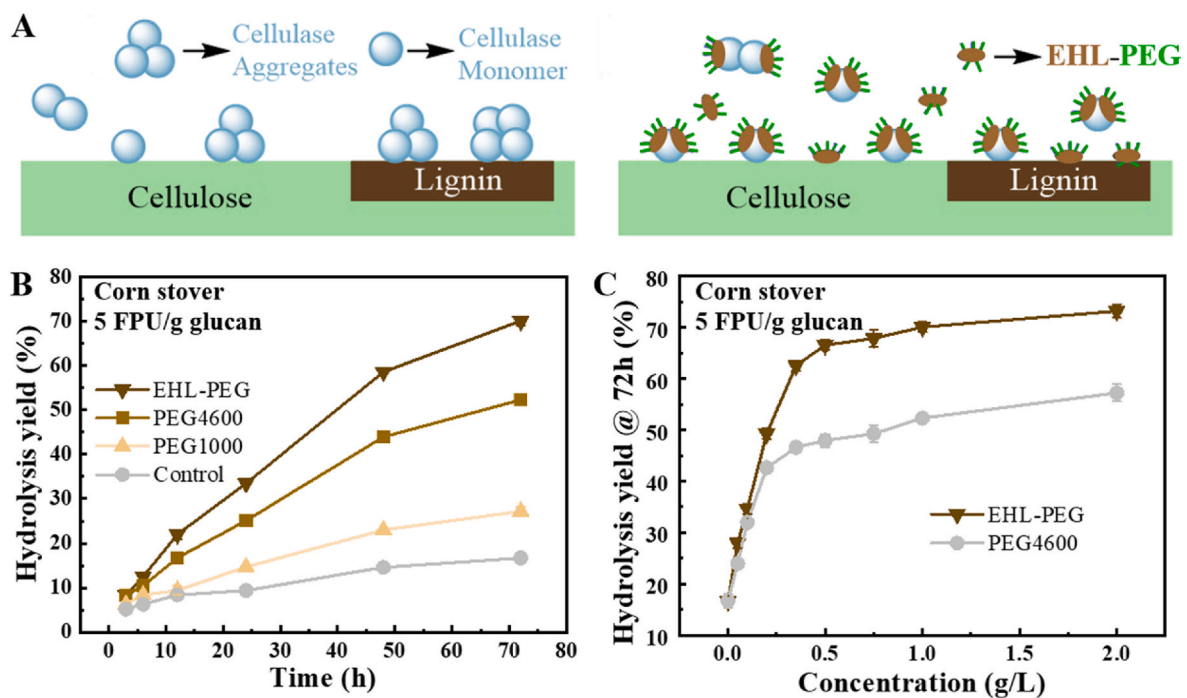


Fig. 16. EHL-PEG enhanced the enzymatic hydrolysis of lignocellulose by dispersing cellulase and reducing its non-productive adsorption on lignin (A); enzymatic hydrolysis yield of corn stover enhanced by different additives (B); effects of different concentrations of PEG and L-PEG on the enzymatic hydrolysis of corn straw (C) [177].

caused by covalent bonding. By recycling the insoluble residue after enzymatic hydrolysis, cellulases could be recovered, because the residual lignin adsorbed part of cellulases [213]. However, lignin without modification has limited ability to immobilize cellulases and usually inhibits cellulase activity. So, appropriate modification of lignin before the immobilization is necessary.

5.1. Lignin-based insoluble carriers

Nonaka et al. [43,44], found that functional phenolic polymers based on lignin (ligno-*p*-cresol) synthesized from lignin and phenol derivatives can be used to immobilize cellulase (Fig. 17). During the enzymatic hydrolysis of carboxymethyl cellulose (CMC) and filter paper, the hydrolysis performance of the cellulases immobilized on ligno-*p*-cresol reached 80–90% and 30–50% of that of free cellulases, respectively [44]. It means that the cellulases adsorbed on ligno-*p*-cresol retained relatively high activity for soluble CMC. Recently, lignin was also modified to lignoresorcinol (LigR) and lignopyrogallol (LigP) to increase the phenolic hydroxyl content for cellulase adsorption [214]. Compared with the lignin reference, the maximum cellulase adsorption capacity was improved from 76.5 mg/g to 842.1 mg/g and 911.4 mg/g, respectively. The activity of the adsorbed cellulases on LigR was higher than that on LigP, because the cellulases adsorbed on LigR could migrate to the fresh substrates during enzymatic hydrolysis.

In addition to immobilizing cellulases on phenolic lignin, Zdarta et al. [215], successfully immobilized cellulases from *Aspergillus niger* on a synthetic TiO₂-lignin hybrid carrier through physical interactions. Under optimized immobilization conditions, the activity of immobilized cellulases reached 80% of that of free cellulases. The immobilized cellulases showed high stability and recyclability. After 10 cycles, the activity of immobilized cellulases still maintained 90% of their initial activity.

Although the cellulases immobilized on insoluble carriers have high stability, the mass transfer barrier between the solid substrate and the immobilized cellulases leads to relatively low hydrolysis yield [44,216]. In addition, after lignocellulose hydrolysis, the immobilized cellulases are mixed with hydrolysis residues, which are not easy to be recycled.

5.2. pH-responsive lignin-based carriers

It is not new to immobilize cellulases on pH-responsive carriers, such as poly(L-glutamic acid) [217], Eudragit L [218], hydroxypropyl methylcellulose acetate succinate [219], N-succinyl chitosan [220], and so

on. However, the research on recovering cellulases with pH-responsive lignin-based carriers has only been reported recently.

Modifying lignin into zwitterionic surfactants can reduce its inhibitory effect on cellulases to a certain extent, and the pH response can be realized by adjusting the proportion of cation and anion groups [176, 221]. A pH-responsive lignin zwitterionic carrier (pH-LZC) with a pI of 2.2 was obtained by grafting an appropriate number of sulfonic acid groups and quaternary ammonium groups on lignin [222]. At enzymatic hydrolysis pH (about 5.0), pH-LZC stayed soluble in hydrolysate to enhance hydrolysis. After hydrolysis, the hydrolysis residue was separated by centrifugation or filtration, and the hydrolysate pH was then adjusted to 3.0 to precipitate pH-LZC. So, cellulases were recovered because they were adsorbed on pH-LZC through electrostatic attraction (cellulase was positively charged, while pH-LZC was negatively charged) (Fig. 18). During the enzymatic hydrolysis of Avicel and corncob residue, pH-LZC recovered more than 70% and 50% of cellulases, respectively.

Grafting lignin with a small amount of PEG could also lead to pH-responsive lignin-based carriers (pH-L-PEG) [223]. The pH response of pH-L-PEG was mainly attributed to the ionization and protonation of lignin carboxyl groups. Grafting too much PEG could reduce the inhibitory effect of pH-L-PEG on cellulases but also affect the interaction with cellulases and pH response sensitivity. Therefore, only the lignin grafted with an appropriate amount of PEG could effectively recover cellulases by adjusting pH [224].

5.3. Magnetism-responsive lignin-based carriers

Compared with the pH response, the magnetic response does not need acid and alkali to adjust pH, which is more environmentally friendly [225,226]. Generally, recycling operation through magnetic response can be completed in a few minutes [227,228]. Since lignin itself has no magnetic response, it needs to be loaded on magnetically responsive particles. As magnetically responsive carriers are generally insoluble, appropriate strategies are required to reduce the mass transfer barrier between immobilized cellulases and cellulose.

Dong et al. [229], prepared lignin-based magnetic nanoparticles by coating quaternary ammonium lignosulfonate (QALS) on the surface of magnetic Fe₃O₄ through coprecipitation. The resultant QALS/Fe₃O₄ had a size of about 100 nm (in fact, the size was not well controlled). The QALS/Fe₃O₄ nanoparticles were then used to adsorb and desorb cellulases by adjusting the pH of.

The solution, and the absorbed cellulases could be recovered by

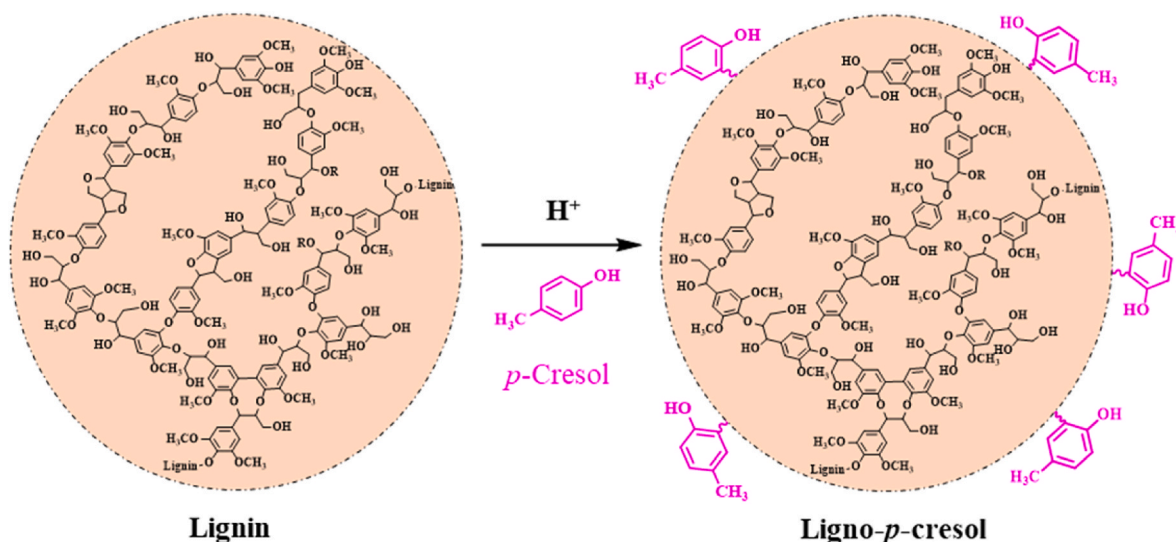


Fig. 17. Lignin-based cellulase carrier (Ligno-*p*-cresol) was synthesized by grafting *p*-cresol onto lignin.

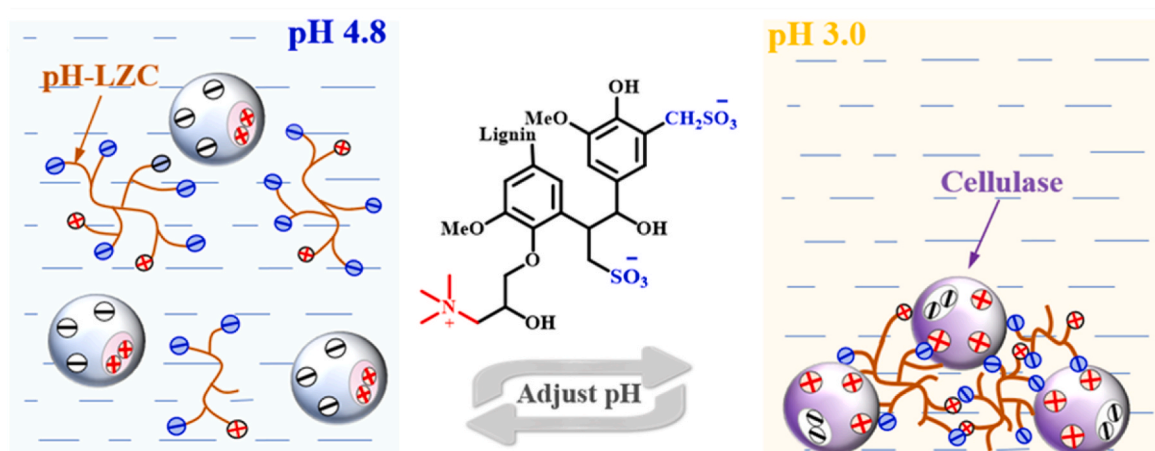


Fig. 18. Schematic diagram of pH-LZC recovering cellulase by adjusting pH [222].

magnetic force (Fig. 19). The results showed that cellulases were effectively immobilized (absorbed) on QALS/Fe₃O₄ at pH 4.8, and 68% of the immobilized cellulases were desorbed when adjusting pH to 6.8. After five cycles, the desorbed cellulases retained 32% of the initial activity.

In Dong's work [229], acids and bases were still needed to adjust the solution pH, because the immobilized cellulases had low mobility and needed to be desorbed and adsorbed during and after cellulose hydrolysis. If the lignin-based carriers were prepared into well-dispersed colloids, the mass transfer barrier between cellulases and substrates could be significantly reduced [200,230]. In this way, it is possible to truly realize the recovery of cellulase by magnetic force without any reagent addition. The advantages and disadvantages of three types of lignin-based additives above are shown in Table 5.

6. Conclusion and outlook

As the most abundant biomass resource, the efficient utilization of lignocellulose has always been an important issue of concern to researchers. Clean and safe enzymatic conversion is an important means of lignocellulose utilization, but the low efficiency and incomplete hydrolysis of substrate limit its industrialization progress. Lignin is an inevitable substance in the enzymatic hydrolysis of lignocellulose and generally has a significant inhibitory effect on the enzymatic hydrolysis

Table 5

Advantages and disadvantages of different types of lignin-based carriers.

Types	Advantages	Disadvantages
Anionic	Easy to synthesize, low price	Promotion effect is limited
zwitterionic	Highly efficient, can be recycled	Difficult to synthesize, expensive
Non-ionic	convenient to synthesis, highly efficient	Relatively high price

of cellulose. How to weaken the inhibitory effect of lignin is critical for establishing a sustainable and efficient sugar production platform.

In the last 40 years, the effect of lignin on the enzymatic hydrolysis of lignocellulose has been extensively researched. Although the presence of lignin in plant cells naturally hinders the biodegradation of cellulose, with the efforts of researchers, the inhibitory effect of lignin on enzymatic hydrolysis can be alleviated. Under certain conditions, lignin can play a positive role in enzymatic hydrolysis. When developing new methods for lignocellulose pretreatment, more attention has been paid to modifying lignin structure and understanding the relationship between lignin structure and hydrolysis performance. Many lignin-based additives for enhancing enzymatic hydrolysis and lignin-based carriers for recovering cellulase have been synthesized. These efforts and successes have significantly promoted the research and development in establishing the lignocellulose-to-sugar platform for green and

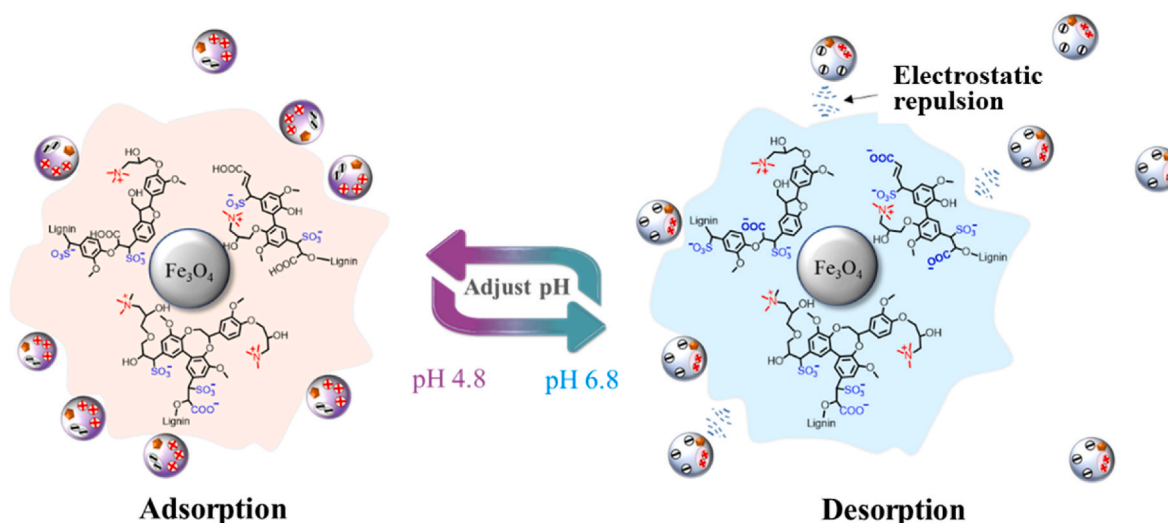


Fig. 19. Adjust solution pH to regulate the adsorption and desorption of cellulase on QALS/Fe₃O₄.

sustainable production of fuels and chemicals from renewable resources.

However, there are still many lignin-related issues and challenges that need to be addressed to make cellulosic sugars more competitive in the market.

- 1) *Engineering plants to manipulate the content, distribution, and molecular structure of lignin in lignocellulosic feedstocks.* Lignin plays an important role in bonding and protecting plant fibers in plants and often covers the outer layer of cellulose bundles. Lowering lignin content, weakening the crosslinking with carbohydrates, decreasing the affinity and impact of lignin to cellulases, and creating easy-to-break lignin are strategies to enhance the enzymatic hydrolysis of lignocellulose. Screening, breeding, and genetic engineering could make breakthroughs in this regard.
- 2) *Understanding and controlling the changes in molecular structure and aggregate morphology of lignin during pretreatment.* The structural changes to lignin could dramatically affect its physicochemical properties, cellulase adsorption capacity, and inhibitory effects on the enzymatic hydrolysis of cellulose. The changes in lignin aggregate morphology affect the removal rate of lignin and substrate accessibility. In this regard, new spectroscopic, NMR, and adsorption detection methods need to be established, especially the means that can in situ characterize the dynamic changes of lignin during pretreatment.
- 3) *Modifying lignin into efficient enzymatic hydrolysis additives (promoters) or cellulase recovery carriers.* Although lignin can be modified into enzymatic hydrolysis additives and cellulase recovery carriers, the modification methods and application conditions still need to be optimized for industrial applications. For example, in situ modification of lignin into enzymatic hydrolysis additives (promoters) and cellulase carriers during pretreatment would simplify operations and reduce costs. Another promising but challenging direction is modifying lignin into temperature-responsive additives or carriers, which can be recovered simply by changing temperature. However, the sensitivity of temperature response is difficult to control due to the heterogeneity of lignin structure.
- 4) *Valorizing enzymatic hydrolysis lignin (EHL) into high-value products.* EHL is the main component of the enzymatic hydrolysis residue and is usually contaminated with enzymes and/or yeast and unhydrolyzed cellulose and/or hemicelluloses. Though it is challenging, high-value utilization of EHL is of great significance for reducing the overall enzymatic hydrolysis cost and realizing the comprehensive utilization of lignocellulose components. The potential of EHL utilization is dependent on its structural characteristics. For example, the EHL with a high β -O-4 content is suitable for the preparation of aromatic chemicals by depolymerization, severely condensed EHL can be used to prepare bio-based carbon materials, and the EHL rich in phenolic hydroxyl groups is suitable for the preparation of antioxidant products. In addition, the near-complete hydrolysis of carbohydrates can result in EHL with high purity, which is beneficial to its subsequent high-value utilization.

Credit authors statement

Cheng Cai: Conceptualization, Investigation, Methodology, Writing–original draft. **Chaofeng Zhang:** Investigation, Methodology, Writing–review & editing. **Ning Li:** Investigation, Writing–review & editing, Visualization. **Huifang Liu:** Investigation, Writing–review & editing, Visualization. **Jun Xie:** Methodology, Supervision. **Hongming Lou:** Methodology, Supervision. **Xuejun Pan:** Methodology, Supervision. **J.Y. Zhu:** Writing–review & editing, Supervision. **Feng Wang:** Methodology, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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