Lignin-enzyme interaction: A roadblock for efficient enzymatic hydrolysis of lignocellulosics

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Abstract

Efficiently producing second-generation biofuels from biomass is of strategic significance and meets sustainability targets, but it remains a long-term challenge due to the existence of biomass recalcitrance. Lignin contributes significantly to biomass recalcitrance by physically limiting the access of enzymes to carbohydrates, and this could be partially overcome by applying a pretreatment step to directly target lignin. However, lignin typically cannot be completely removed, and its structure is also significantly altered during the pretreatment. As a result, lignin residue in the pretreated materials still significantly hindered a complete conversion of carbohydrate to its monosugars by interacting with cellulase enzymes. The non-productive adsorption driven by hydrophobic, electrostatic, and/or hydrogen bonding interactions is widely considered as the major mechanism of action governing the unfavored lignin-enzyme interaction. One could argue this type of interaction between lignin residue and the activated enzymes is the major roadblock for efficient enzymatic hydrolysis of pretreated lignocellulosics. To alleviate the negative effects of lignin on enzyme performance, a deep understanding of lignin structural transformation upon different types of pretreatments as well as how and where does lignin bind to enzymes are prerequisites. In the last decade, the progress toward a fundamental understanding of lignin-enzyme interaction, structural characterization of lignin during pretreatment and/or conformation change of enzyme during hydrolysis is resulting in advances in the development of methodologies to mitigate the negative effect of lignin. Here in this review, the lignin structural transformation upon different types of pretreatments and

the inhibition mechanism of lignin in the bioconversion of lignocellulose to bioethanol are summarized. Some technologies to minimize the adverse impact of lignin on the enzymatic hydrolysis, including chemical modification of lignin, adding blocking additives, and post-treatment to remove lignin were also introduced. The production of liquid biofuels from lignocellulosic biomass has shown great environmental benefits such as reducing greenhouse gas emissions and mitigate climate change. By addressing the root causes of lignin-enzyme interaction and how to retard this interaction, it is our hope that this comprehensive review will pave the way for significantly reducing the high cost associated with the enzymatic hydrolysis process, and ultimately achieving a cost-effective and sustainable biorefinery system.

Keywords: lignocellulosic biomass, pretreatment, lignin, enzymatic hydrolysis, lignin-enzyme interaction, biofuels

Abbreviations

p-hydroxyphenyl (H) ; syringyl (S); and guaiacyl (G); γ-valerolactone (GVL); benzyl phenyl ether (BPE), 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]); 1-butyl-3 methylimidazolium chloride ([BMIM]Cl); 1-hex-cetylpyridinium chloride ([Hpy][Cl]); 1,3-dimethylimidazolium methyl phosphonate ([DMIM][MPh]); Ionic liquids (IL); ammonia fiber expansion (AFEX); organosolv and ammonia pretreatment (OR and AR); lignin-carbohydrate complex (LCC); atomic force microscopy (AFM); quartz crystal microgravimetry (QCM); carbohydrate binding modules (CBM); polyethylene glycol (PEG); sulfite pretreatment (SPORL); laccase-mediator treatments (LMT); maleic acid (MA), Bovine Serum Albumin (BSA); liquid hot water (LHW); tannic acid (TAN); pH-responsive lignin-based polymer (EHL-MPEG); monomethoxy polyethylene glycol (MPEG); enzymatic hydrolysis lignin (EHL); enzymatic hydrolysis lignin-grafted phosphobetaine (EHLPB); Lignosulfonate (LS); cetyltrimethylammonium bromide (CTAB);

1. Introduction

Energy is an essential part of today's society, and petroleum-based oil is considered as one of the most efficient and reliable energy sources. It was showed that EUR/USD exchange rate is strongly affected by the price of oil, which in turn affects the global economy through a variety of channels [1]. Ethanol-blended fuels for transportation have been recognized as a promising avenue of next-generation energy fuels because of their safe, clean, and widely accessible properties [2]. However, corn-based bio-ethanol technology is not practical for wide applications due to high cost associated

with its production. The increased production of corn-based biofuel also generated the "food versus fuel" debate. Because of the rising production of bioethanol and biodiesel, the demand for feedstocks such as sugar cane, corn, and vegetable oils has increased as well, which raises the question if the rising in food prices is caused by an increasing biofuel consumption. A recent study showed that the main driver for food price fluctuations is mainly the oil price shock, however, this did not prove the exact role of biofuel production in this link [3]. Additional research is required to settle the debate. Hence, there is a pressing need and interest to establish a cost-effective strategy to efficiently convert the non-starch based substrates to ethanol [4]. Various lignocellulosic biomass (such as agriculture and forestry residues, energy crops, and wastes from pulp mills) possess great potential in the cellulosic-ethanol industry, while most of them are discarded as waste or managed by burning or landfill [5].

Although the current U.S. biofuel industry is still dominated by corn ethanol and biodiesel produced from soybean or cooking oils, lignocellulosic-based biofuel will play an essential role in the future to make the shift from fossil-based fuels to cleaner energy [6]. The economic and sustainable considerations for the whole bioconversion process of lignocellulosic biomass to biofuel are very important to facilitate their large-scale industrial application [7-10]. It has been suggested that the overall cost of lignocellulosic ethanol could be nearly 20% lower than that of the corn-based ethanol [11]. More importantly, the lignocellulosic biomass does not compete with the food industry, and unlike corn, its price is typically not related to the rising gasoline price. Over the past few years, the quick development in biotechnology, chemical engineering,

genetics, enzyme technology, process chemistry is leading to a new green and sustainable manufacturing concept of converting lignocellulosic biomass to liquid fuels via multiple steps (e.g., pretreatment, hydrolysis, fermentation) [12]. For this kind of bioenergy industry or companies, further research should focus on identifying key elements that are environmentally responsible for ensuring sustainable and responsible economic development [13-15]. Since the first commercial-scale biofuel facility to produce bioethanol from corn stover opens in the U.S. in 2013, the cellulosic ethanol production continues to increase each consecutive year reaching nearly 10 million gallons in 2019. However, this is still far from the Renewable Fuel Standards (RFS)'s target of 16 billion by 2022 [16]. The future success of bioethanol production from renewable lignocellulosic will strongly depend on how to reduce the overall cost, and this would require the development of promising technologies for every stage of bioconversion process.

In the cell wall of lignocellulose, the major components of cellulose, hemicellulose, and lignin render the tight three-dimensional ultrastructure. The tightly arranged cell wall components significantly hinders the conversion of carbohydrate into sugars by enzymes (**Fig. 1**) [17]. Lignin, an amorphous and highly branched aromatic polymer, is largely distributed in the secondary plant cell wall. It primarily composed of syringyl (S), guaiacyl (G), and *p*-hydroxyphenyl (H) subunits, which are synthesized via the free radical polymerization of hydroxycinnamyl monolignols including sinapyl, coniferyl, and *p*-coumaryl alcohols, respectively. Besides these predominant monomers, other naturally occurring hydroxyphenylpropanoid compounds such as tricin [18],

hydroxycinnamaldehydes [19], dihydroxycinnamyl alcohols [20] are also incorporated into the polymer to different extents. These basic phenylpropane units are connected to each other mainly through ether (e.g., 4-O-5, α-O-4, β-O-4) and carbon-carbon (5-5, β-β. β-1) bonds. Lignin macromolecule also contains huge amount of functional groups such as aliphatic and phenolic hydroxyl groups that governs its hydrogen bonding and hydrophobic properties. In the plant cell wall, lignin also linked to hemicellulose via various alkyl/aryl-ether bonds to form the lignin-carbohydrate complexes that blocks the access of enzymes to cellulose during enzymatic hydrolysis. The enzyme hydrolysis process refers to a multistep reaction to convert carbohydrate into simple sugars via a group of enzymes produced from bacterial and fungal species. Unlike other hydrolysis technologies, cellulase enzymes do not leave residues in the hydrolysate that can serve as inhibitors of the downstream fermentation process. During this enzymatic hydrolysis, lignin can non-productively bind to the enzymes thus negatively affecting the enzymatic hydrolysis of biomass [21, 22]. It has been proposed and proved to some extent that this type of non-productive adsorption is driven by a collective of interactions including hydrophobic, electrostatic, and/or hydrogen bonding. In an effort to assess directly the effects of these interactions on lignin-enzyme adsorption, years of research have focused on modifying lignin structure or using phenolic model compound and correlating these structure features to changes in lignin's adsorption capacity toward cellulase enzymes [23]. However, the exact role of individual factors is still unknown and in fact, much of the literature report inconsistent results. The lack of consensus is mainly attributed to the near-impossibility of differentiating these driving

forces as well as the lack of analytical techniques that could directly measure these interactions [24]. For example, hydrophobic interactions are largely represented by the content of phenolic hydroxyl groups of lignin which could be measured via ^{31}P NMR [25]; however, there are also reports in the literature that suggest these hydroxyl groups also significantly affect the lignin's hydrogen binding ability with amino acid residues in cellulase enzymes [26]. Much more research is needed to resolve these challenges.

In order to obtain more fermentable sugars for downstream fermentation, various methods of pretreatment should be carried out to remove lignin to eliminate its negative effect on enzyme and increase the accessibility of cellulose in the pretreated biomass [27]. In recent years, the field of lignin-target pretreatment has advanced tremendously with traditional alkaline based pretreatment technologies originated from the pulp and paper industry being replaced or supplemented by promising lignin-targeted biomass fractionation techniques. Among these pretreatment technologies, renewable solvents derived from biomass such as γ-valerolactone (GVL) [28], cyrene [29], methyltetrahydrofuran [30] and green solvent system includes renewable deep eutectic solvent and ionic liquid have begun to draw significant attention [31, 32]. Yiin et al. introduced a novel green DES system containing malic acid, sucrose, and water, and showed that the mixture was quite effective in terms of disrupting the three-dimensional structure of lignin [33]. The recent advances in choline chloride based green DES for lignocellulosic biomass fractionation were also summarized [34]. For a more elaborate discussion on the introduction of these pretreatments, the reader is referred a recent dedicated review [35].

Most of the above-mentioned pretreatment techniques are not capable of completely remove lignin. It is thus believed that the extents of the residual lignin inhibitions on hydrolysis are highly correlated to the structure of residual lignin, which is significantly affected by the applied pretreatment methods [36]. For example, branched G lignin subunits are typically more inhibitory than linear S type of lignin unit [37], and this type of S lignin units were also reported to be preferentially degraded/removed over G lignin units during flow-through hydrothermal pretreatment [38]. As a result, this structural change of lignin during flow-through hydrothermal pretreatment had an adverse impact on the subsequent hydrolysis process. On the other hand, enhanced enzymatic hydrolysis has been reported by incorporating acid groups [39] and sulfonate groups [40] onto the lignin during biomass pretreatment. Therefore, a comprehensively understanding of the changes of physicochemical properties of lignin during pretreatment is essential to alleviate the negative or promote the positive effects of the lignin on enzymatic hydrolysis.

Efforts towards developing efficient biomass pretreatment are continuously increased in the past 15 years. As shown in **Fig. 2a**, a quick survey of the literature suggests that the number of publications about biomass pretreatment was continuously increased from 2015 to 2020 (search from google scholar website using a keyword of biomass pretreatment), in which China, USA, and Japan were among the biggest publishers (**Fig. 2b**). Among these publications, most of the review literatures focused on summarizing the pretreatment technologies for efficiently enzymatic conversion of pretreated biomass and the influence of lignin on enzymatic hydrolysis without

addressing the structural changes of lignin during various pretreatment processes and how to minimize the adverse impact of lignin on enzymatic hydrolysis. In our opinion, a fundamental understanding of the mechanism behind the lignin chemistry during pretreatment and lignin-enzyme interaction during enzymatic hydrolysis is of great importance for researchers from both academic and industry to finally achieve the economic and feasible approaches for lignocellulosic biorefinery.

The two primary mechanisms of lignin affecting the degradation of carbohydrate to fermentable sugars include limiting the cellulose accessibility and interacting with cellulase enzymes. While biomass pretreatment could partially overcome the physical barrier, it also causes irreversible structural modification to lignin affecting its interaction with cellulase enzymes. Although the relative contributions of these two roles of lignin are still under debate, it is hypothesized that limiting the non-productive binding of lignin to cellulase could significantly reduce the dose of enzyme required for an efficient enzymatic hydrolysis process. Due to recent advances in the development of analytical techniques for investigating the non-productive adsorption of cellulase to lignin, three major driving forces include hydrophobic, hydrogen bonding, and electrostatic have been identified in several studies to govern the lignin-enzyme interaction. However, the relative contribution of each factor as well as how the lignin structure change during biomass pretreatment affects its interaction with enzymes are still not fully understood. In recent years, intriguing reports have shown that lignin with certain physicochemical properties and/or from certain locations of the surface of pretreated biomass had a positive effect on enzymatic hydrolysis [41-44].

The underlying mechanisms of this type of hydrolysis-enhancing impact from lignin remain unclear and need further elucidation. This manuscript aims to offer a comprehensive review of the lignin structure changes during various mainstream pretreatments and their effect on lignin-enzyme interaction during enzymatic hydrolysis process. In addition, the primary mechanisms governing the lignin-enzyme interaction as well as technologies used to minimize the adverse impact of lignin on the enzymatic hydrolysis were also revealed. By elucidating the lignin structure change during various biomass pretreatment and alleviating the lignin-enzyme interaction during enzymatic hydrolysis, the cost associated with the enzymatic hydrolysis process will be dramatically reduced, and ultimately a cost-effective and sustainable biorefinery system could be achieved. The successful production of ethanol fuels from biomass in a cost-effective way will reduce the impact of greenhouse gas emissions as well as the dependence on fossil fuels, lower the level of pollution, and promote the economic security.

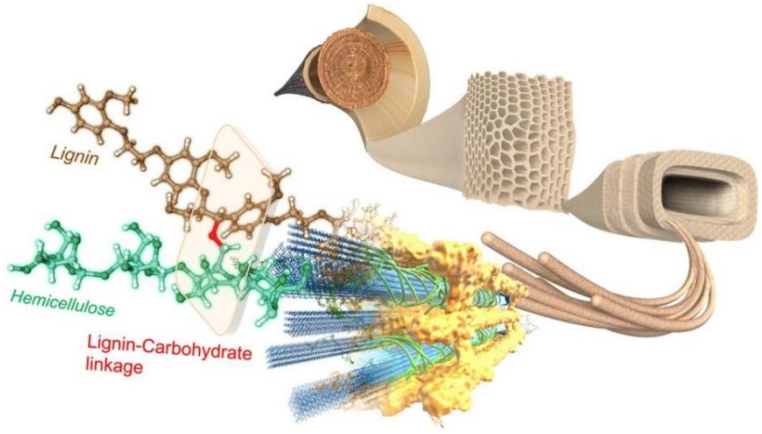

Fig. 1. The recalcitrance of lignocellulosic biomass for bioconversion (reproduced with copyright permission from Springer Nature) [45].

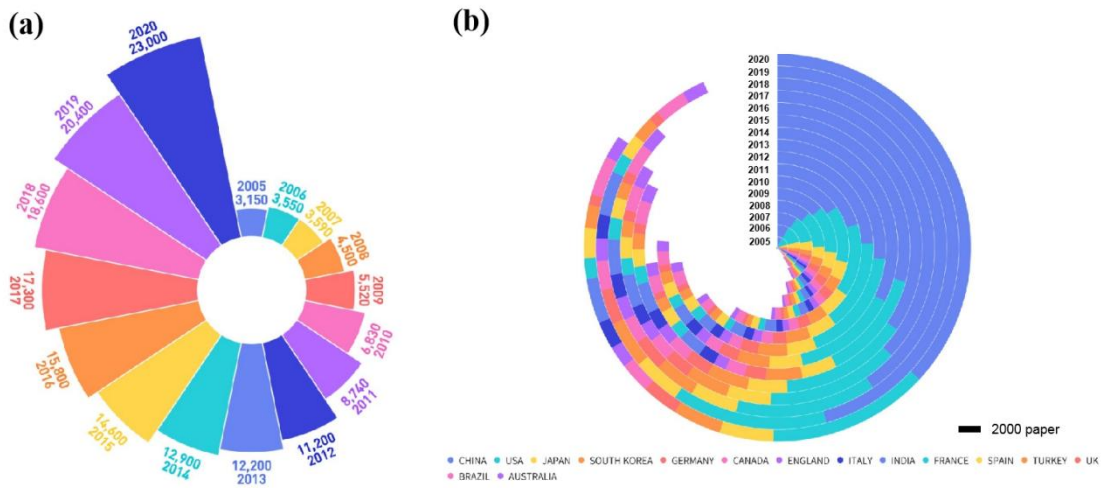

Fig. 2. The published paper about pretreatment from 2015 to 2020 (a); the published paper about biomass pretreatment from different countries during 2015 to 2020 (b).

2. Lignin structural transformation during various biomass pretreatment and its effect on enzymatic hydrolysis

During the pretreatment process, lignin can be removed, redistributed, and chemically modified (e.g., degraded and condensed) by the pretreatment solvent and catalyst. Common lignin modifications during pretreatment include the cleavage of interunit linkages, change of the composition of lignin monomer, and the content of functional groups. Many of these changes in lignin structure are intercorrelated. For example, the cleavage of ether linkages is typically accompanied with the formation of extra phenolic OH and loss of aliphatic OH groups [46]. It is believed that these structural changes can affect the adsorption behaviors between lignin and enzymes and further affect the subsequent enzymatic digestibility of pretreated biomass. A fundamental understanding the changes of lignin structure during various pretreatments is a prerequisite for

building the “lignin structure-enzyme interaction function” relationship, and this will help developing new pretreatment technologies that render lignin inhibiting to enzymes.

2.1. Acid pretreatment

Acidic pretreatment normally refers to the pretreatment using external acids such as sulfuric acid [47], hydrochloric acid [48], phosphoric acid [49], formic acid [50], p-toluenesulfonic acid [51], and oxalic acid [52], as the catalyst. Despite the high effectiveness on saccharification, high concentrated acid pretreatment is less favored industrially duo the requirement of acid-resistant equipment and high-cost acid recovery process [53]. Whereas dilute acid pretreatment is considered a promising industrial method as it is technically mature and has been proven to be cost-effective [54]. During acid pretreatment, lignin undergoes mainly acid-catalyzed degradation and condensation rendering small soluble fragments and insoluble residue solids. These direct chemical changes in lignin can cause a negative effect on the enzymatic hydrolysis of the pretreated biomass. The pretreatment hydrolysate soluble fraction mainly contains monomeric, dimeric, and oligomeric phenolic compounds derived from lignin [55, 56] . These hydrolysate soluble degradation products also have potential inhibition effects on biomass bioconversion and require additional treatment which will be covered later in the review. The solvent extractable lignin fraction usually has a higher molecular weight than the hydrolysate soluble fraction with weight-average molecular weight normally below 3000 g/mol [57, 58]. This fraction of lignin also undergoes degradation and may migrate within and out of the plant cell wall and even redeposits on biomass’s surface [59]. The insoluble residual lignin has the highest

molecular weight (~5000 – 10000 g/mol) and bears most of the condensed structures.

The chemical transformation of lignin under acidic conditions has been well documented. The predominant reaction is acid-catalyzed cleavage (acidolysis) of β-aryl ether which is the most abundant linkage in lignin. The mechanism of acidolysis has been revealed by an extensive model compound study as shown in **Fig.** [60, 61]. The acid catalyzes the rapid formation of benzylic carbocation by cleaving the α-hydroxyl or α-ether. The benzylic carbocation further undergoes β-proton abstraction (route i, enol ether intermediate) followed by protonation, accompanying direct hydride transfer from the β to α position (route iii), yielding a β-carbocation intermediate. The β-carbocation intermediate continues to react causing the cleavage of β-O-4' linkage and formation of Hibbert's ketone. Previous research also reveals that β-elimination of γ-hydroxymethyl groups (as formaldehyde, route ii) becomes dominant when sulfuric acid is used as the catalyst [62, 63].

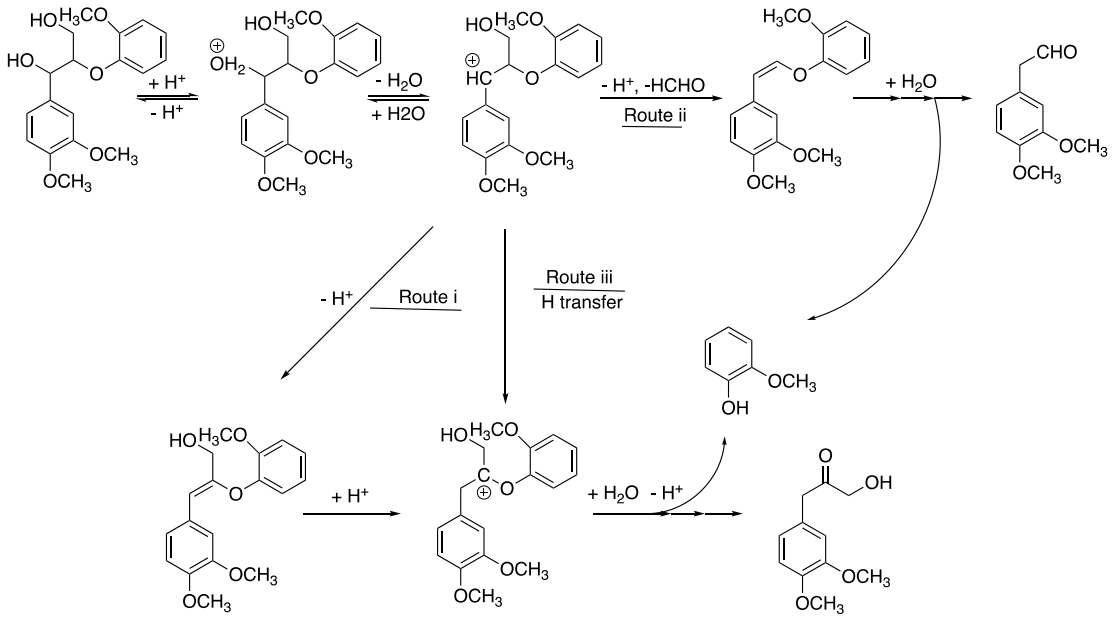

Fig. 3. Mechanism of β-aryl ether acidolysis (reproduced with copyright permission

from Taylor & Francis) [61].

Besides acidolysis, it has been proposed that the homolytic cleavage of lignin is another important reaction of β-aryl ether degradation under acid conditions. The reaction mechanism of homolytic cleavage of β-O-4' is illustrated in **Fig. a**. The β-aryl ether forms a quinone methide intermediate followed by homolysis affording radicals. These radicals could undergo coupling forming β-β', β-5' and β-1' structures or radical exchange forming cinnamyl alcohol. The homolytic cleavage of β-aryl ether has been observed for a long time and is often reported under the moderate acidic conditions of pretreatment. For example, Li et al. reported that only homolytic cleavage of ether linkages occurred when the model compound containing β-aryl ether structure was treated with water, while both acidolysis and homolytic cleavage reactions occurred with 0.2 M acetic acid [64]. Leschinsky et al. also reported the existence of homolytic cleavage of β-aryl ether in the autohydrolysis of *Eucalyptus globulus* wood with the evidence of increase β-β' linkages and appearance of stilbene structures after pretreatment [65]. The homolytic cleavage is also proposed to be responsible for the lignin degradation during steam explosion pretreatment [66, 67]. On the other hand, Sturgeon et al. investigated the mechanism of acid-catalyzed cleavage of aryl-ether linkages using a radical scavenger, and found that the above proposed homolytic cleavage pathway of β-aryl ether linkages is unlikely in acidolysis conditions [68]. The homolytic bond dissociation enthalpies of lignin aryl-ether model compound were reported larger than 60 kcal/mol according to the density functional theory, making this type of cleavage extremely difficult to occur under typical acid pretreatment conditions

[69-71].

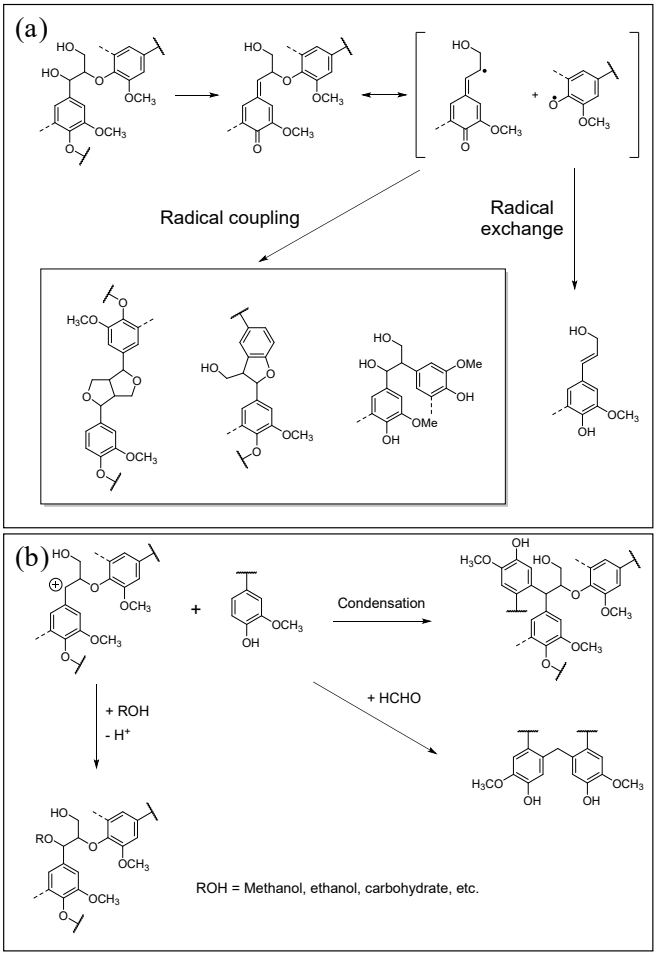

Fig. 4. Homolytic cleavage of β-aryl ether under mild acidic conditions (a); Condensation of lignin during acidic pretreatment and alkoxylation of side chain in lignin during acid-catalyzed organosolv pretreatment (b).

Lignin degradation greatly decreases its molecular weight thus increases its solubility in the pretreatment hydrolysate, facilitating the removal of lignin from the plant cell wall. This delignification is beneficial for the enzymatic hydrolysis of cellulose to produce glucose. However, condensation/repolymerization is inevitable in most of the pretreatments under severe acid conditions, which can hinder the enzymatic conversion. The mechanism of lignin condensation under acidic conditions is shown in Fig. 4b. The benzylic carbocation is very reactive and can be easily attached by a

nucleophile, such as nucleophilic carbon, causing condensation. Under acidic conditions, condensation mainly occurs between an electron rich carbon (C_6 carbon on the aromatic ring) and benzylic carbocation forming α-6' bond. Another type of lignin condensation is the formation of diphenylmethane structures between two C6 carbons and the release of formaldehyde when using sulfuric acid as the catalyst [72]. Various research has shown that S units in lignin tend to depolymerize into small moieties whereas G units are prone to condensation [57, 73].

Besides the above-mentioned structural change, the formation of stilbene types of structures was also reported. The stilbene structures (e.g., β-5 and β-1) could be formed by the coupling of quinone methide and phenoxy radical intermediates. Other minor reactions during acidic pretreatment are the hydrolysis of γ-ester which releases acetic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, and ferulate [57, 74], These occurred chemical reactions during the acid pretreatment ultimately alters the functional groups in lignin, such as decreasing aliphatic hydroxyls, increasing the phenolic hydroxyls, etc. It is believed that the increased amount of phenolic hydroxyl groups in lignin might endow a more inhibitory role towards enzymatic hydrolysis due to the increased hydrophobic nonproductive binding between lignin and enzymes [75]. Hence, various post-treatments should be carried out to reduce the inhibitory role of residual lignin in acid-pretreated biomass to improve the enzymatic conversion.

2.2. Alkaline pretreatment

Alkaline pretreatment is inspired by the pulping technology in paper industry, which has the advantage of high delignification efficiency [76]. Various alkaline

pretreatment processes, such as dilute sodium hydroxide [77], kraft pulping [78], lime [79], green liquor [80], ammonia hydroxide [81], and ethylenediamine [82], have been developed to reduce the biomass recalcitrance. The key lignin reaction during alkaline pretreatment is the base-catalyzed cleavage of ether linkages (α-ethers and β-ethers). The phenolic α-ethers are alkaline liable and ready to be cleaved forming a quinone methide intermediate, whereas the non-phenolic α-ethers are relatively stable under alkaline conditions (**Fig a**). However, it was also reported that this type of non-phenolic α-ethers could be cleaved if an extra hydroxyl group is presented adjacent to β position, in which case the adjacent OH groups could facilitate the cleavage via the formation of epoxide [83]. The reaction of phenolic β-ethers also starts with the formation of quinone methide intermediate (**Fig b**). The quinone methide intermediate is very unstable and further reacts to form more stable products. In addition, the hydroxide ion could deprotonate γ-hydroxyl groups leading to the loss of γ-carbon as formaldehyde through a reverse aldol condensation reaction and forming a vinyl ether (Route I). This is the major reaction pathway during soda pulping. Alternatively, the hydroxide ion could deprotonate β-carbon and the quinomethide rearomatizes forming another vinyl ether product, which is stable under alkaline conditions (Route II). The quinone methide intermediate could also be attacked by other nucleophiles from alkaline reagents, such as SH^-, SO_3^{2-}, etc., leading to lignin fragmentation (Route III). The formation of epoxide (or episulfide) is the key step that facilitates the cleavage of β-ethers. The epoxide is further hydrolyzed to verarylglycerol, whereas episulfide further undergoes complex redox reaction giving rise to cinnamyl alcohols and other fragments with one or two

carbons side chain. Generally, the enhanced cleavage of β-ethers can facilitate the removal of lignin from biomass, which is benefit for the enzymatic hydrolysis.

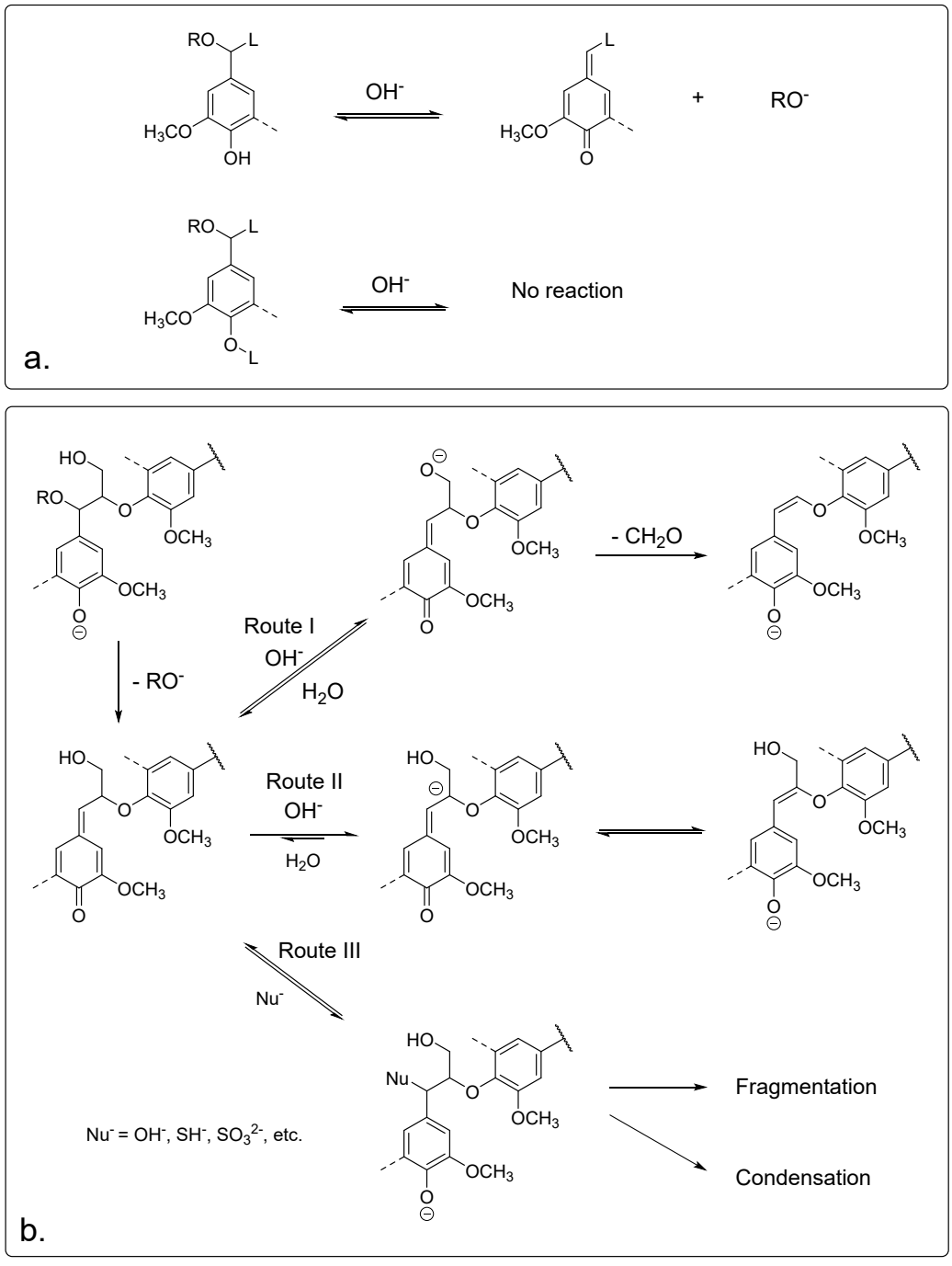

Fig 5. The reactions of α-ethers (a) and phenolic β-ethers (b) under alkaline conditions.

The lignin condensation reaction also occurs under alkaline conditions resulting in

new alkaline stable C-C bonds. The possible pathways for alkaline induced lignin condensation between phenolic substructures are shown in **Fig**. The α-carbon of quinone methide resulted from the liberation of the OH^- from the α-position of phenolic structure could be nucleophilically attacked by C_5 or C_1 carbon of another phenoxide substructure, forming α-5 or α-1 type of condensation product, respectively. During the process, formaldehyde is usually released from a γ-hydroxymethyl group. Subsequently, the two phenolic guaiacyl units could react with the released formaldehyde forming diphenylmethane structures. These irreversible condensations could cause an inevitable increase in lignin molecular weight. According to Zhao et al. (2020), lignin with larger molecular weights has more non-productive adsorption with enzymes compared to those of lower molecular weights, due to the increase in hydrophobic interactions between lignin and enzymes [84]. Hence, low sugar release from enzymatic hydrolysis of alkaline pretreated biomass was still observed despite significant delignification, which might be due to the increased molecular weight of residual lignin in pretreated biomass.

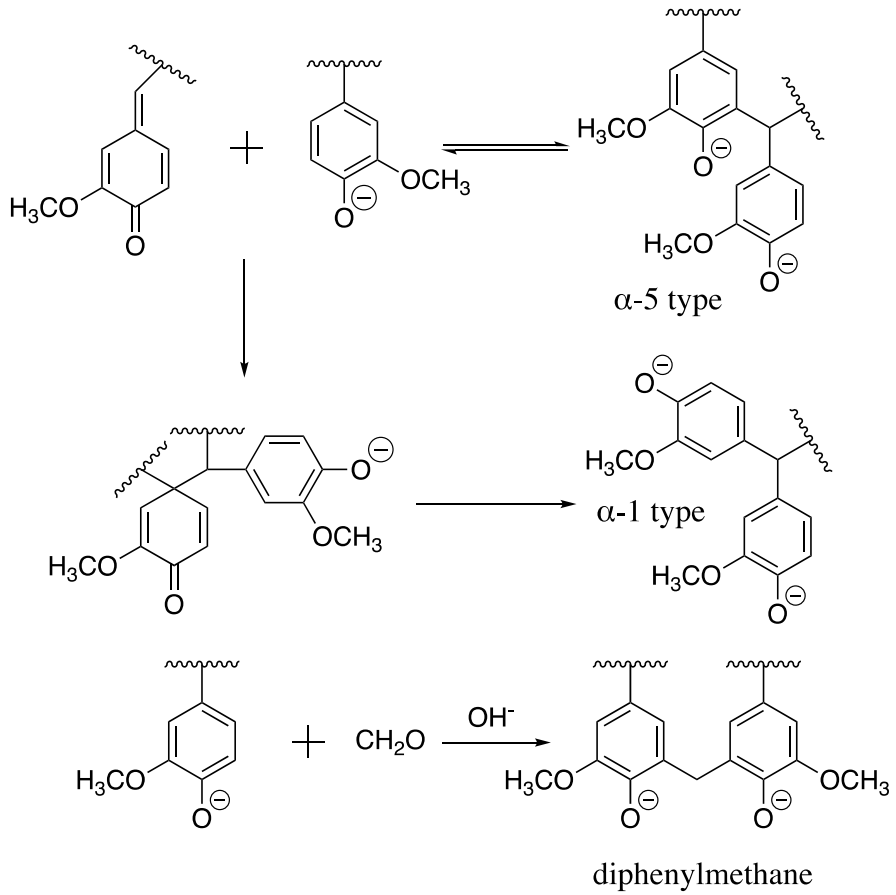

Fig. 6. Pathways of lignin condensation under alkaline conditions

2.3. Organosolv pretreatment

Organosolv pretreatment also can be dated back to the concept of organosolv pulping, which achieves high delignification by physically dissolve lignin without firstly degrading it. Various solvents have been employed in organosolv pretreatment, such as ethanol [85], acetone [86], methyl isobutyl ketone [87], glycerol [88], ethylene glycol [89], GVL [28], cyrene [29], tetrahydrofuran [90], 1,4-butanediol [91], 1-pentanol [92], butanol [93], sulfolane [94]. Catalysts, such as sulfuric acid [95], sodium hydroxide [96], and $FeCl_3$ [97], were also used to facilitate the organosolv pretreatment to improve the enzymatic hydrolysis of biomass by removing the lignin with enhanced yield. Organosolv pretreatment is recognized as a high-cost pretreatment method

compared to dilute acid pretreatment as it requires more energy for solvent recovery [98]. The advantage of organosolv pretreatment is the production of pure lignin and other products [99]. The major reactions of lignin under organosolv pretreatment are similar to the reactions under acid or alkaline pretreatments depend on the catalyst used. Besides major lignin reactions, the alkylation of lignin side chain (mainly at Cα position) also occurs under acidic organosolv pretreatment with alcohols. This trapping of the reactive cationic intermediate by the alcohol significantly suppressed the further lignin degradation/condensation. Dong et al. also reported that a diol pretreatment could quench the benzyl carbocation intermediate and form an ether linkage with a hydroxyl tail at the α position therefore increased its solubility in the organic solvent [91]. Berlin et al. reported that the lignin with 8-10/100 C9 units of ethylated structures can be isolated from ethanol pretreatment of coastal Douglas-fir (*Pseudotsuga menziesii*) [100]. The ethylated structures were also observed in the isolated lignins from other species with ethanol organosolv pretreatment, such as cottonwood, black willow, aspen, eucalyptus, and sweetgum [101]. The extent of α-OH substitution depends on the lignin composition with G units reported to be more reactive than S [102]. The alkylation is believed to reduce the binding affinity of the enzyme onto lignin [101]. Recently, the organosolv pretreatments using tetrahydrofuran and GVL have drawn growing interest due to their effectiveness in delignification and promoting enzymatic hydrolysis. The addition of co-solvent such as water into the organosolv pretreatment system had a significant impact on the lignin structure. The addition of water could alter the co-solvent system's Hildebrand solubility parameter (δ), which can cause it close to that

of lignin. Thus, lignin's solubility in the mixture of water and different organic solvents such as ethanol, tetrahydrofuran, 1,4-dioxane, acetone, and butanediol could be greatly enhanced [102]. Patri et al. analyzed the effect of tetrahydrofuran-water cosolvent on lignin structure via molecular simulations, and results suggested that the tetrahydrofuran/water could form a "θ" solvent and lignin could be dissociated from the plant cell wall and expands to form a random coil type of structure, rendering them more susceptible to degradation by acid-catalyzed cleavage of ether linkages [103]. Jasiukaitytė-Grojzdek et al. used an α-O-4 model compound benzyl phenyl ether (BPE) and beech wood to show that the addition of water dramatically affects both the lignin isolation pathway and the structure of lignin [104]. The addition of water into the GVL system slowed the lignin depolymerization over the ether bond cleavage. However, only negligible condensation reactions were detected within the aromatic region in the GVL/water system. The model compound study showed that this type of inhibition (e.g., on the formation of dimeric or trimeric structures) is due to the fact that water as an external nucleophile could stabilize the benzyl carbocation-type intermediates, introducing reactive OH groups (**Fig. 7**).

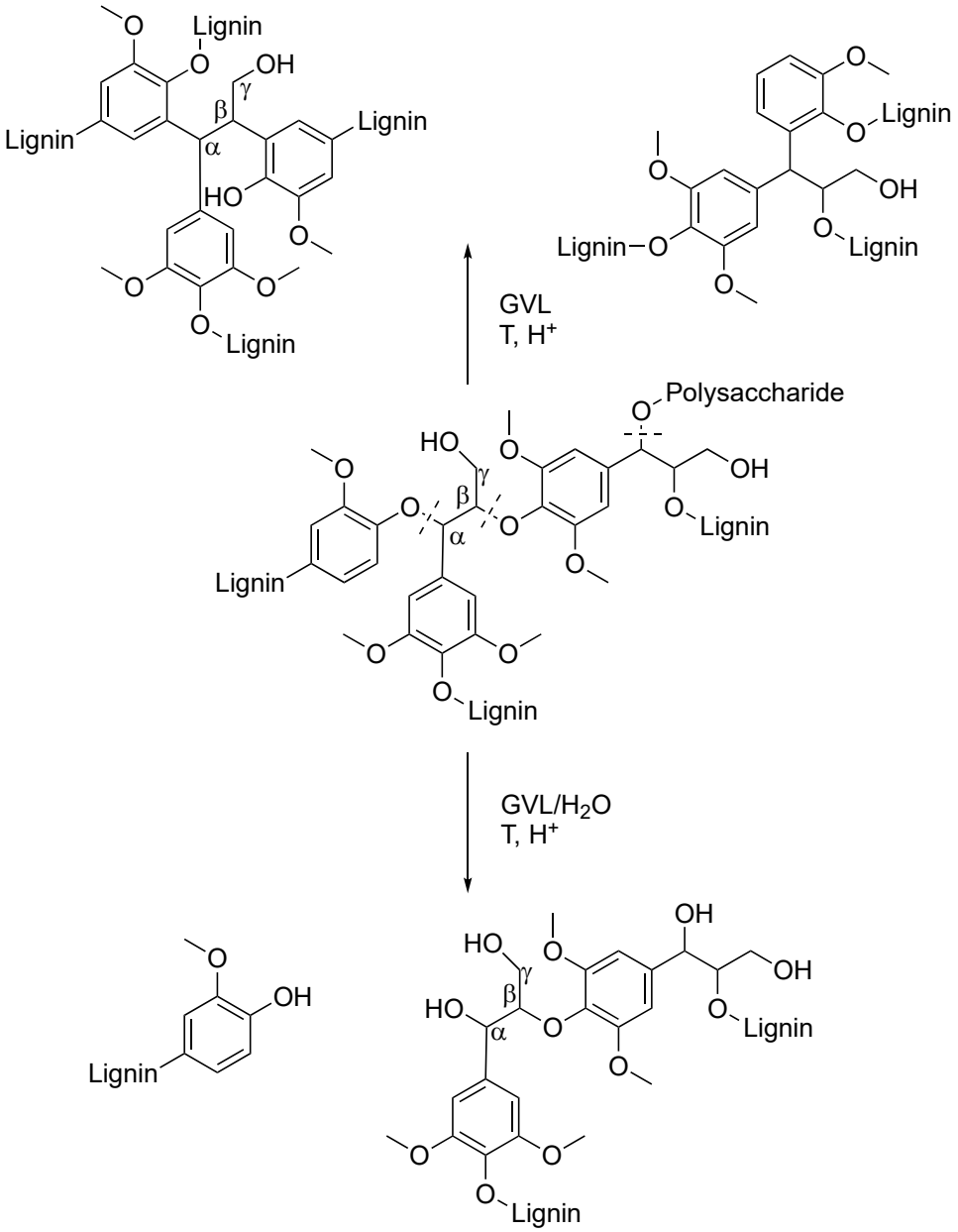

Fig. 7. Mechanism of the α-Ether and β-Ether Bond Cleavage in Lignin during Organosolv Pulping in GVL and GVL/Water (reproduced with copyright permission from ACS Publications) [104].

2.4. Oxidative pretreatment

Oxidative treatment has been recognized as one of the most commonly used technologies for lignin depolymerization and degradation in modern pulping. It has also been proven that oxidative treatment is an effective pretreatment technique to

simultaneously remove hemicellulose and lignin, which can destroy the structural barriers of biomass for improving its enzymatic digestibility. The common oxidants used for biomass pretreatment include hydrogen peroxide [105], ozone [106], oxygen [107], peracetic acid (peroxyacetic acid) [105, 108], sodium chlorite [108, 109], persulfate [110], and in combination (e.g., oxygen-enhanced hydrogen peroxide treatment) [111]. The reaction of lignin under oxidative pretreatment can be classified into three categories: 1) oxidating lignin to aromatic carbonyls or carboxylic acids, 2) oxidating aromatic rings, and 3) oxidation limited to specific groups [112]. Lignin oxidation under hydrogen peroxide pretreatment falls into the first two categories and reacts differently depends on the catalyst (acid or alkaline) as shown in **Fig**. The alkaline used to catalyze hydrogen peroxide pretreatment includes sodium hydroxide [113] and ammonia [114]. The phenolic lignin unit reacts through Dakin-like reaction pathway under alkaline catalyzed hydrogen peroxide pretreatment resulting fragmentation. The α-carbonyls also reacts with hydrogen peroxide under alkaline conditions causing cleavage between C_1-C_α (non-etherified unit) or C_α-C_β (etherified unit). The cleavage of these bonds is expected to facilitate the fragmentation and dissolution of lignin under alkaline conditions. Lignin oxidation undergoes different pathways under acid-catalyzed hydrogen peroxide pretreatment (acetic acid [105] or phosphoric acid [115]). Lignin could be degraded through cleavage of β-aryl ethers (**Fig. 9a**, Route I and II to produce guaiacol and o-benzoquinones, respectively), cleavage of the alkyl-aryl carbon–carbon bond (**Fig. 9a**, Route III to produce guaiacoxy aldehydes), α-hydroxyl oxidation (**Fig. 9a**, Route IV to α-carbonyl group) and oxidative cleavage

of aromatic rings (**Fig. 9b** to dicarboxylic acids) [116, 117]. The hydrogen peroxide pretreatment also can be catalyzed by transition metal ions, such as iron (also known as Fenton reaction) [118].

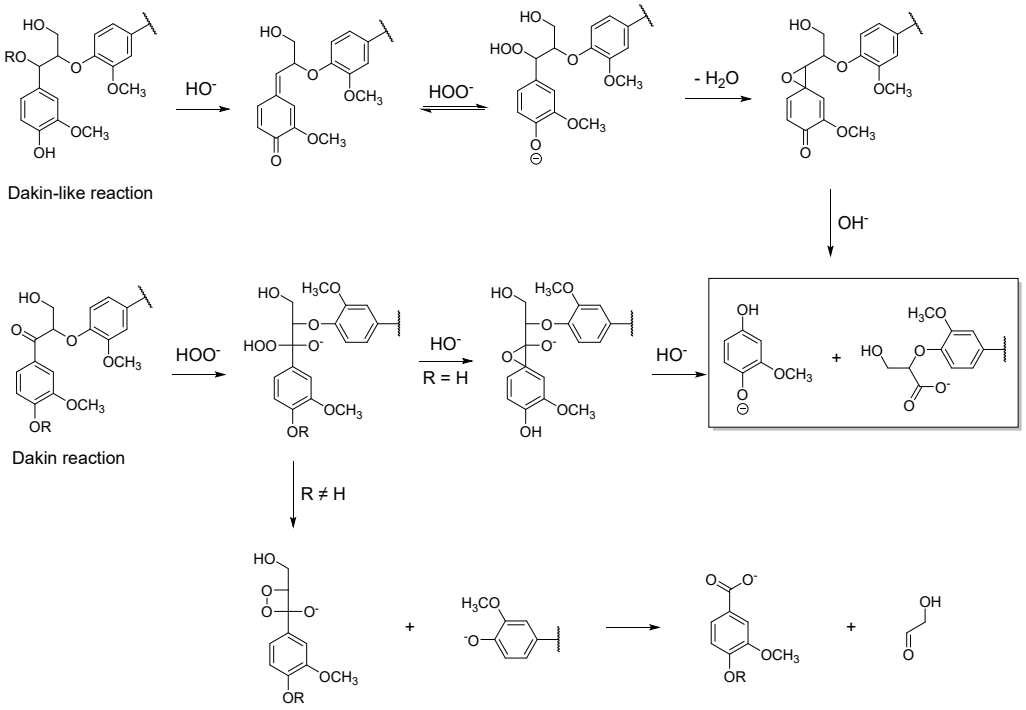

Fig. 8. Reaction mechanism of alkaline catalyzed lignin oxidation by hydrogen peroxide

Ozone is another common oxidant that has been used for oxidative biomass pretreatment. It could oxidize the lignin side chain, causing cleavage of C_1-Cα affording acids. It also could oxidize phenolic end units into muconic acids. Another possible reaction pathway is that ozone direct attacks C_β carbon causing the cleavage of β-aryl ether and lignin fragmentation. Bule et al. characterized the wheat straw lignin before and after ozone pretreatment and confirmed that both cleavage of β-O-4' linkages and ring-opening reaction occurred during the pretreatment [106]. Other types of oxygen-containing groups that are frequently introduced to lignin side chain are ketone and aldehyde, and these groups are expected to impair the π-π interactions among the

phenylpropane units [119]. For a more elaborate discussion on the depolymerization and fragmentation of lignin via oxidative route, the reader is referred to these dedicated reviews [119, 120].

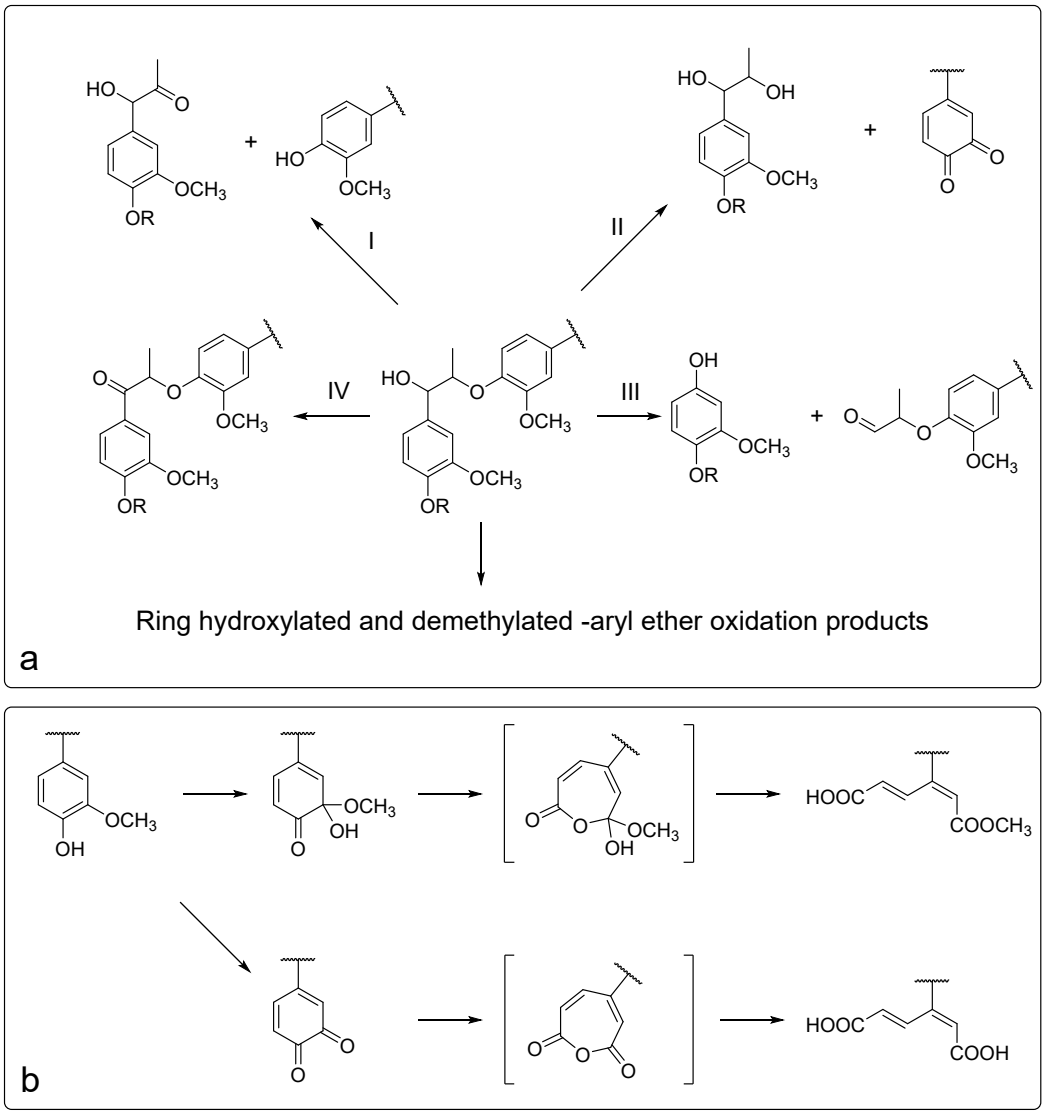

Fig. 9. Reaction pathways of peracetic acid degradation of β-O-4' lignin dimers (a) and oxidation of aromatic ring (b) (reproduced with copyright permission from Elsevier) [116].

2.5. Ionic liquid pretreatment

Ionic liquids (ILs) as emerging promising green pretreatment solvent have drawn great attention, because of their low volatility, flammability, and high chemical stability.

They can selectively or simultaneously dissolve cellulose, hemicellulose, and lignin, therefore IL pretreated lignocellulosic substrates typically have excellent enzymatic digestibility [121]. However, the commercial utilization of IL for biomass pretreatment is hindered due to its high cost, purity requirement as well as biomass loading [122]. Various ILs have been proposed and screened as feasible solvent candidates in pretreatment, and these solvents include but not limited to 1-ethyl-3-methylimidazolium acetate ([EMIM][Ac]) [123, 124], 1-butyl-3 methylimidazolium chloride ([BMIM]Cl) [125, 126], 1-hex-cetylpyridinium chloride ([Hpy][Cl]) [127], and 1,3-dimethylimidazolium methyl phosphonate ([DMIM][MPh]) [128]. The nature of IL anions was reported to have a significant impact on lignin solubility [129] and structure [130], and the hydrogen bonding basicity rather than strength of ILs appears to be a vital factor as well [131]. Although the detailed lignin reaction mechanism in ILs is still not well reported and requires more investigation, it is proposed that the nucleophilic anion of ILs could attack the highly electron deficient protonated ether bonds of lignin under acid conditions [132]. Depends on the pretreatment pH, both lignin degradation and condensation reactions could occur. One of the advantages of some ionic liquids in lignin degradation relates to their abilities to act as both an effective lignin solvent and an acidic catalyst [133]. Torr et al. reported a reduction of β-O-4' linkages in radiata pine lignin after pretreatment with [EMIM][Ac] [134]. NMR study also suggested the cleavage of β-O-4 and condensation of birch lignin after pretreatment within the same ILs system [135]. They also proposed that ILs could promote acidolytic cleavage of aryl ether linkages. Sathitsuksanoh et al. investigated

the structural change of lignins from wheat straw, miscanthus, and loblolly pine during the pretreatment with [EMIM][Ac] and found that the content of lignin native linkages (e.g., β-O-4, β-5, and β-β) decreased with the increasing pretreatment temperature [136]. This type of lignin structural transformation showed a positive effect on the enzymatic hydrolysis of the pretreated biomass. Interestingly, the β-O-4 linkage's quantity was higher in the residual lignin, whereas the content of β-5 and β-β was higher in the isolated lignin (solubilized lignin during pretreatment) [136]. Although the lignin reaction mechanism during IL pretreatment is still not clear and strongly depends on the nature of solvent system and pH, it is reasonable to speculate that it should be similar to that proposed under typical acid or alkaline-catalyzed conditions. For example, model compound studies confirmed the formation of Hibbert's ketone, lignin self-condensation, and acid-catalyzed dealkylation during IL pretreatment [137]. A more detailed investigation is still needed to have a fundamental understanding of the exact mechanisms of lignin reaction in different ILs.

2.6. Physico-chemical pretreatment

Physico-chemical pretreatment, using heat and mechanical energy, can also deconstruct lignin for improving the enzymatic conversion of biomass. The common physico-chemical pretreatment includes steam explosion, ammonia fiber expansion (AFEX) [138, 139], and CO_2 explosion [140]. Among which, the steam explosion might be the most cost-effective one since the high energy demand can be allocated from waste energy [141-143]. Lignin reactions during the steam explosion and CO_2 explosion are expected to include acid-catalyzed degradation and condensation type of

reactions. Whereas the ammonia fiber expansion only leads to a mild lignin degradation with the basic lignin structure remaining intact except to the formation of acetamide or phenolic amide due to the ammonolytic cleavage of ester and/or ether linkage [144, 145]. The AFEX pretreatment transforms lignin into fragments that be extracted by organic solvents. Hence, it has been considered as an effective pretreatment to improve enzymatic hydrolysis of different biomass feedstocks, achieving a good cellulose conversion yield of 60-90%. For example, Sousa et al investigated the cell wall deconstruction during AFEX pretreatment and concluded that the cleavage of esters affording amides is a major cell-wall-disrupting action, results in a good enzymatic digestibility of corn stover even with low enzyme loading and high solids loading [146].

2.7. Other types of pretreatments

Besides the aforementioned methods, other types of pretreatment techniques also have been employed to reduce the recalcitrance of biomass for its enzymatic conversion. These techniques include but not limited to deep eutectic solvent pretreatment [147], ultrasonic pretreatment [148], electron beam irradiation [149, 150], γ-irradiation pretreatment [151], pressure shock waves pretreatment [152, 153], and combination of aforementioned pretreatments. Torrefaction represents another promising thermochemical process for solid bioenergy production (e.g., biomass-derived biochar), and it is typically performed between 200 and 300 ^{o}C under inert atmosphere [154-157]. Although liquid fuel (e.g., bioethanol and bio-oil) are typically not major products from the traditional torrefaction process, a recent study by Yu et al. did show that microalgal could be converted into bioethanol and biochar simultaneously via a

one-pot microwave/acid-assisted wet torrefaction technology at low temperatures (~160 °C) [158]. Even these pretreatments have been proven to be effective in facilitating the enzymatic hydrolysis, however, the lignin physicochemical properties (e.g., monolignol composition, surface charges, molecular weight, content of hydroxyl and carboxylic groups, degree of condensation, etc.) still require more detailed characterization.

In conclusion, high costs associated with biomass pretreatment and enzymatic hydrolysis are major challenges for an economically viable bioconversion of biomass to ethanol. It has been reported that the minimum ethanol selling price (MESP) strongly depends on biomass pretreatment [11]. Dilute acid pretreatment and hot water pretreatment are considered two of the most viable pretreatment techniques to commercialize bioethanol in the short term. Techno-economic analysis of different pretreatment processes for bioethanol production showed that liquid hot water pretreatment had the best values for the MESP around $1.78/L, while the MESP of ethanol produced via ammonia fiber expansion (AFEX) could reach as high as $5.76/L [159]. Thus understand the structure change of lignin during biomass pretreatment and how it impacts the enzymatic hydrolysis is essential to reduce the overall cost of bioconversion process.

2.8. Effects of pretreatment on lignin and their impacts on enzymatic hydrolysis

As summarized above, lignin structural change during pretreatment can be generally classified into degradation and condensation. These changes include cleavage of native lignin ether linkages, elimination of aliphatic hydroxyls, alternation of S/G

ratio accompanying with the formation of phenolic hydroxyls, carboxylic acids, and new carbon-carbon bonds [121, 144]. The solubilized lignin during pretreatment has minimal impact on enzymatic hydrolysis assuming they are not precipitated back on the surface of biomass, whereas the residual lignin in the pretreated biomass with various features plays an important role in governing the rate and extent of enzymatic hydrolysis [160]. Meanwhile, a complete removal and degradation of lignin during pretreatment may not always necessary to achieve high biomass digestibility [161]. In fact, a near-complete removal of lignin sometimes could cause cellulose aggregation which ultimately decreased cellulose accessibility and digestibility [162, 163]. Significant removal of lignin by various thermal chemical pretreatments typically required severe reaction conditions, which could result in unnecessary loss of carbohydrate and condensation of lignin fragments. From these perspectives, rather than achieving a complete removal of lignin, the structure changes and location of the lignin left in the pretreated solid residue are particularly important. Taking organosolv and ammonia pretreatments for example, the lignin structure left in the pretreated materials could be significantly different from the native lignin and the solubilized lignin in the liquid phase. Yoo et al. found that organosolv pretreatment caused dramatic depolymerization of poplar lignin and increased the content of condensed S and G units as well as the phenolic hydroxyl groups, which increased the maximum adsorption capacity of lignin toward cellulases compared to native [164]. For hydrothermal and dilute acid pretreatment, it was also reported that the increase in the combined severity factor of pretreatment correlated well with the increased inhibition on enzymatic

hydrolysis of Avicel [165]. This is mainly due to the increase of free phenolic hydroxyls and condensed aromatic structures in lignin residues as pretreatment severity increased, which subsequently increased the hydrogen bonding and hydrophobic interactions between lignin and cellulases [166]. Steam explosion and hydrothermal pretreatment also changed the lignin structure leading to increased cellulase adsorption [165, 167]. A strong association of condensed phenolic moieties in lignin with their inhibition of enzymatic hydrolysis was also reported [166]. To better view the effect of lignin's characteristics on enzymatic hydrolysis, the residual lignin characteristics from the aforementioned pretreatments and their impact on enzymatic hydrolysis are summarized in Table 1. It is worth mentioning that increasing evidence has started to show lignin with certain physicochemical properties or from certain location of biomass surface could enhance the enzymatic hydrolysis process [41-43]. To better understand how these lignin substrates positively impact the enzymatic hydrolysis, the molecular-level driving forces in lignin-enzymes interaction need to be fully understood.

Table 1 Effects of pretreatment on residual lignin structure and their impact on enzymatic hydrolysis

Pretreatment	Biomass	Lignin description	Substrate of Enzymatic hydrolysis	Impact on Enzymatic hydrolysis	Reference
Acid – 80% acetic acid with 0.3% sulfuric acid	sugarcane bagasse	β-O-4 (decrease) Phenolic OH (decrease) Aliphatic OH (decrease)	micro-crystal cellulose	Negative to negligible	[168]
Dilute sulfuric acid sodium hydroxide Hydrothermal	sugarcane bagasse	S/G ratio (decrease) Phenolic OH (decrease/increase) Hydropholicity (decrease /increase)	Avicel	Negative	[169]
ammonia fiber expansion (AFEX)	Switchgrass	S/G ratio (increase)	Avicel	Negative	[138]
Hydrothermal / Dilute acid	Spruce / Wheat straw	β-O-4 (decrease) β-5’ (increase)	Avicel	Negative	[165]
Hydrothermal	Bamboo	S/G ratio (decrease) Hydrophobicity (increase) Phenolic OH (increase)	Avicel	Negative	[170]
Organosolv-ethanol	Loblolly Pine and Sweetgum	Residue lignin content	Pretreated Loblolly Pine and Sweetgum	Negative	[95]
Organolsolv-ethanol	Loblolly Pine and Sweetgum	Alkylation of side chain	Avicel	Positive	[101]
Organolsolv - ethanol	Bamboo	β-O-4 (decrease) Carboxylic (increase) Hydropholicity (increase)	Avicel	Positive	[171]
Fenton oxidation	Bamboo	Surface negative charges (increase) β-O-4 (decrease) S/G ratio (decrease) Phenolic OH (decrease)	Avicel	Positive	[172, 173]
Ionic liquid	Rice straw	Phenolic OH (increase) Carboxylic (increase)	Microcrystalline cellulose	Negative	[174]

3. Mechanism of lignin inhibition on enzymatic hydrolysis

The residual lignin, dissolved lignin (to a lesser degree), and lignin-derived phenolic molecules in the pretreated biomass can all trigger the decrease in stability and activity of cellulases for the enzymatic hydrolysis process, as well as the unfavorable enzyme recyclability, thus reducing the effective enzyme concentration for hydrolysis [175]. Dissolved lignin can precipitate back on the surface of biomass as droplets, making the biomass less accessible for enzymes to cellulose [176]. During enzymatic hydrolysis, the lignin will produce non-productive adsorption with cellulase through hydrophobic, electrostatic, and hydrogen bonding interactions, causing a large amount of cellulase loses its activity, as shown in **Fig. 10** [177]. The mechanism of lignin inhibition on enzymatic hydrolysis will be reviewed in this section.

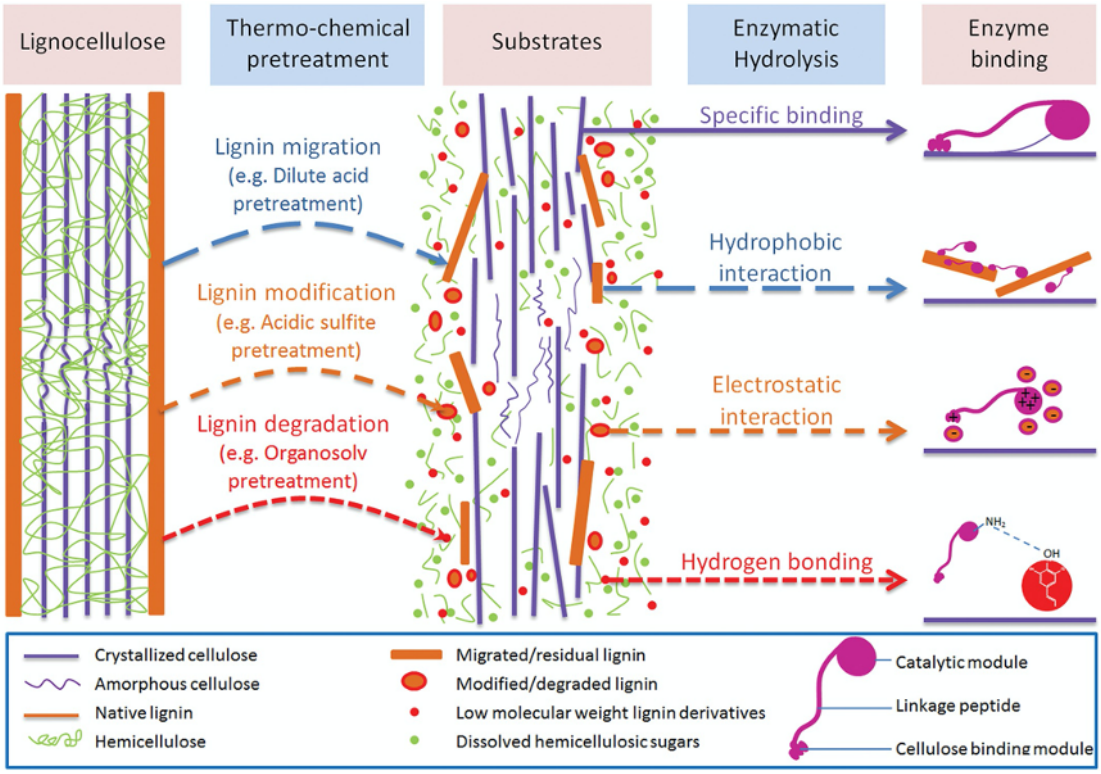

Fig. 10. Schematic illustration of cellulase-lignin interactions (reproduced with copyright permission from Wiley Company) [177]

3.1. Steric hindrance to hinder reaction between cellulose and cellulase

During the biomass pretreatment process, lignin can be redistributed back to the surface of biomass [176], and even the solubilized lignin can repolymerized to form droplets [178]. These actions could facilitate enzyme adsorption onto the non-carbohydrate fraction and impair the hydrolysis of cellulosic substrates. Chandra et al. found that the degree of enzymatic hydrolysis of cellulose increased with the increase of the steam pretreatment severity, which is believed to be due to that the reduced distribution of lignin in the cell wall of lignocellulosic materials and further increased the accessible surface area of cellulose to enzymes [179]. Donaldson et al. showed that the effect of alkali pretreatment on the redistribution of lignin provides negative effect for cellulose hydrolysis, because the lignin was transferred from the fiber wall to the fiber surface, which causes a physical obstacle to the binding of cellulase and cellulose [180]. In the cell wall of biomass, the lignin in lignocellulosic biomass is covalently linked with the carbohydrate (mainly hemicellulose) to form the lignin-carbohydrate complex (LCC), which can also show physically prevent cellulase for enzymatic hydrolysis [181] [182]. For example, Huang et al., reported that degrading the remained LCCs in the pretreated bamboo by enzymes could improve its enzymatic digestibility, as the enzymes can disrupt the chemical bonds between lignin and carbohydrates to eliminate the steric hindrance of lignin for cellulose [183]. In addition, a large amount of lignin residue could inhibit the swelling of lignocellulose if the degree of

pretreatment is not sufficient, limiting the increase of specific surface area of lignocellulosic substrates and subsequently the cellulose accessibility [184, 185]. To eliminate the physical hindrance of lignin for enzymatic hydrolysis, various technologies have been carried out to reduce the content of lignin during pretreatment process or remove the surface lignin by post-extraction, which is aimed to increase the pore size and volume of the voids and the accessible surface area of pretreated lignocellulosic biomass for improving the final hydrolysis efficiency [162].

3.2. Non-productive adsorption between lignin and cellulase

As shown in **Fig. 10**, three main non-covalent interactions between cellulase and lignin have been proposed, namely hydrophobic adsorption, electrostatic adsorption, and hydrogen bonding adsorption [186, 187]. The relative contributions of these individual interactions on the final non-productive adsorption behavior between lignin and enzymes are still under debate. Hence, it is necessary to further update the carried-out work to recognize these theories.

3.2.1. Hydrophobic adsorption theory

Lignin contains lots of hydrophilic hydroxyl groups, but the three-dimensional aromatic structure and the widely distributed methoxy groups makes lignin naturally hydrophobic. On the other hand, cellulase contained hydrophobic amino acids residues from tryptophan, phenylalanine, and tyrosine. Therefore, hydrophobicity has been proposed as one of the major driving forces governing the non-productive adsorption between lignin and cellulase [186].

Recently, various advanced analytical techniques have been proposed to identify

the hydrophobic adsorption between lignin and enzymes during enzymatic hydrolysis process [188-191]. Qin et al. immobilized cellulase on a silicon chip and used atomic force microscopy (AFM) to measure the force involved in the non-productive binding of cellulase and lignin. The test results show that the adhesion force between cellulase and sulfate lignin is ~45% higher than that between cellulase and hydroxypropyl cellulose, and the interactions between hydrophobic probes and cellulase are 43% and 13% higher than those of probes with -OH and COOH groups, respectively [189]. Fritz et al. used quartz crystal microgravimetry (QCM) along with an AFM technique to assess the interactions between enzymes and different lignin films and indicated that hydrophobic interactions play an essential role in the lignin affinity to enzymes [190]. *Börjesson* et al. pointed out that compared with the exocellulase (*Tr*Cel7A), the endocellulase (*Tr*Cel7B) is easier to bind to the separated lignin due to its strong hydrophobicity, but the catalytic domains of the two enzymes have similar binding degrees to lignin [192]. Lignin has stronger hydrophobicity than cellulose, so according to the hydrophobic interaction theory, cellulase is more likely to bind to lignin. Research by Lu et al. showed that the combination of cellulase and lignin due to the hydrophobic interaction leads to a decrease in enzyme activity, which is also related to the components of cellulase [193]. During dilute acid and liquid hot water (especially at high pretreatment severity) pretreatments, extensive degradation of carbohydrate could lead to the formation of pseudo lignin (lignin-like aromatic material), which precipitated on the surface of the pretreated biomass causing an even more detrimental effect on the enzyme hydrolysis of cellulose than real lignin substrate [194]. Hu et al.

suggested that the hydrophobic nature of pseudo lignin was probably responsible for its non-productive interaction with the cellulase enzymes, resulting in the inhibitory effect on enzymatic hydrolysis of cellulose [194]. Hence, it can be known that both residues lignin and new-formed pseudo lignin in the biomass after acid pretreatment can bind with the cellulase via hydrophobic interaction and further negatively affect the enzymatic hydrolysis of cellulose. It was also showed that soy bean protein exhibited a lower extent of adsorption on pure synthetic hydrophobic surface (e.g., self-assembled monolayers of dodecanethiol) than on lignin substrate, suggesting interaction originated from lignin other than hydrophobic could play some roles [195]. Similarly, Kellock et al. also reported that the surface hydrophobicity of cellulase enzymes could not alone explain the adsorption behavior of various enzymes toward lignins isolated from steam pretreated spruce and wheat straw [196]. Factors such as electrostatic force and hydrogen bonding need to be considered as well.

3.2.2. Electrostatic adsorption theory

There are multiple amino acid residues on the structure of enzymes, and their electrical properties are related to pH of the environment [197]. The residual lignin in the pretreated lignocellulosic biomass also contains functional groups such as carboxyl, phenolic/aliphatic hydroxyl, and carboxylic acid [100]. The association and dissociation of functional groups of lignin (carboxyl and hydroxyl groups) and enzyme (amino acid residues) in an aqueous environment can lead to potential electrostatic interactions between them [24, 198]. Hence, the interaction between lignin and enzyme via electrostatic adsorption is highly pH-dependent.

According to the difference of adjacent substituent groups, the ionization pH of lignin phenol hydroxyl is between 6.2 and 11.3 [199]. The cellulase enzymes (e.g., endoglucanases and cellobiohydrolase) have their own unique isoelectric point (pI) with pH ranging from 4.3 to 6.2 [200]. Enzymatic hydrolysis experiments of lignocellulosic biomass are typically carried out at a pH around 5, thus these different types of enzymes could be either positively or negatively charged. Lignin contains carboxyl group and the sulfonic acid groups that are in ionized states, therefore can form an electrostatic attraction with the positively charged groups of the enzymes. Nakagame et al. [201] and Lou et al. [202] have confirmed that the pH of the solution had an important influence on the interaction between lignin and cellulase, which further affected the enzymatic hydrolysis of cellulose. At a pH value of 6.0, the adsorption between lignin and cellulase in the substrate is relatively weak, which might be attributed to the mutual electrostatic repulsive forces [187, 203]. In addition, different pretreatment technologies can change or adjust the charge of the chemical group of the substrate lignin molecule. Fritz et al. [190] compared the effect of cellulolytic enzyme lignins (CEL) [204] and kraft lignin on the lignin-cellulase adsorptions, and found that the electrostatic interaction between cellulases (both Cellic CTec 2 and CBH I from *Trichoderma reesei*) and CEL was more significant than that between cellulase and other types of lignin. However, the contributions of the electrostatic forces to protein binding are not the same in all cases. For example, Cel7A strongly binds to lignin under a pH that is higher than its pI, which indicates that the Coulombic repulsion can be overcome by the hydrophobic interactions [188, 190, 205].

This has been proved by the measurement of attractive force between cellulase and functional groups using AFM instrument and QCM adsorption studies [24].

3.2.3. Hydrogen bond adsorption theory

Functional groups on lignin surfaces (especially hydroxyls and carboxylic acid groups) contain hydrogen atoms that could interact with enzymes through hydrogen bonding, thus cannot be ignored in lignin-enzyme interactions. It has been hypothesized and confirmed to some extent that phenolic hydroxyl groups of lignin were mainly responsible for enzyme adsorption [167], as evidenced by the fact that hydroxypropylation of phenolic sites could mitigate the inhibition of lignin on cellulose hydrolysis [206]. Yang et al. used the isolated organic solvent lignin from poplar and pine to study the inhibitory effect of lignin on cellulase in the hydrolysis process and found that phenolic hydroxyl is the key factor affecting the inhibitory effect of lignin via hydrogen bond adsorption. Qin et al. used AFM instrument to measure the forces involved in the non-productive binding of cellulase and lignin, and confirmed that hydrogen bonding can promote the binding of cellulase and lignin, although it is not the dominant force [205].

To study the hydrogen bonding adsorption between lignin and enzyme, different technologies have been developed and utilized. For example, blocking free phenolic hydroxyl groups through chemical reactions can significantly reduce the hydrogen bond adsorption between lignin and enzyme [207]. In addition, surfactants, such as Tween 80 and polyethylene glycol (PEG) that could form hydrogen bonds with phenolic groups of lignin, can prevent lignin-enzyme adsorption and further enhance the

enzymatic hydrolysis of cellulose by improving the amount of free enzymes in the enzymatic hydrolysis system [208]. Unfortunately, the relative contributions of the hydrogen bonding in lignin-enzyme interactions remain uncertain to some extent due to the paucity of research as well as the interactive effects of individual factors. Therefore, extra study needs to be performed to clarify the effect of these individual factors on lignin-enzyme interactions.

4. Technology to minimize the adverse impact of lignin on the enzymatic hydrolysis

To overcome the adverse impact of lignin on enzymatic hydrolysis, many technologies have been carried out, such as lignin modification by biomass pretreatment, blocking the nonproductive binding by additives, and post-treatment to remove or modify lignin. A brief overview of these strategies is provided in this section.

4.1. Modification of lignin during pretreatment

How to develop new or optimize current pretreatment technologies with reduced lignin content or modified lignin properties remains a great challenge for the production of cost-competitive biofuels. It has been proposed that using specific solvent in the pretreatment can modify the lignin to improve the enzymatic hydrolysis of biomass. For example, it is suggested that a deacetylation step (i.e., via alkaline extraction) prior to typical acid pretreatment is capable of reducing the generation of acetic acid, which has the potential to reduce the extent of lignin condensation and formation of phenolic hydroxyl groups for improving the enzymatic hydrolysis [209]. For the sulfite pretreatment, the sulfite in pretreatment system can sulfonate the lignin in biomass into

lignosulfonate (LS), which can reduce the nonproductive lignin-enzyme interactions to improve the amount of free enzyme to hydrolysis cellulose. A novel process using sulfite pretreatment to overcome recalcitrance of lignocellulose (SPORL) to improve the bioconversion of softwoods was proposed, and a schematic flow diagram of this process is shown in **Fig. 11** [210]. In brief, biomass wood chips were firstly pretreated in the aqueous sulfite solution. After appropriate size reduction via disk refining, the pretreated biomass was then washed with DI water and subjected to enzymatic hydrolysis and fermentation. After the SPORL pretreatment, more than 90% of cellulose from pretreated spruce chips can be converted to glucose during enzymatic hydrolysis with low enzyme loading, which is mainly due to the sulfonation of lignin into lignosulphonate. In addition, the pretreatment hydrolysate could be collected to recover the hemicellulose and lignosulfonate. Wu et al. [151] also reported that alkaline sulfonation pretreatment can predominantly occur within the secondary-cell-wall lignin and endow the residual lignin in pretreated softwood with high content of sulfonic acid group, which can increase cellulose accessibility to the enzymes for enzymatic hydrolysis.

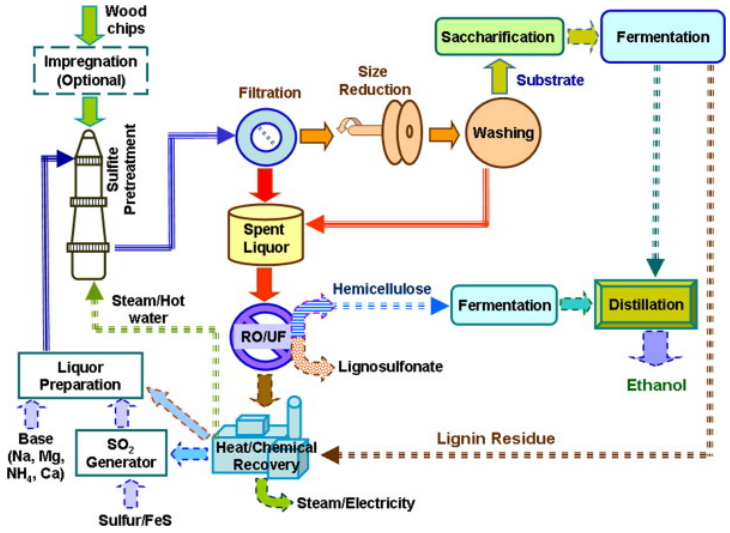

Fig. 11. Schematic process of the sulfite pretreatment to overcome recalcitrance of lignocellulose (reproduced with copyright permission from Elsevier) [210].

Changing the surface chargers of lignin by incorporating certain chemical groups into lignin during biomass pretreatment is another way to intervene the lignin-enzyme adsorption by affecting the electrostatic interaction. For example, Lai et al. reported that the addition of 2-naphthol-7-sulfonate as a carbocation scavenger during dilute acid pretreatment could improve the enzymatic hydrolysis of pretreated mixed wood sawdust by altering the surface charges and limit the condensation of lignin [211]. During that pretreatment, sulfonyl groups were incorporated into the lignin and result in an increase of negative surface charges and hydrophilicity of the pretreated substrate. These actions suppressed the cellulase-lignin interaction, as a result, the enzymatic hydrolysis yield was increased. The unproductively binding of lignin to enzymes could be also suppressed by changing the hydrophobicity of residual lignin and adding surfactant during biomass pretreatment. In situ lignin modification by PEG during alkaline pretreatment was reported to increase the enzymatic saccharification due to its ability to reduce the non-productive binding of enzymes [212]. Lignin modification

could be also done by laccase-mediator treatments (LMT) to increase its hydrophilic property. Zwan et al. showed that laccase-mediated hydrophilization of steam pretreated lignin via in situ phenolic compound grafting could decrease the unfavorable enzyme binding [213]. Lignin and cellulose oxidation caused by LMT reduced the binding of cellulases on lignin and consequently enhanced the cellulose digestibility [214]. Lignin sulfomethylation represents another way to increase the hydrophilicity of residual lignin. Ying et al. proposed a sequential Fenton oxidation and sulfomethylation pretreatment for facilitating the introduction of the sulfomethyl group on the lignin aromatic ring, which simultaneously accelerated the delignification and increased the hydrophilicity of lignin [215]. Besides controlling the content of phenolic hydroxyls in lignin, increasing the content of carboxylic acid or aliphatic hydroxyls in lignin could also decrease the surface hydrophobicity of lignin thus alleviating the non-productive binding [216]. Dong et al. discovered a new type of diol pretreatment that could introduce a hydroxyl tail at the α position of the lignin side chain thus increases its solubility [91]. Compared to conventional monohydric alcohol pretreated lignin, diol pretreated lignin had a higher amount of aliphatic OH and a lower amount of phenolic OH, thus had a lower cellulase binding strength and adsorption affinity. Recently, a new type of hydrotropic fractionation biomass pretreatment was developed in USDA using maleic acid (MA) as an acid hydrotropic [217, 218]. NMR analysis suggested that MA carboxylated the lignin in the pretreated residue, which dramatically enhanced the digestibility of MA pretreated materials by reducing the lignin-cellulase interaction through pH-mediated electrostatic repulsion. Lignin isolated from pretreated softwood

is generally considered more detrimental to cellulase performance than that isolated from pretreated grasses [219, 220]. This is mainly because the branched G type of lignin has a higher affinity for the cellulase enzymes, particularly β-glucosidase, than linear S lignin [221]. As a result, modifying the S/G ratio of lignin or selectively degrading G type of lignin via pretreatment represents another way to alleviate the cellulase-lignin binding. For example, Kim et al. showed that oxalic acid-catalyzed DA pretreated hardwood poplar had a higher content of G-type of lignin, therefore had lower enzymic hydrolysis yield (~83 – 88%) than that of the sulfuric acid pretreated one (~94 – 96%) [52]. Yoo et al. showed that *Populus* wood sample with higher lignin S/G ratio had higher bioethanol yield after enzymatic hydrolysis and fermentation [222]. Several pretreatments include dilute acid [223], ionic liquid [224], and laccase treatment [225] have all shown the ability to increase the S/G ratio in lignin, thus could be used to alleviate the lignin-enzyme binding.

4.2. Adding lignin blockers to reducing adsorption between lignin and cellulase

Besides altering the physicochemical properties of lignin in the plant cell wall via thermo-chemical pretreatment, adding "lignin blockers" during enzymatic hydrolysis represents another effective way to minimize the adverse impact of lignin on enzymatic hydrolysis. This has become a topic of great interest due to its operational feasibility. Effective adsorption of additives onto the surface of lignin left in the pretreated lignocellulosic substrate could reduce the nonproductive binding of cellulase or xylanase to lignin [226]. Up to date, additives including metal ions, peptides, proteins, and various types of surfactants/polymers have been used to promote the enzymatic

hydrolysis by reducing the nonproductive adsorption of cellulase on lignin.

Bovine Serum Albumin (BSA), a non-enzymatic protein, is widely known for its ability to bind to lignin via hydrophobic interaction due to its large hydrophobic patches. Hence, it could be used as a lignin blocker during enzymatic hydrolysis, ultimately leading to an increased amount of free enzyme activity [227]. In a recent study, hydrogen bonding was also suggested as the strongest binding force between lignophenols and BSA as compared with the traditionally recognized hydrophobic and electrostatic interactions [228]. Regardless of the interactions, BSA treatment can significantly reduce the adsorption of cellulase and particularly β-glucosidase on lignin [229]. Several studies also suggested that BSA addition could improve the sugar release of biomass with high lignin content, even with reduced cellulase loadings during enzymatic hydrolysis. For example, the addition of BSA during the enzymatic hydrolysis of liquid hot water (LHW) pretreated mixed hardwood significantly reduced the amount of cellulase in order to achieve an 80% of cellulose conversion [230]. The beneficial effect of BSA on enzymatic hydrolysis also depends on the type of pretreatment and nature of the pretreated substrates. It was reported that BSA had a larger effect on enzymatic hydrolysis of flowthrough dilute acid pretreated biomass than batch LHW pretreated substrate [231]. This is probably attributed to the differences in hydrophobicity of the lignin residue in different pretreated biomass. Because lignin can physically block the accessible surface area of reactive cellulose as well as reduce enzyme non-productive binding, the relative contribution of these two different roles could be estimated based on the differences between the overall lignin

inhibition and the inhibition of enzyme binding which could be assessed by a lignin removal and BSA addition, respectively [232]. Jia et al. concluded that physical blocking of cellulose accessibility rather than lignin-induced enzyme binding appeared to play a more detrimental role toward limiting the enzymatic hydrolysis rate of acid pretreated softwood [232]. Nevertheless, the relative contributions of these two factors are still under debate and need to be analyzed in further study. In summary, the addition of non-enzymatic protein such as BSA offered an alternative to high-severity pretreatment for achieving reasonable sugar release. Instead of performing severe pretreatment which requires intensive energy input and adding cost to an already expensive conversion process, a mild pretreatment could be utilized along with addition of non-enzymatic protein like BSA for enhancing the glucose and xylose yield (**Fig. 12**).

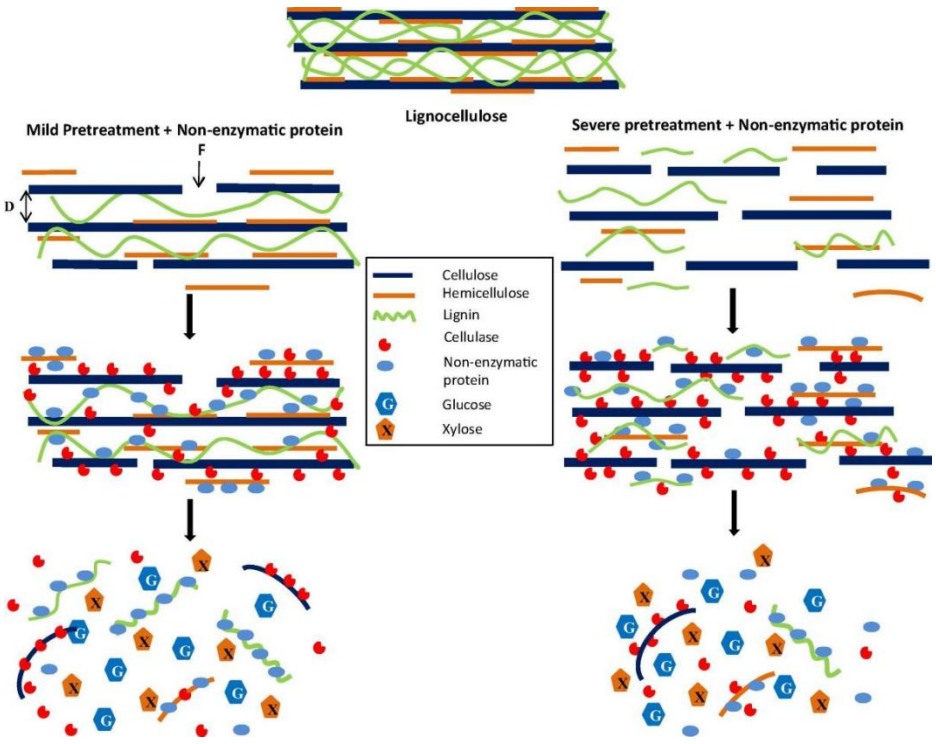

Fig. 12. The proposed model of non-enzymatic protein for improving enzymatic

hydrolysis of pretreated biomass (D: distance; F: fracture) (reproduced with copyright permission from Elsevier) [233].

Non-ionic surfactants, such as Tween and PEG, can also increase the hydrolysis efficiency by preventing non-productive binding of cellulase on lignin [234]. They also do not affect the charge of the hydrolysis system, which could prevent cellulase flocculation that might be caused by electrostatic interactions. The effects of non-ionic surfactants on enzymatic hydrolysis have been elucidated in several studies, and results indicated that surfactants were able to influence both the adsorption and desorption behavior of cellulase on lignin or biomass [208]. Li et al. reported that the addition of Tween 80 could enhance the desorption of cellulases from lignin because of the competitive adsorption between cellulase and Tween 80 on lignin and biomass [235]. In addition, Tween 80 could also alleviate the adsorption of cellulase on biomass surface due to the occupation of surfactant on the hydrophobic surface of lignin left in the biomass. For example, Agrawal et al. evaluated 21 commercial surfactants in terms of their ability to enhance the enzymatic hydrolysis of pretreated wheat straw and found that the saccharification yield of steam exploded wheat straw could be increased by 55% with the addition of 0.5% (w/w) of surfactant EOPO5 [236] [237]. Just like the interaction between lignin and enzymes, the interaction between surfactant tail and lignin is also believed to be hydrophobic, ultimately causing steric repulsion of cellulase from the lignin surface that favors the enzymatic hydrolysis process [192, 238, 239]. Olsen et al. used tannic acid (TAN) as a polyphenolic model compound to represent lignin and measured the interactions between different types of cellulases, TAN, and

surfactant Triton X-100 [240]. Their results supported the hypothesis that TAN-lignin type of interaction was indeed disrupted by the presence of surfactant by a mechanism of stronger TAN-Triton interaction. The hydrogen bonding ability and surface charges of lignin could be also altered by the addition of surfactants [241]. However, the exact mechanism of these nonionic surfactants accelerating the enzymatic hydrolysis is still under debate in recent years. While major of literature appeared to suggest that the improvement of nonionic surfactant on enzymatic hydrolysis was mainly due to the hindrance of the surfactant on the cellulase adsorption onto lignin [192, 242] [243]. However, the lignocellulosics' structure, enzymatic hydrolysis condition, and types of cellulase are all believed to affect the beneficial action of these surfactants. For example, the surfactant of Tween 20 and 80 at high concentration greatly facilitated the hydrolysis of dilute acid pretreated biomass, while showed notable inhibition to pure cellulose conversion especially in the late phase of hydrolysis [244]. Lou et al. also showed that PEG was not capable of improving the enzymatic hydrolysis of Avicel at a low agitation rate but could significantly facilitate the enzymatic hydrolysis of Avicel at a high agitation rate [245]. Similarly, Bhagia et al. showed that amphiphilic additives such as BSA and Tween 20 only had prominent effects on enzymatic hydrolysis performed in flasks that were shaken [246]. This suggested that the addition of surfactants could protect cellulase against the shear-induced deactivation at the air-liquid interface (**Fig. 13**).

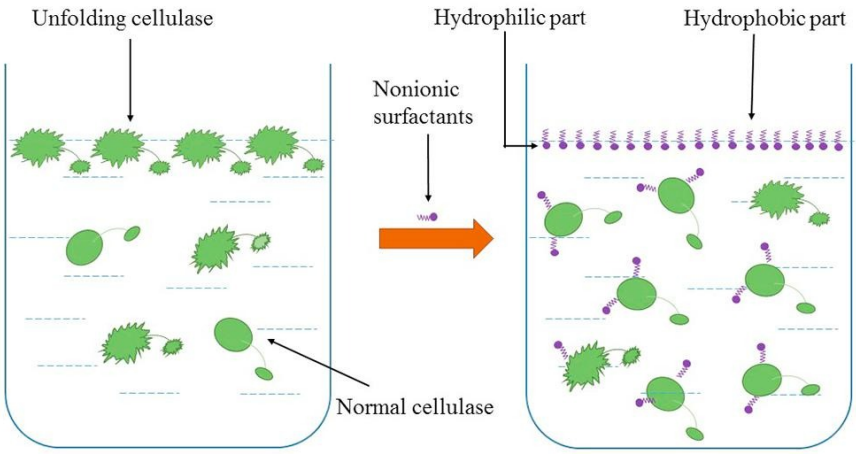

Fig. 13. A mechanism of nonionic surfactants on protecting cellulase against the shear induced deactivation at the liquid-air interface (reproduced with copyright permission from Elsevier) [245].

Lignin itself could be also incorporated into surfactants to form lignin-based surfactant that can be used to promote the enzymatic hydrolysis of lignocellulosic biomass. Lai et al. synthesized a lignin-based surfactant by grafting poly(ethylene glycol) diglycidyl ether on lignin and evaluated its effect on enzymatic hydrolysis [247]. Results showed that the binding rates of the modified PEG on lignin surfaces were dramatically increased due to the incorporation of hydrophobic lignin moieties onto the surfactants. In addition, the modified lignin-based PEG could also disperse cellulase aggregates, thus weakening the binding strength between lignin and cellulase [248]. Consequently, the lignin-based surfactant exhibited better performance in terms of promoting enzymatic hydrolysis than unmodified PEG (**Fig. 14a**). The isoelectric point of the lignin-based surfactant was also reported to be an important factor affecting its effectiveness on enzymatic hydrolysis [249]. Cai et al. synthesized a pH-responsive lignin-based polymer (EHL-MPEG) by grafting monomethoxy polyethylene glycol

(MPEG) onto enzymatic hydrolysis lignin (EHL) and found that the enzymatic hydrolysis yield of pretreated eucalyptus could be increased to >90% with the addition of appropriated amount of EHL-MPEG [250]. In addition, Li et al. grafted phosphobetaine on the enzymatic hydrolysis lignin to prepare the lignin-based surfactant of enzymatic hydrolysis lignin-grafted phosphobetaine (EHLPB) [251]. They found that EHLPB showed a pH-sensitive response with a good ability to improve enzymatic hydrolysis of sulfite pretreated eucalyptus and corncob residue with the maximum degree of 38% and 19%, respectively, which is due to the EHLPB's ability to reduce the nonproductive adsorption of cellulase on lignin (**Fig. 14b**). In another study, a pH-responsive lignin-based amphoteric surfactant was synthesized via quaternization of lignin aiming to simultaneously improve the enzymatic hydrolysis and recycle the surfactant after the enzymatic hydrolysis. The addition of this recyclable lignin-based surfactant at 2 g/L could increase the digestibility of pretreated corncob residues and eucalyptus from 37.8 and 36.7% to 90.5 and 84.3%, respectively, and up to 90% of surfactant could be recycled by adjusting the pH of the solution after enzymatic hydrolysis [252].

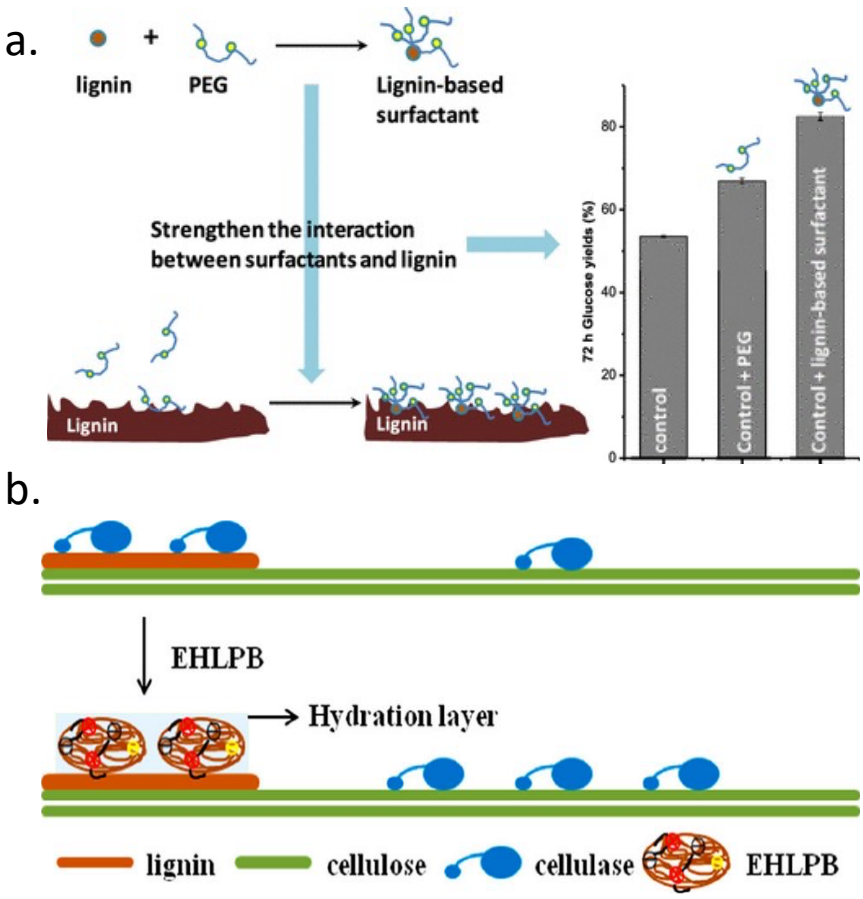

Fig. 14. Lignin-based polyoxyethylene exhibited better performance in terms of enhancing enzymatic hydrolysis compared to PEG (a) [247] and Lignin-Grafted Phosphobetaine on Enzymatic Hydrolysis of Lignocelluloses (b) [251](reproduced with copyright permission from ACS Publications).

LS, a type of lignin byproduct from sulfite pretreatment of lignocellulosics, could be also used to enhance the enzymatic hydrolysis as a nonionic surfactant [41, 253]. By directly mixing the sulfite pretreatment hydrolysate with the corresponding sulfite pretreated substrate, a high cellulose conversion (>90%) could be achieved. In addition, adding LS during the pretreatment can also enhance the enzymatic hydrolysis of biomass, which is due to the adsorbed lignosulfonates on the substrate to enhance cellulose accessibility [254]. Besides reducing the enzyme dosage, because LS is a side

product of sulfite pretreatment, it should be able to eliminate the washing step of the sulfite pretreated solid fraction for a direct simultaneous enzymatic saccharification and even a combined fermentation of enzymatic and pretreatment hydrolysates [254]. The mechanism of LS-induced reduction of nonproductive binding of lignin to cellulase enzyme is illustrated in **Fig. 15** [40]. Without the addition of LS, lignin could be bind to cellulase via a normal electrostatic attraction. With the addition of LS, its acid groups could bind to the oppositely charged groups of enzymes, forming a highly negatively charged LS-cellulase complex. This complex could alleviate the non-productive binding of cellulase to lignin as a result of electrostatic repulsion. In another study, cetyltrimethylammonium bromide (CTAB) was used to facilitate the hydrolysis of sulfite pretreated pine by increase the adsorption efficiency of LS on lignin via a mechanism that CTAB could neutralize the negative charge of LS thus decrease the electrostatic repulsion between LS and lignin [255].

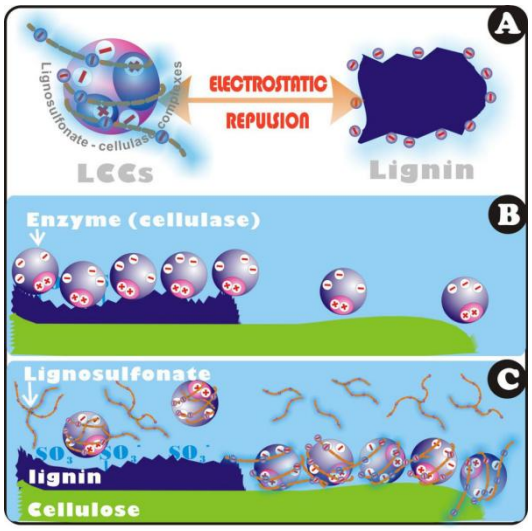

Fig. 15. A schematic representation of LS-cellulase complex induced reduction of nonproductive cellulase binding to lignin; (a) electrostatic repulsion between LS-cellulase complex and lignin, (b) Binding of cellulase to lignin in the case of

enzymatic hydrolysis of lignocellulosic substrate without LS. (c) Reduced cellulase binding to lignin with the addition of LS. (reproduced with copyright permission from Springer Nature) [40].

Although BSA, PEG and Tween have been all proved to be effective additives for improving enzymatic hydrolysis, the high cost and non-biocompatibility associated with these additives still limit their potential applications on an industrial scale. Other cheap additives need to be developed. Soybean, one of the cheapest proteins available on the market, has a price of ~$1.25/kg of protein [256]. It is much cheaper than other possible lignin-blocking proteins or surfactants such as BSA (~$10/g of protein) and Tween 80 (~$80/L). Luo et al. reported that a soluble soy protein extracted from defatted soy powder showed excellent performance in promoting the sugar release from LHW pretreated bamboo, which could be significantly reduced the enzyme dosage by nearly 8 times by adding 80 mg soy protein/g glucan to achieve 80% enzymatic yield. The mechanism of the soy protein promoted enzymatic hydrolysis of pretreated lignocellulosic biomass is proposed in **Fig. 16** [257]. The cost of soybean as a lignin blocking additive during enzyme hydrolysis has also been estimated, and results showed that a loading of ~12 mg soybean /g glucan was only equivalent to 1.2 FPU/g glucan. Similarly, Florencio et al. also reported the soybean protein led to approximately a 2-fold increase in the hydrolysis yield of steam-exploded pretreated sugarcane bagasse [258]. Other non-enzymatic proteins or agricultural waste such as corn steep liquor, year extract, peptone, and tea waste also had a similar effect on the saccharification of pretreated biomass [233, 259]. Last but not least, metal ions could

be also used to block the non-productive cellulase adsorption on lignin due to its ability to alter the lignin's surface charge. Akimkulova et al. screened eleven salts in terms of their ability to block non-productive adsorption of cellulase onto lignin and reported that Mg^{2+} was a promising candidate [260]. The addition of these metal ions could weaken the lignin-cellulase interaction by altering the surface charges as well as the hydrogen bonding ability of the lignin.

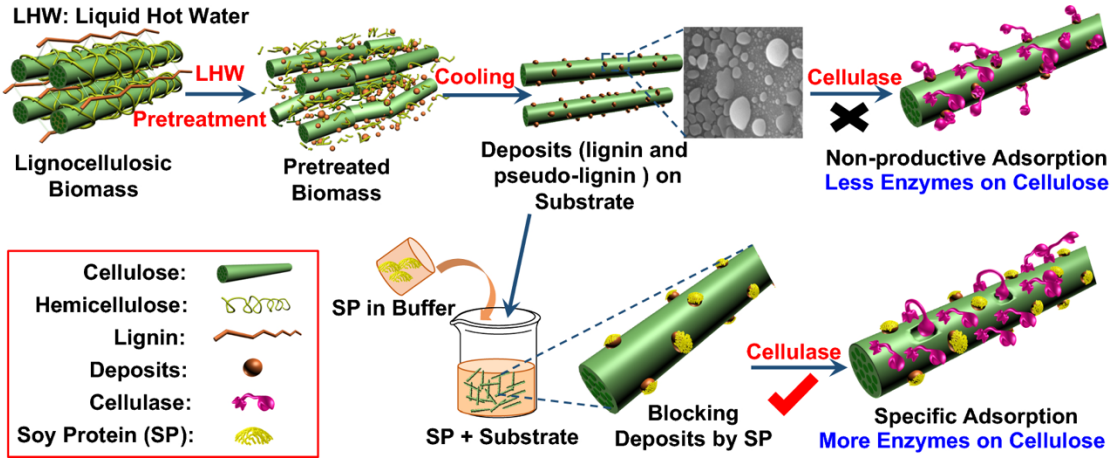

Fig. 16. A proposed mechanism of the soy protein enhanced enzymatic hydrolysis of liquid hot water pretreated lignocellulosic biomass [257].

In conclusion, various types of non-ionic surfactants, lignin-derived surfactant, proteins, and metal ions can reduce the nonproductive adsorption of cellulase on lignin, which can further promote the enzymatic hydrolysis. The effect of different additives on the enzymatic hydrolysis of various pretreated lignocellulosic biomass are summarized in Table 2.

Table 2. Positive effect of different additives on the enzymatic hydrolysis of various pretreated lignocellulosic biomass.

Additive	Substrates	Enzymes	Summary	References
BSA	NaOH pretreated poplar	Cellulase from *Trichoderma viride*	After BSA addition, cellulose conversion increased by 13.3% with ~33% reduction of cellulase dosage.	[261]
BSA	LHW pretreated mixed hardwood	Novozymes 188 and Cellic™ Ctec 2	For 80% cellulose conversion, adsorption of BSA to pretreated substrates prior to enzymatic hydrolysis enabled reduce enzyme dosage.	[230]
BSA	Steam pretreated Douglas fir	Celluclast 1.5L and Novozymes 188	Cellulose conversion increased from 16 to 66% with the addition of BSA.	[262]
Tween 80	Cellulolytic enzyme lignin from pine	Celluclast 1.5 L and Spezyme CP	The adsorption of cellulase onto lignin from pine was reduced by 60% with addition of 0.1-0.2% Tween 80.	[263]
Lignin-PEG	Steam explosion pretreated corn stover	Cellic CTec2	Lignin based PEG interacted with cellulose and dispersed cellulase aggregates to smaller aggregates, which subsequently reduced the nonproductive adsorption of cellulase on lignin.	[248]
Soy protein	LHW pretreated bamboo	Celluclast 1.5 L® and Cellic CTec2	The loading of cellulase could be reduced by ~8 times from 96.7 to 12.1 mg protein/g glucan with the addition of 80 mg/g glucan SP.	[264]
Lignosulfonate	Sulfite pretreated pine	Celluclast 1.5 L and Novozyme 188	~90% of cellulose conversion could be achieved with the presence of lignosulfonate at a cellulase loading of just 13 FPU/g glucan.	[254]
Lignosulfonate	Sulfite pretreated poplar and pine	Cellulase, β-glucosidase and hemicellulase	Lignosulfonate could behave as a polyelectrolyte to form a negatively charged lignin-cellulase complex with enzymes, which weakened the nonproductive binding of cellulase to lignin.	[40]
Metal ions	DAP wheat straw	Cellic CTec2	~20% improvement of glucan conversion was achieved by addition of Mg^{2+}	[265]

4.3. Post treatment to remove lignin

Besides modifying the lignin structure during biomass pretreatment and adding additives during enzymatic hydrolysis, post-treatment can also remove lignin or modifying the lignin structure after pretreatment for mitigating the negative effects of lignin [266-268]. Post mechanical refining treatment could reduce the particle size and improve hydrolyzability of pretreated biomass [269]. This section will mainly focus on post treatments such as simple solvent wash, organic solvent extraction, or chemical treatment under a relatively mild condition.

During biomass pretreatment, a series of hydrolysis and fermentation inhibitors could be formed including furan type of structure (e.g., furfural and hydroxymethylfurfural) and phenolic compound resulting from the degradation of carbohydrate and lignin, respectively [270]. Maroušek et al. reported that removing the most labile organic matter from biomass could significantly minimize the inhibitor formations in the subsequent high-pressure pretreatment process such as steam-explosion [271]. In addition, a simple wash of pretreated biomass with water could remove hydrolysis and fermentation inhibitors include sugars (xylo-oligomers), furan derivatives (hydroxymethyl furfural), and acid (formic acid and acetic acid) and various phenolic compounds [272-275]. Frederick et al. showed that rising the sulfuric acid pretreated *Populus* with 1.5 volumes of water resulted in glucose yields that were nearly seven times greater than the untreated biomass [276]. Several studies suggested that hot washing especially at a temperature above the transition temperature of lignin (130-150 ^{o}C) recovers more hydrophobic and inhibitory phenolics from the pretreated substrates,

therefore significantly improved the cellulose digestibility [277, 278].

Alkaline post-treatments using dilute sodium hydroxide have been applied to carbohydrate-rich cellulosic material to further increase the yield of enzymatic hydrolysis due to its ability to cause cellulose swelling and hydrolysis of acetyl group [28, 29]. Alkali post-treatment is capable of removing a considerable amount of lignin from lignin-rich pretreated biomass, thus increasing the enzymatic hydrolysis [279-282]. However, its use does not always facilitate enzymatic hydrolysis because the nature and location of lignin also have significant influences. Alkali treatment could redistribute the spherical lignin droplets formed during high severity hydrothermal pretreatment across the surface of biomass more evenly thereby decreasing the cellulose accessibility [283]. Several studies also confirmed that alkaline treatment decreased the enzymatic hydrolysis yield of different types of pretreated substrates [284-286]. Carboxylated, oxidized, and sulfonated lignin during post-extraction process could all weaken the nonproductive adsorption between enzymes and lignin [287]. Kumar et al. assessed the effect of hydrogen peroxide, neutral sulfonation, chlorite delignification, and alkali post-treatment on the enzymatic hydrolysis of stream pretreated Douglas fir softwood. They found that chlorite and hydrogen peroxide treatment both significantly enhanced the enzymatic hydrolysis of steam pretreated Douglas fir because of the high degree of lignin removal and increased amount of acid groups caused by lignin oxidation [288].

The surface lignin and solvent extractable lignin from pretreated biomass have low molecular weight and likely play a different role in the enzymatic hydrolysis process

compared to the bulk lignin residue in the pretreated material that has highly condensed and high molecular weight. While it has been widely reported that the removal of residual bulk lignin could enhance the hydrolysis yield, whether those solvent extractable lignins located at the surface of biomass should be removed is still under debate. Huang et al. reported that post extraction of dilute acid pretreated bamboo with phosphoric acid, urea, and ethanol at room temperature, which could remove a decent amount of lignin by disrupting the covalent and hydrogen bonding among lignin-carbohydrate complexes to increase the sugar release from 15 % to 61%, 59%, and 43%, respectively [289]. On the contrary, solvent extractable lignin removal from dilute acid and organosolv pretreated sweetgum reduced their enzymatic hydrolysis from 38 and 70% to 32, and 49%, respectively [43]. This is because the low molecular weight lignin could counter the non-productive binding between the high molecular weight residual bulk lignin and cellulase enzymes, as confirmed by the protein adsorption experiment and quartz crystal microbalance [44]. Thus, post treatment should be focused on removing or modifying the inhibitory lignin fraction while maintain the stimulatory fraction.

5. Conclusion

Lignin negatively affects the enzymatic hydrolysis process by blocking the access of cellulase to cellulose and binding to cellulase non-productively. Biomass pretreatment reduces the recalcitrance of lignocellulosic biomass by degrading and removing (partially) lignin from the plant cell wall. To achieve high delignification, high pretreatment severity is required. During these pretreatments, lignin also undergoes

irreversible depolymerization and repolymerization through cleavage of native ether linkages and formation of new carbon-carbon bonds, respectively. The structural transformation of lignin occurs via different pathways depends on the employed pretreatment solvent and severity, and the resulting pretreated lignin has different functionalities and characteristics that dictates its interaction with enzymes. The net effect of lignin residue on enzymatic hydrolysis strongly depends on the combined influence of hydrophobicity, hydrogen binding, and electrostatic interaction between lignin and cellulase. The detrimental lignin-cellulase interaction could be partially alleviated by chemical and surface modification of lignin by adding certain chemicals and additives during the pretreatment or enzymatic hydrolysis process. During the chemical modification process, lignin's hydrophilicity is typically increased. Among the available lignin-blocking additives, soybean as a cost-effective non-catalytic protein has huge industrial potential. Performing enzymatic hydrolysis process at an elevated pH (>5) or increase the zeta potential of lignin surface represents another effective way to preventing the non-productive binding mainly through increasing the electrostatic repulsive forces between lignin and cellulase. Last but not least, genetically modified lignin with adjusted monolignol composition (e.g., high H and S units, low G units) that have low cellulase binding affinity may also result in significantly enhanced enzymatic hydrolysis. With detailed review of lignin structural change, lignin-enzyme interaction mechanism and approaches to reduce the adverse impact of residual lignin, this manuscript will provide insights of selecting proper process to achieve a satisfying enzymatic digestibility of biomass for bioethanol production.

Acknowledgments

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