Lignin Interaction with Cellulase during Enzymatic Hydrolysis

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Abstract: The conversion of lignocellulosic biomass into biofuels or biochemicals typically involves a pretreatment process followed by the enzyme-catalyzed hydrolysis of cellulose and hemicellulose components to fermentable sugars. Many factors can contribute to the recalcitrance of biomass, e.g., the lignin content and structure, crystallinity of cellulose, degree of fiber polymerization, and hemicellulose content, among others. However, nonproductive binding between cellulase and lignin is the factor with the greatest impact on enzymatic hydrolysis. To reduce the nonproductive adsorption of enzymes on lignin and improve the efficiency of enzymatic hydrolysis, this review comprehensively summarized the progress that has been made in understanding the interactions between lignin and enzymes. Firstly, the effects of pretreatment techniques on lignin content and enzymatic hydrolysis were reviewed. The effects of lignin content and functional groups on enzymatic hydrolysis were then summarized. Methods for the preparation and characterization of lignin films were assessed. Finally, the methods applied to characterize the interactions between lignin and cellulase were reviewed, and methods for decreasing the nonproductive binding of enzymes to lignin were discussed. This review provides an overview of the current understanding of how lignin hinders the enzymatic hydrolysis of lignocellulosic biomass, and provides a theoretical basis for the development of more economical and effective methods and additives to reduce the interaction of lignin and enzymes to improve the efficiency of enzymatic hydrolysis.

Keywords: lignocellulosic biomass; lignin; cellulase; nonproductive binding; interaction

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1 Introduction

For the conversion of lignocellulose to bioethanol, the polysaccharides in the lignocellulose must be decomposed into monosaccharides, which are subsequently fermented into ethanol by microorganisms[1-2]. The resistance of lignocellulosic biomass to enzymatic and microbial decomposition is one of the main factors hindering the production of cheap biofuels[3]. The biochemical processing of lignocellulose includes enzymatic depolymerization of the structural plant cell wall. Lignocellulose is predominantly composed of three biopolymer components, cellulose, hemicellulose, and lignin, all of which have different chemical and physical properties and structures. The inhibition of the interaction of enzymes with the lignocellulosic substrates and the degradation of the enzyme efficiency by lignin during the reaction process are significant. The resistance of lignocellulosic biomass toward enzymatic hydrolysis has been directly correlated to the lignin content in the plant cell wall[4].

The physicochemical characteristics of lignin can vary significantly depending on the source of the feedstock[5], chemical pretreatment used, and lignin isolation methodology[6]. The purpose of this work is to review the nonproductive adsorption of cellulase on lignin and to discuss the binding of lignin with enzymes during hydrolysis processing, with the aim of reducing nonproductive binding between cellulase and lignin and further improving the enzymatic hydrolysis of lignocellulose. These goals will be accomplished by describing the changes to the structure of lignin during various chemical pretreatment processes and the effect of the feedstock source, giving an overview of the properties of lignin that affect enzymatic hydrolysis, and summarizing the preparation methods and properties of ultrathin biomass films. Additionally, the interactions of cellulase with lignin and the corresponding adsorption mechanism are discussed. These insights could be used to develop more efficient methods to reduce the inhibition of cellulase hydrolysis activity and stability due to lignin, and to enhance the understanding of the complex process of lignocellulosic biomass bioconversion.

2 Lignin characteristics

Lignin is a three-dimensional structure composed of natural polymers, and is found widely in highly vascular plants, especially woody plants. The lignin contents of different raw materials vary, ranging from 25% to 35% and 20% to 25% in softwood and hardwood, respectively, and from 15%~25% in monocotyledon grasses. Lignin is an aromatic polymer found in the cell wall, where it forms chemical bonds with carbohydrates and imparts rigidity and moisture and microbial resistance. Three main lignin units exist: guaiacyl (G), syringyl (S), and hydroxyphenyl (H)[7]. Softwood lignin is mainly composed of G type lignin units, whereas hardwood is rich in both S and G type lignin. The lignin in grasses contains G, S, and H type lignin units.

3 Effect of pretreatment on lignin content and enzymatic hydrolysis

Pretreatment is a key process in the preparation of ethanol from biomass. Pretreatment is used to modify the lignocellulose structure and lignin content of the biomass to improve enzymatic hydrolysis. The different lignin contents of different materials lead to variations in their hydrophilicity and affect the enzymatic hydrolysis process[8]. Dilute acid pretreatment (DAP) can reduce the recalcitrance of lignocellulose and enhance enzymatic hydrolysis via the effective solubilization of hemicellulose, which in turn improves the efficiency of the enzymes during the subsequent decomposition of cellulose[1]. Additionally, DAP can lead to the dissolution of lignin from the material and slight delignification. The content of acid-insoluble (Klason) lignin in acid-pretreated material has been reported to increase with increasing residence time at high temperature in a batch system[9-11]; however, flow-through pretreatment has been found to remove a large fraction of the original lignin, thus avoiding the adhesion of acid-soluble lignin to the cellulose surface[12]. Additionally, the redistribution and self-aggregation of lignin has been observed to result in

the formation of large lignin droplet particles with various morphologies[10, 13]. Pretreatment using a higher acid intensity was found to be undesirable, as the redeposition of lignin and pseudo-lignin on the cellulose surface impacts enzymatic hydrolysis[14]. Dilute acid pretreatment can also cause equipment corrosion.

Liquid hot water (LHW) pretreatment enhances the enzyme accessibility of cellulose by removing most of the hemicellulose from the lignocellulosic material, increasing the porosity of the material, and changing its structure[15]. However, the majority of lignin still remains in the biomass, increasing the possibility of non-productive adsorption of cellulase onto the lignin, especially for lignin derived from more severely pretreated hardwoods, which has a high glass transition temperature value and more strongly inhibits enzymatic hydrolysis[16]. Furthermore, a change in the hydrophobicity of lignin was found to enhance the adsorption of cellulase from Penicillium oxalicum onto lignin isolated from corn stover by LHW pretreatment[17]. Although LHW pretreatment can destroy the cell wall structure to some extent, increasing the enzyme accessibility of the lignocellulose substrates, the extraction process produces byproducts (formic acid, acetic acid, furfural, etc.) that reduces the activity of cellulase and inhibits the efficiency of the enzymatic hydrolysis of cellulose.

Alkaline pretreatment weakens the recalcitrance of the cell wall and breaks the glycoside bond linkages in the polysaccharides, further dissolves and depolymerizes the lignin structure, and improves enzymatic hydrolysis. The application of alkaline treatments for pre-swelling could alter the structures of protolignin in the plant cell wall. Alkaline chemical pulping techniques have also been applied to prepare a set of biomass substrates and reduce the effects of the surface lignin content on enzymatic hydrolysis[18].

Organic solvent pretreatment (OSP) has been used as an effective pretreatment to reduce carbohydrate degradation, and has been shown to improve the conversion rates of cellulose saccharification, enhance enzyme efficiency, and inhibit by-product formation compared to those of acid and alkaline pretreatments[19]. During the OSP process, lignin and hemicellulose can be dissolved by the solvent, decreasing the recalcitrance of the lignin and increasing the surface area of cellulose, therefore enhancing enzyme hydrolysis rates[20]. OSP is an advantageous method for the fractionation of lignocellulosic biomass into purified cellulose, hemicellulose, and lignin; moreover, the reclamation and reutilization of the organic solvent is easy. OSP can be considered as a green pretreatment for enzymatic hydrolysis in the future. Compared to organic solvent pretreatment, ionic liquid pretreatment of lignocellulosic feedstock is more environmentally friendly, and can also degrade the cell wall to allow the lignin to dissolve out. In one study, lignin was selectively extracted from wood meals using [Emim][CH_3COO] to enhance the efficiency of enzymatic hydrolysis. Additionally, the reusability of the ionic liquid was found to be beneficial in terms of reducing the cost of the hydrolysis procedure[21].

Therefore, pretreatment techniques enhance the enzymatic hydrolysis performance by altering the lignocellulose structure. Different pretreatments have different impacts on the lignin structure and different impacts on the adsorption of cellulase on lignin[22-23].

4 Effects of the lignin content and lignin functional groups on enzymatic hydrolysis

Lignin prevents enzymes from coming into contact with the substrates, which inhibits enzymatic hydrolysis[24]. The type of lignin present in a material, its surface content, and its distribution can all affect its inhibition of the enzymatic degradation of lignocellulosic biomass. Therefore, a better understanding of the effect of the lignin content and lignin functional groups on the interactions between the cellulase and lignin is required.

4.1 Lignin content

High lignin content plays a more important role than fiber swelling in restricting the enzyme accessibility of the substrates. The removal of lignin increases the population of pores and enhances hydrolysis significantly[25]. A negative correlation between the

lignin content and the amount of sugar released via enzymatic hydrolysis was identified in one study. The extraction of 40% of lignin from wood meal using 1-ethyl-3-methylimidazolium acetate decreased the index of cellulose crystallinity, thus resulting in more than 90% of the cellulose being hydrolyzed by cellulase[21]. In another study, the adsorption of cellulase onto cellulolytic enzyme lignin (CEL) derived from steam-exploded Lodgepole pine (SELP) and ethanol-pretreated Lodgepole pine (EPLP) was studied. The times required for the adsorption of cellulase onto CEL-EPLP and CEL-SELP were longer than those for SELP and EPLP, demonstrating the even lignocellulosic substrates with high lignin contents could be hydrolyzed quickly during the initial stage[26].

4.2 Lignin functional groups

The chemical composition of the lignin is an important factor affecting enzymatic hydrolysis. The content of aliphatic and phenolic hydroxyl groups in cellulolytic enzyme lignin has been reported to be higher than that in swollen residual enzyme lignin, because cellulolytic enzyme lignin contains more degraded lignin fragments[27-28]. The negative correlation of the methoxyl group content of lignin with fiber digestion has been explained in terms of the possible formation of quinone methide intermediates from guaiacyl and syringyl lignin units[29]. However, the formation of quinone methide intermediates can be hindered by the hydroxypropylation of lignin, which also reduces the inhibitory effect of lignin and improves cellulose degradation with lignin blending in the incubation medium[30]. The methoxyl and free phenolic hydroxyl group contents of lignin are important factors in the inhibition of cellulose saccharification and fiber degradation. The degree of protein adsorption by lignin is dependent upon the phenolic hydroxyl content and the lignin preparation method, which also affects the distribution and flexibility of the phenolic hydroxyl groups. Similarly to hydroxypropylation, ammonization pretreatment almost eliminated the inhibition effect of lignin to cellulase[31]. Pan et al also reported that hydroxypropylation could significantly reduce the inhibition of enzymes by lignin[32-34]. Additionally, the inhibition of enzymatic hydrolysis by phenolic hydroxyl groups may be more significant than that caused by physical barriers and non-specific adsorption, but no clear evidence has been provided showing the relationship between methoxyl groups and their inhibitory effect. The common effect of different lignin groups on lignin adsorbing cellulase process needs to be further studied.

Lignin degradation products in pretreatment process also contain some phenol compounds that inhibit cellulase hydrolysis and deactivate cellulase[35]. Furthermore, syringyl (S) lignin has a lower enzyme adsorption capacity than guaiacyl (G) lignin; in particular, the adsorption of β-glucosidase onto lignin indirectly inhibited the enzymatic hydrolysis of cellulose[36]. Corn stover lignin has a stronger adsorption affinity than kenaf lignin or aspen lignin due to its higher content of phenolic hydroxyl groups and lower content of carboxylic acid groups. The higher adsorption capacity of pine lignin than stover lignin may be due to its lower number of aliphatic hydroxyl groups, which reduces hydrophobic interactions[37]. The adsorption of different lignins onto cellulase is usually evaluated by studying the protein content and enzyme activity of cellulase; however, cellulase contains various enzymes, such as endoglucanase and exoglucanase, and the lignin is adsorbed differently onto the enzymes. Moreover, the protein structure of cellulase also affects the adsorption of lignin onto cellulase.

5 Effect of cellulase selectivity on enzymatic hydrolysis

Celllulases are members of the glycoside hydrolase family of enzymes, and their dominant function is to hydrolyze lignocellulose into oligomers and polysaccharides. They catalyze the cleavage of the β-1,4 glucosidic bonds that link the glycosyl units of cellulose. Different cellulolytic enzymes act on different sites of cellulose. Exo-β-(1,4)-D-glucanases or cellobiohydrolases (CBHs) hydrolyze cellulose chains

from the chain ends to produce soluble cellobiose as the main product; endo-β-(1,4)-glucanases (EGs) hydrolyze internal β-1,4 glucosidic bonds of the cellulosic chains and β-D-glycosidase hydrolyzes the soluble disaccharide cellobiose and soluble cello-oligosaccharides into monomeric glucose[38-39]. These enzymes have a three-dimensional structure, and most are composed of multiple domains, which are structurally and functionally discrete protein units. In particular, they contain a catalytic domain, a cellulose-binding domain (CBD) or carbohydrate-binding modules (CBMs), and an interdomain[24]. The binding interaction between the CBMs and the cellulose surface brings the catalytic domain in proximity to the substrate for insoluble cellulose, presumably facilitating cellulose hydrolysis.

Cellulase systems exhibit a synergistic effect, with the system having a higher collective activity than the sum of the activities of the individual enzymes[39]. The removal of the CBD of an enzyme has little impact on its cellulase activity towards soluble substrates, but clearly decreases its binding with insoluble cellulose[40-41]. Such binding domains improve the binding and facilitate the activity of the catalytic domain on the insoluble substrates[42-43]. The CBD is hydrophobic and may be involved in lignin binding, but enzymes lacking CBDs also have a high affinity for lignin, indicating that the lignin binding sites exist in the catalytic domain[44]. The extent to which lignin is bound to cellulase differs for different varieties of cellulase, with the endoglucanase EG-I exhibiting distinctively higher affinity for lignin than the exoglucanase CBH-I. In the absence of a CBD and a linker, the exoglucanase CBH-I core adsorbs slowly and is not able to penetrate the lignocellulose thin films, reducing the effectiveness of hydrolysis[38]. During enzymatic hydrolysis, the catalytic domain and cellulose-binding domain play different roles, and the different enzyme components work synergistically to efficiency hydrolyze cellulose. Additionally, the selectivity of cellulase plays an important role in the enzyme hydrolysis reaction and in reducing nonproductive binding with lignin. Due to the complexity of the structures of lignocellulose, lignin, and cellulase, the adsorption process of lignin on cellulase is complicated. Therefore, advanced technologies are required to study the adsorption process of lignin on cellulase.

6 Preparation and characterization of lignin films

In order to more directly monitor the interactions between cellulase and lignin in real time, film substrates composed of lignin, two components, or multiple components have been prepared. There are two main established methods for the preparation of flat and smooth lignin model surfaces: Langmuir-Blodgett (LB) deposition and spin-coating. Both techniques involve dissolving the coating materials before their deposition on the substrate.

6.1 Langmuir-Blodgett lignin films

The first Langmuir-Blodgett lignin films were fabricated by dissolving lignin in tetrahydrofuran and subsequently spreading the solution on an ultrapure water surface and evaporating the solvent. The film was characterized using surface potential measurements and ellipsometry, which indicated that films derived from *Pinus* and bagasse had positive potentials and negative potentials, respectively, and that the thickness of the film was proportional to number of layers deposited[45]. Lignins obtained from different feedstocks produce films with different surface morphologies and properties. LB films have been analyzed using AFM and FT-IR to determine their surface characteristics. LB films from ethanol lignin were found to have a flatter surface than those from saccharification lignin, and lignin aggregates were preferentially oriented perpendicular to the substrate[46]. In another study, AFM analysis revealed that the extraction process affected the lignin content and surface pressure-area isotherms and lowered the surface potentials, as well as the root mean square roughness, of the resulting LB films[47]. An FT-IR analysis demonstrated that phenyl groups were preferentially oriented parallel to the substrate surface

in homogeneous LB lignin films, and that LB films could be deposited onto gold electrodes[48]. However, the use of the LB technique to prepare films is limiting. LB technology is time-consuming, the preparation process is complex, and the film properties are difficult to control. Spin coating, on the other hand, is a well-established technique for industrial applications and can provide a quick and easy alternative to produce thin organic films[49].

6.2 Spin-coated lignin films

Spin-coating is another important method of preparing lignin films. A study using AFM and water contact angle indicated that the roughness of Kraft lignin films remained relatively low independent of the film thickness; that is, no relationship was observed between the film thickness and the water contact angle. The Kraft films were also stable in air and aqueous solutions[50]. The stability of lignin films in the presence of water is an important factor in enzymatic hydrolysis. In a study using water-penetrated Norway spruce lignin films, no swelling of the films was detected using a quartz crystal microbalance with dissipation (QCM-D), indicating the stability of lignin films prepared by spin-coating[51]. Additionally, the surface energy and wettability of lignin are important for a number of natural feedstocks. The total surface energy of lignin is of a similar magnitude to that of cellulose. Water may only be considered a partially wetting liquid on a lignin surface[52]. Norgren et al investigated the properties of seven solvents for dissolving Kraft lignin, and showed that uniform lignin films were dissolved by aqueous NH_4OH[50]. Milled wood lignin from acid-pretreated *Miscanthus* was dissolved in tetrahydrofuran (THF) and then spin-coated on gold Q-Sense sensors[53], showed that the discrepancies among the solvent-dissolved lignin were attributed the different pretreatments and feedstocks. In order to further explore the role of lignin in lignocellulose hydrolysis, lignocellulose films composed of the biocomponents acetylated lignin (LAc) and trimethylsilyl cellulose (TMSC) dissolved in chloroform were prepared by spin-coating on silicon wafers to investigate the inhibition of lignin on the cellulase activities[54]. In addition, amphiphilic bicomponent thin films composed of different ratios of hydrophilic TMSC and a hydrophobic lignin ester were prepared by spin-coating[55]. Multi-component biomass thin films containing lignocellulosic nanofibrils (LCNFs) were spin-coated on a gold QCM-D sensor to investigate the effect of lignin on enzymatic hydrolysis[56]. The fabrication of lignin films is thus a well-established method to investigate the interaction between lignin and cellulase.

7 Kinetic studies of interaction between lignin and cellulase

In order to fully comprehend cellulase hydrolysis processing, the distinct impacts of the substrate composition, recalcitrance, and environmental conditions on the mechanisms and dynamics of the adsorption of cellulase on the lignocellulose substrate and hydrolysis must be considered. The quartz crystal microbalance with dissipation Quartz Crystal Microbalance with Dissipation (QCM-D) method has been used to investigate the binding and activity of enzymes on cellulose/lignin thin films deposited on the sensors. Changes in the resonance frequency Δf and energy dissipation ΔD are measured simultaneously in QCM-D. The energy dissipation is associated with friction and viscoelastic energy losses due to changes in the sensing surface. The dissipation is quantified by the damping of the oscillating signal, because its vibration amplitude is exponentially attenuated. The adsorption of material on the crystal can be regarded as equivalent to a change in the mass of the crystal itself. The change in mass Δm is proportional to the change in frequency Δf; the linear relationship between Δm and Δf is given by the Sauerbrey equation[57-59]:

$$\Delta m = \frac{-\rho_q t_q \Delta f}{f_0 n} = \frac{-\rho_q v_q \Delta f}{2 f_0^2 n} = \frac{-c\Delta f}{n} \quad (1)$$

Where, ρ_q and v_q are the specific density and the shear wave velocity in quartz, respectively, t_q is the thickness of the quartz crystal, and f_0 is the elementary resonance frequency (when n=1). The constant c

determined by the crystal substrate is 17.8 ng/(cm·Hz). The change in energy dissipation during adsorption reflects the energy dissipation of the adlayer or the interfaces. The shift in the dissipation factor in a liquid environment can be calculated according to the following formula[57, 59]:

$$\Delta D = \frac{1}{\rho_q t_q} = \sqrt{\frac{\rho_1 n_1}{2\pi f}} \quad (2)$$

Where, η_1 and ρ_1 are the viscosity and density of the fluid, respectively, and tq and ρ_q are the thickness and density of the quartz plate, respectively.

QCM or QCM-D can be used for *in situ* and real-time monitoring of the adsorption of cellulase on lignin, allowing investigation of the influence of lignin on cellulase accessibility and hydrolysis efficiency. In one study, lignin was dissolved in an organic solution and then spin-coated onto a QCM-D crystal to determine the stability and physical properties of lignin, which did not change with ionic strength[51]. The reduced affinity of cellulases with acetylated and partially deacetylated lignin films compared to that of lignin has been ascribed to the effect of electrostatic repulsion, which prevents the nonspecific binding of cellulases[54]. Furthermore, the adsorption of partially hydrophobic protein residues onto lignin has been shown to increase the wettability of the lignin surface[60-61]. When the mono-component cellulase CBH-I was injected into a QCM flow module in which substrate films were mounted, a rapid increase in Δf3 was observed for all the films, irrespective of their lignin content, in the initial stages. The CBH-I core was adsorbed onto lignin films more slowly and to a lesser extent compared to the case of native CBH-I due to the lack of a binding domain. QCM-D monitoring revealed that the binding of CBH-I onto isolated lignin films involved a two-phase process with a rapid initial phase and slower second phase. On other hand, the adsorption of enzymes onto the surface of lignin as measured by QCM-D was found to correlate to the hydrophobic cluster scores, allowing prediction of the degree of binding. The hydrophobic patch score shows a strong correlation with binding capacity for enzymes and monomeric proteins, which suggests that hydrophobic interactions are the dominant interactions between the proteins and lignin films[62]. Three kinetic models, namely, a single-site transition model (A), a transition model with a changing adsorbate footprint (B), and a two-site transition model involving reversible-irreversible binding (C), were evaluated to describe the binding of cellulase Cel7B to lignin detected using QCM-D[63], as shown in Fig.1. The results indicated that the adsorption of cellulase Cel7B on lignin was best described through a multiple binding site transition model, and that the lignin surface displayed binding sites with varying affinity for Cel7B due to the wide variety of chemical functionalities present in lignin. However, other models may be required to describe the adsorption of cellulase on lignin when the discrete surface sites have varying adsorption rate constants[53]. Although QCM-D can effectively elucidate the kinetic process of the adsorption of lignin on cellulase, the adsorption kinetics for multicomponent lignocellulose and cellulase should be further explored. In addition, further study of the driving force for the adsorption of lignin on cellulase is necessary.

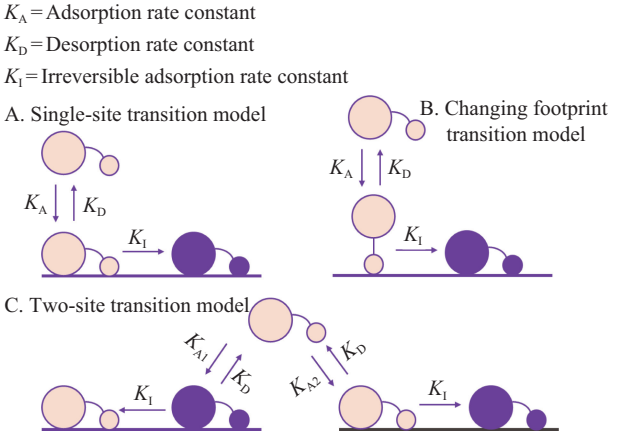

Fig.1 Models of protein adsorption, desorption, and irreversible adsorption

8 Interactions between lignin and cellulase

The binding of cellulase with lignin is a critical factor in the development of enzyme mixtures and lignocellulose hydrolysis processes. Nonproductive enzyme adsorption onto lignin inhibits the enzymatic hydrolysis of lignocellulosic biomass. Hydrophobic interactions

have been identified as a major driving force for the adsorption of enzymes on lignin; electrostatic interactions and hydrogen bonding also play important roles. Additionally, the interactions between cellulase and lignin are influenced by the steric hindrance of the reaction surroundings.

8.1 Hydrophobic interactions

Hydrophobic interactions play a significant role in the adsorption of cellulase and related enzymes to lignocellulose, leading to the irreversible adsorption of enzymes on lignin[31, 44, 64]. Xylanase complexes have been found to be more strongly inhibited by organic dissolved lignin (DL) than by enzymatic residual lignin (ERL), because DL has a lower content of carboxyl and aliphatic hydroxyl groups and thus a relatively higher surface hydrophobicity than ERL[64-65]. The hydrophobic interactions between cellulases and substrates, and their critical role on irreversible adsorption, were elucidated using acetylated lignins and trimethylsilyl cellulose films with different ratios[54]. Additionally, using the enzyme surface hydrophobicity calculated using the software program Rosetta to predict the adsorption of cellulase on lignin, the proteins were ranked by a hydrophobic patch score based on the size and number of hydrophobic zones. These results showed that hydrophobic interactions are a dominant part of the interaction energy between the proteins and lignin films[62].

8.2 Electrostatic interactions

Protein surfaces contain ionized side chains and have a net charge. Accordingly, for each protein, there is a pH at which the net charge on the surface is zero; this is known as the isoelectric point (pI). The pI of a protein is specific, and is related to the structure of the protein, but not to the environmental pH value. Different cellulases have different pI values. In one study, the electrostatic properties of cellulases were modified to give them more negative charges, which probably decreased the non-productive binding of cellulases to protease-treated lignin[32]. The optimal reaction pH value of cellulase varies with different substrates. The enzymatic hydrolysis of cellulose to achieve the saccharification of lignocellulosic substrates occurs at a substrate suspension pH value of 5.2~6.2, which is different than the corresponding values for pure cellulose substrates (pH value 4.8 and 5.0). This is because variation in the pH triggers changes in the surface charge depending on the surface functional groups. Changing the electrostatic interactions between lignin and cellulase can therefore reduce the non-productive binding of cellulase to lignin and enhance cellulase hydrolysis[66]. In order to understand the relationships between the protein structure and the protein adsorption on lignin, 16 model protein variants spanning the physiological range of net charges were designed and characterized to investigate cellulase adsorption and inactivation in the presence of lignin. A weak correlation was identified between the net charge of the protein and its binding capacity to lignin[67].

8.3 Hydrogen bond interactions

Hydrogen atoms covalently bond with highly electronegative atoms (X). When a hydrogen atom bound to X comes near a highly electronegative atom A with a small radius (O, F, N, etc.), the hydrogen is used as the medium between X and A to form an X-H...A interaction. This type of intermolecular or intramolecular interaction is known as a hydrogen bond, in which X-H acts as proton donor to A[68]. The secondary structure of proteins involves the formation of hydrogen bonds between carbonyl groups and amide groups; this hydrogen bonding is the main stabilization force of the secondary structure[69]. The presence of carboxyl, hydroxyl, or, to a lesser extent, carbonyl functional groups on both the lignin and enzyme surfaces could interfere with ionic-type lignin binding, which determines its secondary structure[65]. The inhibition caused by the phenolic hydroxyl groups through the quinone methide intermediates may have a more important effect on the enzymatic hydrolysis of lignocellulose than physical barriers or nonspecific binding[31, 33]. The effect and mechanism of hydrogen bond interactions between lignin and

cellulase is difficult to explore due to the dominance of hydrophobic interactions and electronic interactions, and still requires further investigation and confirmation.

8.4 Steric hindrance

Lignin restricts cellulose hydrolysis through either binding cellulase or limiting the swelling of cellulose, and therefore reducing its accessibility. Cellulose swelling can expand the surface area of the substrate, providing more cellulase binding sites, which in turn leads to more complete hydrolysis of the lignocellulose. Cellulase loadings lower than 10 FPU/g on lignocellulose result in poor hydrolysis yields. These are mainly due to the nonproductive binding of the enzymes and limited cellulose accessibility. When lignin does not restrict cellulose swelling and the enzymes can reach the cellulose surface more easily, the impact of unproductive binding on hydrolysis is reduced, and the hydrolysis efficiency of cellulose is enhanced, even at a high lignin content[70-71]. The kinetics of lignin-induced adsorption and the steric hindrance factor can be used to quantify the unproductive binding of enzymes adsorbed on lignin and the steric hindrance of lignin. The binding of cellulase to the cellulose portion of a lignocellulosic biomass has been evaluated by subtracting the theoretical adsorption of lignin from the total adsorption capacity of a given lignocellulosic biomass sample[71-72]:

$$P_{[cellulose]}=\frac{P_{[solids]}-P_{[lignin]}L_w}{C_w} \quad (3)$$

Where, $P_{[cellulose]}$ is the actual specific adsorption capacity of cellulase for cellulose, $P_{[solids]}$ is the total observed specific adsorption capacity of a lignocellulosic biomass sample, $P_{[lignin]}$ is the maximum specific adsorption capacity of lignin, and L_w and C_w are the weight fractions of lignin and cellulose in the lignocellulosic biomass, respectively. The above formula indicated that a higher lignin content resulted in more cellulase being adsorbed on lignin for a cellulose substrate. However, the adsorption of the enzyme on the lignin surface reached saturation, and thus the effect of cellulase binding by lignin decreased as the amount of enzyme was increased past the saturation point. Pretreatment can change the distribution of steric hindrance in the lignocellulosic biomass. To characterize the accessibility of cellulose and assess the effectiveness of pretreatment, a steric hindrance factor (H) was defined as follows[71]:

$$H=\frac{1-P_{[solids]}}{P_{max(C)}C_w+P_{max(L)}L_w} \quad (4)$$

Where, $P_{[solids]}$ is the total observed specific adsorption capacity of the biomass sample, and $P_{max(C)}$ and $P_{max(L)}$ are the maximum adsorption capacities of cellulose and lignin, respectively, which are determined by the adsorption of cellulase on pure cellulose and lignin. The H factor can range from 0 to 1, with 1 indicating complete blocking of the cellulose by lignin, and lower values signifying less blocking. Furthermore, calculation of the H factor revealed that the steric hindrance of lignin was the dominant factor restricting the enzymatic hydrolysis of pretreated lignocellulosic biomass. Thus, the adsorption of cellulase on lignin is affected by different types of interactions; however, accurate quantification of these interaction forces requires advanced technology.

9 Interaction force between lignin and cellulase

Atomic force microscopy (AFM) has the unique ability to directly measure specific interaction forces. AFM measures the force between a tip located on the end of a thin cantilever and a surface, as shown Fig.2.

Furthermore, the AFM probe can be manipulated or designed to have specific chemical or even biochemical

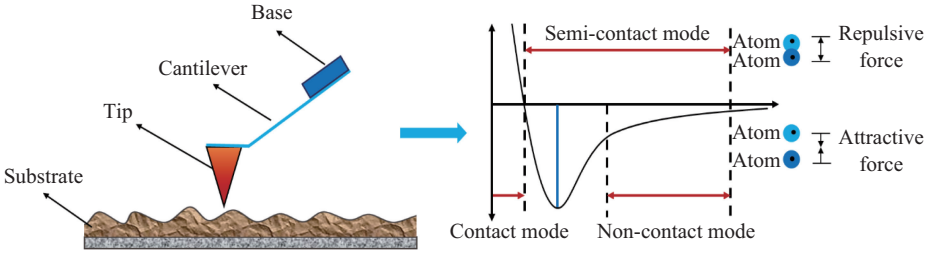

Fig.2 Schematic diagram of the working principle of AFM

characteristics, endowing AFM with the unique ability to measure the interaction forces between specific materials and molecules and obtain the reaction image[73-74]. Using an advanced version of high-speed AFM, the degradation of crystalline cellulose by individual cellulase enzymes was visualized in real-time. The observation of the reaction state of the enzyme molecules blocked on the crystalline cellulose surface revealed that the roughness of the crystalline cellulose surface could lead to the formation of traffic jams of productively bound cellulases; the synergistic use of pretreatment or enzymes could potentially reduce the molecular congestion[75]. The interaction between cellulase and lignin was directly measured using AFM to determine the adhesion forces between cellulase and lignin. The results indicated that the hydrophobic interaction was a dominant attraction force in the binding of cellulase to lignin. Other interactions may also influence the irreversible binding, but more investigation is required in this area[76]. The effect of the surface lignin coverage and type of lignin on the carbohydrate-binding module of CBH on lignocellulosic substrates was measured using AFM. The results indicated that the adhesion force of the biomass to the CBM increased linearly with the surface coverage of Kraft lignin. The adhesion forces were decoupled into specific (hydrophobic) and nonspecific (Lifshitz-van der Waals (LW)) component forces via the Poisson statistical model, whereas electrostatic forces were found to facilitate the interactions of lignosulfonates with CBM. The attractive forces between the CBM and lignin-free substrates increased with the content of xylan and acetone extractives, most likely due to hydrogen bonding forces. The modification of the surface hydrophobicity and the surface energy of lignin to facilitate LW forces to avoid nonproductive binding of cellulase to Kraft lignin has been reported previously[76-77].

The various technologies that have been applied to research the interaction of cellulase and lignin, and to some extent, to explore the effect of lignin and cellulase mechanism, have indicated that the main interaction forces between lignin and cellulase originate from hydrophobic interactions. However, electronic interactions and hydrogen bonding between cellulase and lignin require further investigation with other new research methods and technology, for example, *in situ* analysis of the adsorption of cellulase on the lignocellulose surface and the hydrolysis process at nanoscale using AFM and laser scanning confocal microscopy (LSCM).

10 Reduction of the adsorption of lignin to cellulase

Therefore, it is necessary to take measures to mitigate the nonproductive binding between enzymes and lignin. Surfactants or chemical additives have been applied to reduce the interactions between lignin and cellulase, and have successfully reduced nonproductive enzyme binding and enhanced the enzymatic hydrolysis of lignocellulose biomass to some extent.

Anionic and non-ionic surfactants reduced the adsorption of cellulase Cel7A (CBHI) to lignocellulose and delignified substrates. The surfactant interacted with the lignin on the surface of the lignocellulose, reducing the unproductive adsorption between the enzyme and the lignin part of the substrate and improving the conversion of lignocellulose[78]. The adsorption of the water-soluble polymer polyethylene glycol (PEG) and non-ionic surfactants on lignin through hydrogen bonding and hydrophobic interactions led to a reduction of the nonproductive binding of lignin and cellulase via steric repulsion of the enzyme from the lignin surface. Additionally, the hydrophobic interaction of enzymes on lignin induced the denaturation of enzymes on lignin surfaces[60, 79-80]. The adsorption of cellulase onto steam-exploded Lodgepole pine lignin decreased due to the saturation of the lignin surface by NaCl and Tween 80 at low salt concentrations, possibly due to the competitive adsorption of salt and Tween-80 onto the lignin surface[81]. Tween 20, a nonionic surfactant, was found to significantly overcome the nonproductive adsorption of cellulase and β-glucosidase on lignin by reducing their adsorption affinity, and had no effect on

the adsorption of cellulase on Avicel[82].

The surface energy of the lignin upon adsorption could be modified by the interaction of proteins with lignin. Soy proteins have been found to spread over the surface of lignin and change its water contact angle, resulting in water wettable lignin surfaces; the adsorption of the undenatured proteins onto lignin was more extensive than that of the denatured biomolecules[61]. Adding bovine serum albumin (BSA) lead to obviously increased hydrolysis levels via the adsorption of BSA onto the lignin surface, which reduced the amount of enzyme binding sites on lignin. Furthermore, the incorporation of hydrophobic lignin onto films will result in a further reduction in the relatively low nonspecific adsorption of BSA on cellulose[55, 83].

Lignin can also act as an adsorbent to adsorb metal ions and form lignin-metal complexes; the phenolic sites on the lignin surface have a higher affinity for metal ions than carboxylic sites[84-85]. Based the ability of lignin to adsorb metal ions, nonproductive enzyme adsorption was reduced by using Ca(II) and Mg(II) to form lignin-metal complexes, with a Ca(II) and Mg(II) concentration of 10 mmol/L almost completely eliminating nonproductive enzyme adsorption onto the lignin[86]. In another study, Mg^{2+} was found to be the best choice among eleven salts to improve the enzymatic hydrolysis of dilute acid-pretreated wheat straw. The phenolic hydroxyl group (Ph—OH) was the main active site blocked by Mg^{2+}; the strength of the interaction between Mg^{2+} and the Ph—OH groups of lignin monomeric structures followed the order *p*-hydroxyphenyl > guaiacyl > syringyl. The adsorption of Mg^{2+} on the lignin surface made the surface less negatively charged, which impaired the interaction between lignin and cellulase, leading to a reduction in the nonproductive adsorption of cellulase, as well as possibly weakening the hydrogen bonding and electrostatic attraction between lignin and cellulase[87].

In addition, lignin, as a renewable aromatic polymer, can be modified to obtain hydrophilic lignin additives, which are beneficial to improve the efficiency of enzymatic hydrolysis. Lou et al[88-89] demonstrated that sodium lignosulfonate and the copolymer of lignin and polyethylene glycol not only improved the cellulase activity on cellulose, but also decreased the nonproductive adsorption of cellulase on lignin. In another study, sodium lignosulfonate (SLS) was produced by the direct sulfonation of the spent liquor from the alkali pretreatment of lignin, and was used as a surfactant to improve the enzymatic saccharification of corn stover[90]. The effect of water-soluble lignin as an additive during the enzymatic saccharification of alkali-pretreated wheat straw was studied, and the enzymatic hydrolysis of pretreated solids was improved by the addition of the soluble fraction and sulfomethylated insoluble fraction of alkaline lignin[91]. The addition of sodium lignosulfonate (SL) and cetyltrimethylammonium bromide (CTAB) to pre-hydrolysates was found to enhance the enzymatic hydrolysis of lignocellulose, as CTAB increased the adsorption of SL onto lignin to provide more effective steric hindrance and reduce the non-productive adsorption of cellulase on lignin[92].

Amphoteric lignin additives can also improve the enzymatic hydrolysis of lignocelluloses. Lignosulfonate quaternary ammonium salt (SLQA), an amphoteric lignin surfactant, and dodecyl dimethyl betaine (BS12) were applied during the production of cellulosic ethanol, and were found to effectively enhance enzymatic hydrolysis and the subsequent ethanol yield[93]. Lin et al[94] also reported that the enzymatic hydrolysis of lignocellulose was improved by the use of lignin-based polyoxyethylene ether, a lignin-based amphiphilic polymer.

The role of surfactants, BSA, metal ions, and lignin-based hydrophilic surfactants in attenuating the nonproductive adsorption of enzymes on lignin supports the hypothesis that enzymes interact with lignin through hydrophobic interactions or hydrogen bonding and electrostatic interaction. All these additives could reduce the nonproductive binding between lignin and cellulase to some extent. However, there is a lack of research into the mechanism by which additives reduce

the adsorption of cellulase on lignin, which is necessary to provide a theoretical basis for the development of new additives. For example, in situ AFM could be used to analyze the interaction between additives and cellulase and lignocellulose.

11 Perspective and future direction

Most leading pretreatments of lignocellulosic biomass contribute to reducing its recalcitrance, which is beneficial to isolate lignin from the biomass and decrease the impact of lignin on cellulase hydrolysis. The different pretreatment methods also alter the structure and surface groups of lignin, resulting in different extents of nonproductive binding of cellulase with lignin. Although many technologies have been used to clarify how the interactions between cellulase and lignin change with reaction temperature, pH value, and time, the carbohydrate-binding modules (CBMS) and catalytic domain of cellulase also play important roles in the enzymatic hydrolysis of lignocellulose. Additionally, hydrophobic interactions, electrostatic interactions, and hydrogen bonds play various roles in the interaction between cellulase and lignin. The dominant interaction force between lignin and cellulase has been revealed to be hydrophobic interactions via AFM analysis. Lifshitz-van der Waals forces control the binding of CBM to Kraft lignin, but the role of hydrogen bonding and other forces or synergies among these forces in the inhibition of cellulase by lignin in lignocellulose hydrolysis requires further exploration and research. Furthermore, the structure of lignin is complex, and the synergy between the various functional groups of lignin plays an important role in the adsorption of lignin on cellulase. Therefore, the effect of lignin functional groups on the interaction between lignin and cellulase should be further explored. In addition, *in situ* AFM and LSCM could be used to better explore the interactions between lignin and cellulase.

Although researchers have invested significant efforts into reducing the nonproductive adsorption of lignin on cellulase, the fundamental mechanism behind these optimizations is still not fully understood. It is thus essential to develop a comprehensive understanding of the interactions between cellulase and lignin in order to develop higher efficiency pretreatment methods and more efficient surfactants to further improve the activity of cellulase and the conversion efficiency of biomass, decrease the inhibition of cellulase by lignin, and ultimately reduce the cost of lignocellulose conversion.

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