

Activation of Cellulosic Ethanol Lignin by Laccase and its Application as Plywood Adhesive

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To reuse biorefinery waste, cellulose ethanol lignin was treated with a laccase system and used as an adhesive for plywood panel preparation. The effects of the amount of laccase, the pH of the reaction system, the reaction temperature, and the reaction time on the bonding strength were studied. The reaction characteristics of the lignin were analyzed by Fourier transform infrared spectroscopy (FT-IR), scanning electron microscopy (SEM), and nuclear magnetic resonance (NMR). The results showed that the amount of laccase and the pH value of the reaction system had a significant effect on the bonding strength, and the addition of Tween-80 and polymeric diphenylmethane diisocyanate (PMDI) could improve the wet strength of the cemented system. FT-IR indicated that the lignin had been etherified and NMR analysis showed the partial ether bond in the β -O-4 structure was cleaved so that the lignin fragment was involved in the gluing of the small molecules. Microscopically speaking, the SEM analysis did not observe the activation of lignin adhesives infiltrating the wood substrate, indicating a weak mechanical gluing.

Keywords: Laccase-mediator system; Biorefinery residue; Wood adhesives; Lignin; Biomaterials

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INTRODUCTION

Lignin is a heterogeneous aromatic biopolymer that accounts for nearly 30% of the organic carbon on Earth, and it is one of the few renewable sources of aromatic chemicals. Basically, the highly-functionalized and aromatic nature of lignin is composed of *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, which are cross-linked by the carbon-carbon bonds, ether bonds, and other highly cross-linked chemical bonds (Rahimi *et al.* 2014). With a well-defined sequence of monomeric units, lignin is intimately interlaced with hemicelluloses in the plant cell wall, forming a matrix to cover the crystalline cellulose microfibrils. Its aromatic nature and complex structures make lignin degradation very difficult. Nonetheless, motivated significant research activity into the production of value-added chemicals, alternative fuels, and platform compounds from lignin has grown rapidly, and the preparation of value-added polymers derived from the lignin will also address the problem of waste lignin disposal (Li *et al.* 2015).

Cellulosic ethanol enzymatic hydrolysis lignin mainly comes from the by-products of bioethanol. Its molecular structure has the C3~C6 hydrophobic skeleton as well as phenol hydroxyl, alcoholic hydroxyl, carboxyl, and other active functional groups (Rahimi *et al.* 2014). This kind of residual lignin is different from traditional alkali sulfite and soda boiling pulp lignin, and it preserves the C-O structure because of avoiding the chemical processing (Ragauskas *et al.* 2014). This process is applicable for all wood, grass straw, rice husk, and other agricultural waste fermentation; thus, enzymatic hydrolysis lignin has

very broad applications (Bonawitz *et al.* 2014). After modification, the new desirable properties make it possible for lignin to be widely applied to surface-active agents, concrete admixtures, industrial adhesives, and many other fields. Nevertheless, the process of ethanol refining partly destroys the nature structure, which makes the lignin molecules lack strong hydrophilic functional groups, resulting in poor chemical reaction performance and limited application (Carrasquillo-Flores *et al.* 2013). To improve the application performance of lignin from cellulose ethanol enzymatic hydrolysis, modification methods are used. Commonly used modifications are purification and chemical polycondensation (Li *et al.* 2008). In recent years, the enzymatic method using laccase has been found to be advantageous because of its milder conditions and environmental friendliness, and applications of the laccase mediator system (LMS) have been studied extensively.

Laccase (EC1.10.3.2) is a polyphenol oxidoreductase (Halaburgi *et al.* 2011), which can react with lignin through single electron transfer. After the reaction, the produced free radicals are capable of disproportionation, coupling, and other enzymatic reactions. Laccase can also oxidize the phenolic hydroxyl group into phenoxy radicals. In this way, the lignin molecules generate a crosslinking structure, which is effective to produce self-bonding board (Mayer and Staples 2002). In this context, non-conventional processes based on the treatment of lignin with enzymes have been investigated for fiberboards production at laboratory and pilot scale (Widsten and Kandelbauer 2008). But for various reasons, the level of achievable auto-adhesion is insufficient to warrant the water resistance of conventional adhesives. Addition of a surfactant such as Tween 80 can positively impact lignin and increase the laccase activity, which is promotable for the bonding behavior of laccase-lignin system (Eriksson *et al.* 2002). On other hand, PMDI has been proved to give improvement of modulus of elasticity of the glyoxalated lignin and tannin adhesives which have low wood joint strength (Ping *et al.* 2012).

In this study, the cellulosic ethanol lignin was treated by the laccase mediator system, and then, the activated lignin mixed with the conventional additives to develop a plywood. The effect of laccase amount, the reaction pH, temperature and time were discussed. Tween-80 and PMDI were added to increase the wet shear strength, and the possible bonding mechanism was discussed.

EXPERIMENTAL

Materials

Laccase was commercial grade (Denilite II S) from Novozymes Inc., Guangzhou, China. Cellulosic ethanol lignin was purified from the corn stalk residue from an industrial biorefinery process and obtained from Henan Tianguan Group Co., Ltd., Zhengzhou, China. Poplar veneers (1.6 mm thickness) were purchased from Dunhua Forestry Industry Co. Ltd, Jilin, China. NaOH, acetic acid, sodium acetate, vanillin, and ABTS were analytical grade reagents and purchased from GUOYAO Group Chemical Reagents Co., Ltd, Beijing, China.

Lignin Adhesive Preparation

Lignin was added in the amount of 20 g per 100 mL of acetic acid/sodium acetate buffer with different pH values; 1 g vanillin and 5 mL of 0.28 mm/L ABTS were added. Different amounts of laccase were added to a 500 mL beaker in 40 to 60 °C thermostatic reaction for 2 to 4 h, with the air pump continuing to blow air and with continued stirring at a speed of 240 to 380 r/min.

Specimens Preparation and Test Standards

In this study, 50 parts of 20% solid polyvinyl alcohol, 20 parts of flour, and 5 parts of furfural were added to 100 parts of lignin adhesive. The cross-band veneer of poplar with a moisture content of 6 to 10% was spread on both sides with 0.04 to 0.05 g/cm² adhesive. The three-layer plywood was pre-pressured for 1 h and then hot pressed at 150°C and 1.5 MPa for 7 min. After hot pressing, the plywood was stored under ambient conditions for at least 12 h before testing, twelve plywood specimens (2.5 cm × 10 cm) were cut from the plywood. The dry shear strength was determined according to the GB/T 17657 (1999) standard, and the shear strength was calculated by Eq. 1.

$$\text{Shear Strength (MPa)} = \frac{\text{Tension force (N)}}{\text{Gluing area (m}^2\text{)}} \quad (1)$$

FT-TR

Infrared spectra were obtained using the KBr pellet method with a WQF-520 type infrared spectrometer (Beijing Rayleigh analytical instrument company, Beijing, China). The wave number range was 400 to 4000 cm⁻¹, the resolution was 4 cm⁻¹, and the scanning number was 16.

¹H- NMR and ¹³C- NMR Spectrometry Analysis

Lignin generally does not dissolve in chloroform, deuterated dimethyl sulfoxide (DMSO), or other common solvents. Therefore, the lignin was acetylated to enhance its solubility. For the acetylation of lignin, 1 g of lignin was combined with 30 mL of pyridine and acetic anhydride (1:2, v/v). The mixture was reacted in a dark place for 72 h. Then, the acetylated lignin was filtered and washed several times with 0.1N HCl to remove pyridine; then, they were washed with distilled water to remove HCl and then vacuum dried with P₂O₅. NMR spectra were recorded with a Bruker-Advance III 500 MHz spectrometer (Karlsruhe, Germany) at 25 °C while using DMSO-d₆ as the solvent under the optimal conditions reported by Capanema *et al.* (2004, 2005). The methoxyl group content of the lignin preparation was determined by using quantitative ¹H-NMR spectra (Zhao *et al.* 2017). The signal of aromatic protons is registered between 6.0 and 7.7 ppm, while the signals of the methoxyl group are registered between 3.3 and 4.1 ppm. The methoxyl content can be obtained from the following equation,

$$\% \text{OCH}_3 = 28.28436 - 19.750047x \quad (2)$$

where $x = \text{H (aromatic)}/\text{H (methoxyl)}$. The H (aromatic) and H (methoxyl) are integration values of the aromatic and methoxyl signals in ¹H-NMR spectra.

RESULTS AND DISCUSSION

Characterization of Chemical Properties

FT-IR analysis

Lignin infrared spectroscopy in the range of 1700 to 1000 cm⁻¹ is mainly reflected in the carboxyl, benzene ring, and ether bond, as well as other groups of the absorption peak. Figure 1 shows that the C = O vibration absorption intensity of lignin near to 1338 cm⁻¹ was gradually enhanced, indicating the phenolic hydroxyl groups of lignin etherified under the reaction of laccase. The increased methoxy group content was observed by the absorption peak generated near 1267 cm⁻¹. The absorption peak of the secondary alcohol

in the range of 1093 to 1080 cm^{-1} was reduced, and it was mainly due to the hydroxyl chain oxidizing to α -carboxy. Also, the phenolic hydroxyl absorption peak near 1219 cm^{-1} was weakened, which was due to the polymerization of phenol hydroxyl.

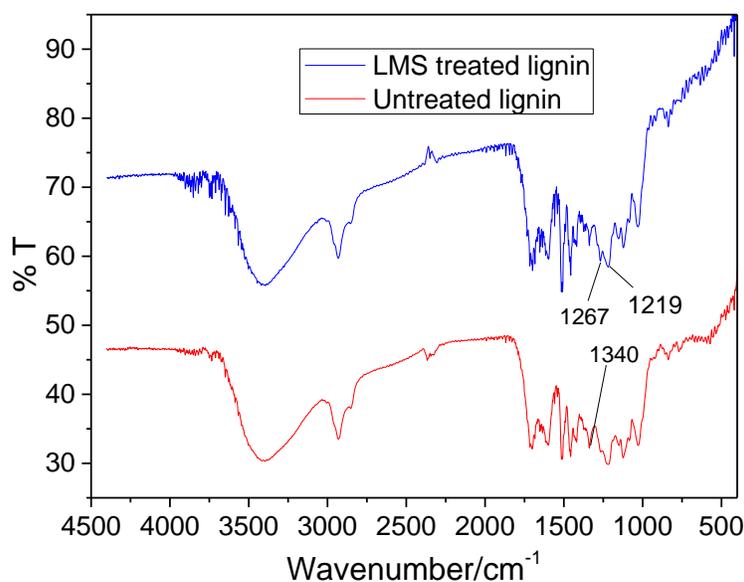


Fig. 1. The FT-IR spectrum of the laccase treated lignin

NMR analysis

After the acetylation, both ^1H -NMR and ^{13}C -NMR analysis of untreated and LMS treated cellulose ethanol enzymatic hydrolysis lignin samples were performed. According to the assignment of each respective signal, the corresponding functional groups were determined. The spectrum is shown in Figs. 2 and 3, the chemical shift assignment is shown in Table 1, and the commonly found interunit linkages are shown in Fig. 4.

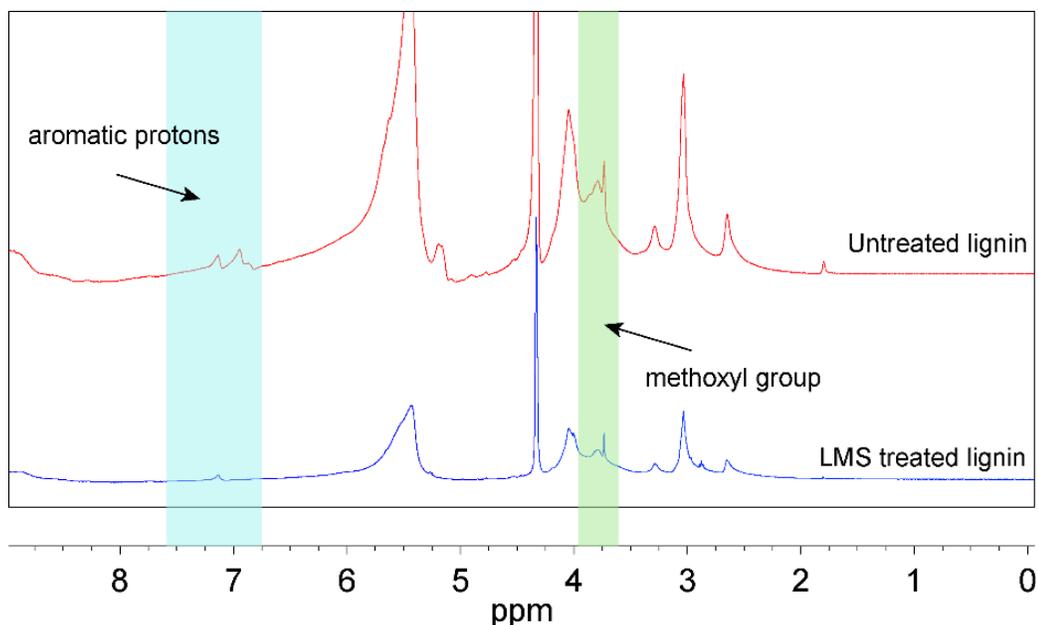


Fig. 2. The ^1H -NMR spectrum of the control sample and LMS treatment with acetylated cellulose ethanol enzymatic hydrolysis lignin sample

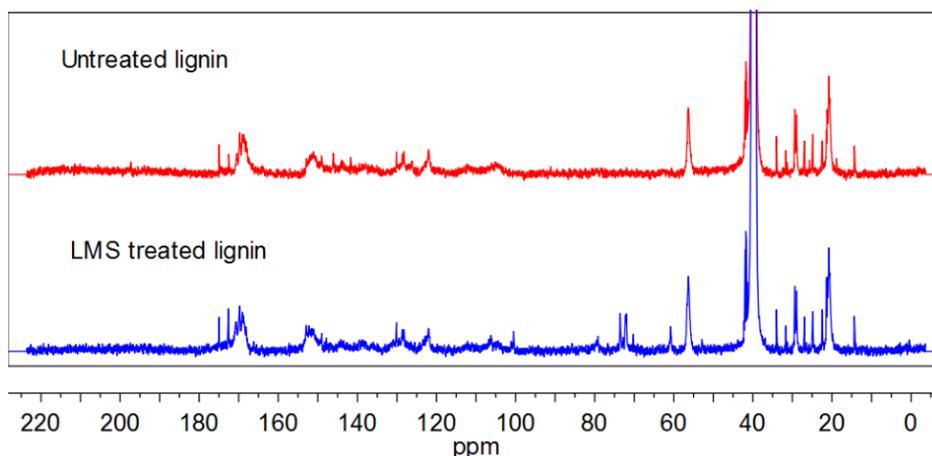


Fig. 3. The ^{13}C -NMR spectrum of the control sample and LMS treatment with acetylated cellulose ethanol enzymatic hydrolysis lignin sample

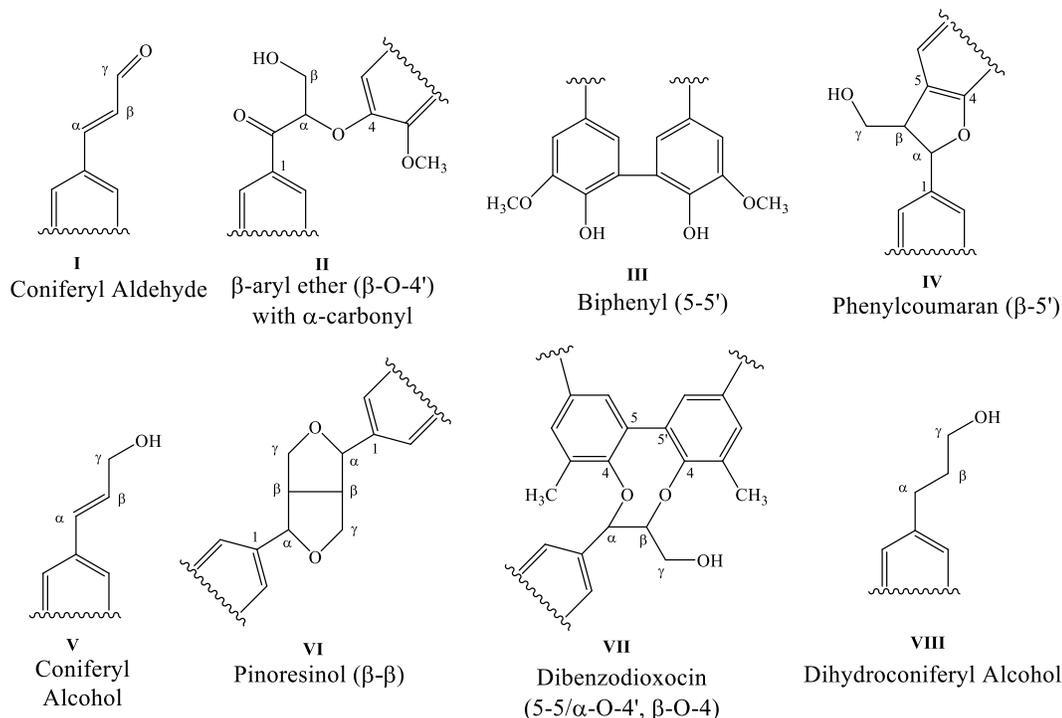


Fig. 4. Commonly found interunit linkages in acetylated cellulose ethanol enzymatic hydrolysis lignin preparations

The methoxyl group content on the benzene ring was decreased from 28.1 to 23.0%, as calculated by Eq. 2, and it was especially important for the application of lignin as an adhesive. After the laccase treatment, the lignin content of C=O increased, and this phenomenon might be due to the oxidation of the hydroxyl in the side chain. The signal intensity of C_β in β - β became weak, and the integral value of C_α and C_γ in β - β was reduced accordingly. This decrease in signal intensity reflected the β - β structure reduction. Because the reaction order of guaiacyl units and syringyl units was different, the carbon numbers of the G_5 - and G_6 - lignin were decreased but the S_2 - and S_6 - lignin increased (Martin-

Sampedro *et al.* 2011). After the laccase treatment, the relative integral values of C α in the β -O-4 structure were significantly decreased, which suggested that the LMS treatment was more advantageous to the partial degradation of the β -O-4 structure. As can also be seen in the NMR data, the signals of -CH $_2$ and -CH $_3$ in the unsaturated alkyl were greatly abated. It could be the fracture of the aliphatic side chain but also may be due to the methylation and oxidation of the hydroxyl side chain.

Table 1. Chemical Shift Assignment for the Non-Acetylated Spectrum of the Quantitative ^{13}C -NMR Experiment*

Chemical Shift δ (ppm)		Assignment
Control	LMS	
174.9	175.0	C=O in (II)
170.0	170.0	C=O in aliphatic ester
169.1	169.1	C=O in —OAc
152.8	153.0	C $_3$ /C $_3'$ in etherified (IV) C α in (II) C $_5$
149.1	149.0	C $_3$ in etherified G units
130.1	130.1	C $_1$ in nonetherified G units C $_5$ /C $_5'$ in etherified (IV)
129.2	129.2	C β in (I) C α and C β in (V)
122.1	122.0	C $_1$ and C $_6$ in α -C=O units
103.7	103.7	C $_2$ and C $_6$ in syringyl
-	72.1	C α in (VII)
60.7	60.7	C γ in (IV) and (VII)
56.3	56.3	—OCH $_3$
34.0	34.0	C α in (VIII)
31.7	31.7	C β in (VIII)
20.8	20.8	CH $_3$ group in acetylated xylan

*Data from Holtman *et al.* (2006)

Effect of Processing Parameters on Bonding Strength of Plywood

Effect of laccase addition on plywood strength

The amount of laccase added directly affected the degree of lignin activation and the shear strength of the plywood. As shown in Fig. 1, the shear strength of plywood showed a volcano-shaped tendency with the increase of laccase dosage. When the amount of laccase was little, the lignin reaction was not complete and there were not enough phenoxy free radicals to react with cellulose and hemicellulose to the produce lignin-carbohydrate complex. As a result, the plywood had poor shear strength performance, and evidence of debonding was observed.

When too much laccase was added, the lignin had an over-reaction, and the plywood came unglued in the hot pressing. As a consequence, the maximum of shear strength was 9.6MPa when the laccase additive amount was 9 g.

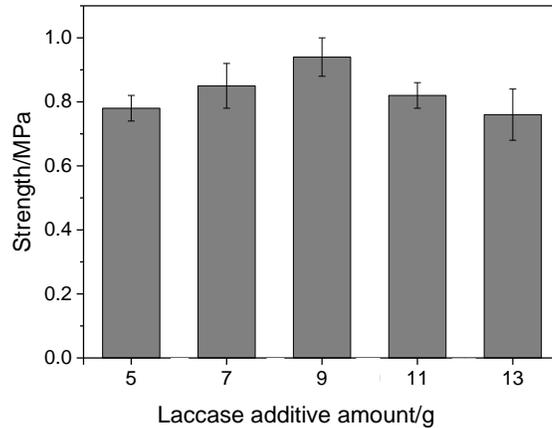


Fig. 5. The relationship between the amount of laccase and the bonding strength (pH = 4, reaction temperature = 50 °C, reaction time = 3 h, error bars represent standard deviations from twelve replicates)

Effect of pH on plywood strength

Extremely high or low pH values generally result in complete loss of activity for most enzymes. Laccase can have markedly different optimum pH ranges, depending on its source. At the optimum pH, the laccase is most suitable for the dissociation of the substrate, and the catalytic action takes place to maximize the reaction rate. Therefore, the pH value affects the enzyme activity and the gluing performance. As shown in Fig. 6, the optimum pH laccase used in the present work was 4.0, and the bonding strength reached the maximum of about 0.9 MPa.

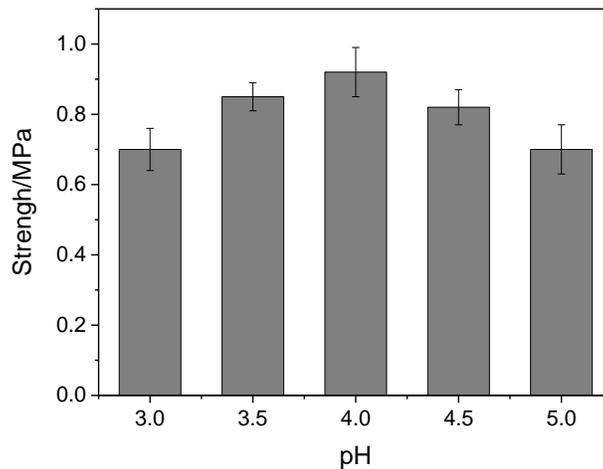


Fig. 6. The effect of pH value of reaction system and the bonding strength (laccase amount = 9 g, reaction temperature = 50 °C, reaction time = 3 h, error bars represent standard deviations from twelve replicates)

Effect of reaction temperature on plywood strength

As shown in Fig. 7, when the reaction temperature was 45 °C, the bonding strength reached the maximum of about 0.9 MPa. To some degree, a higher temperature resulted in a faster reaction rate. However, laccase as a copper protein, and excessively high temperature inactivates laccase, thus affecting the activation effect. At a low temperature, the temperature and the reaction rate showed a positive correlation. After the temperature

rose to a certain range, the laccase activity was dominant, and the reaction rate decreased with the increased temperature. Generally, the maximum reaction rate of laccase is different from the strains. The optimum temperature of laccase is also related to the reaction time. The laccase can withstand higher temperatures in a short time, but with the prolongation of reaction time, the optimum temperature will drop accordingly.

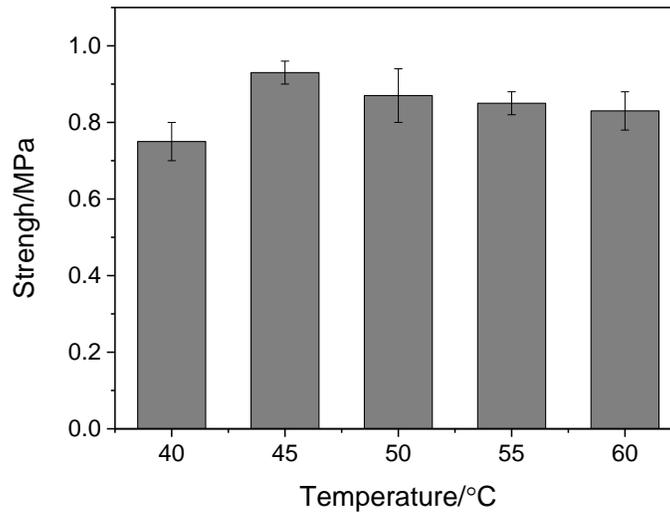


Fig. 7. The relationship between reaction temperature and bonding strength (laccase amount =9 g, pH = 4, reaction time = 3 h, error bars represent standard deviations from twelve replicates)

Effect of reaction time on plywood strength

As shown in Fig. 8, when the reaction time was 2, 2.5, 3, 3.5, and 4 h, the bonding strength increased first and then gradually approached a constant. The effect of reaction time for gluing strength was not significant, and the reaction time of the laccase was related to the catalytic substrate concentration and the catalytic efficiency. Consequently, the reaction time had no effect on the catalytic effect after the lignin reacted completely.

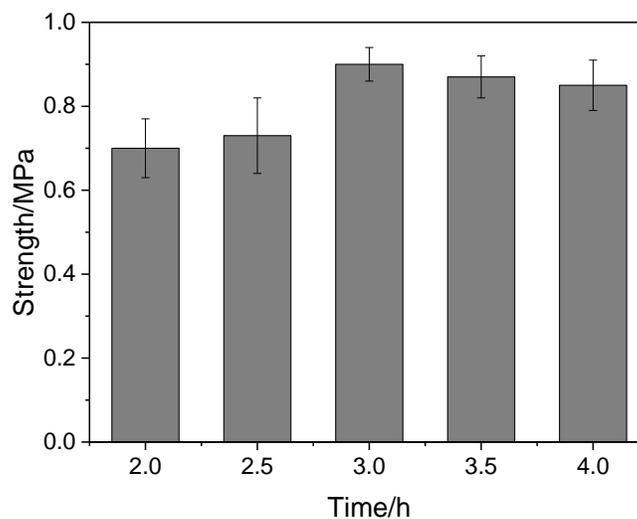


Fig. 8. The relationship between reaction time and bonding strength (laccase amount =9 g, pH = 4, reaction temperature = 50 °C, error bars represent standard deviations from twelve replicates)

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Enhanced water resistance experiments

The above discussion of lignin adhesive was based on the dry shear strength of wood specimens. When the laccase amount was 9 g, the pH was 4, the reaction temperature was 50 °C, and the reaction time was 3 h, the optimal shear strength of the plywood was 0.97 MPa. When the test pieces were put into 63 ± 3 °C water and boiled for 3 h, most of the specimens cracked and loosened the glue effect. To increase the water resistance of lignin adhesives, Tween-80 was added in the adhesive reaction phase and PMDI was added in the glue phase. Figure 9 shows that that the addition of Tween-80 and PMDI increased the wet strength of the lignin-based plywood. At low concentrations of Tween-80 (4 to 8%), the wet intensity changed elementarily and remained at a relatively high position. With increasing concentration, the wet strength of plywood decreased gradually. Actually, the low concentration of Tween-80 promoted the reaction between laccase and lignin. On the other hand, with increasing concentration, Tween-80 inhibited the reaction, which decreased the final wet strength. Furthermore, with the addition of PMDI from 4% to 16%, the gluing strength of plywood increased gradually from 0.4 MPa to 0.72 MPa, mainly due to the alcoholic hydroxyl groups contained in the lignin structure, which could be reacted condensation with the isocyanate compounds, resulting in the formation of lignin-polyurethane complex. The reaction between isocyanate and lignin is shown in Fig. 10.

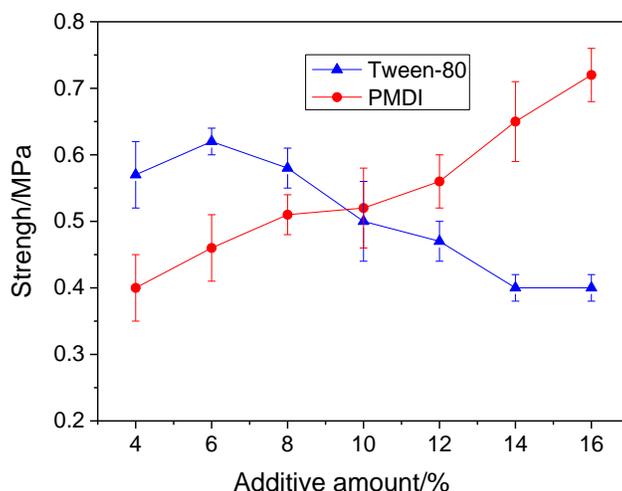


Fig. 9. The influence of different dosage of Tween-80 and PMDI to wet shear strength (The amount of laccase, the pH of the reaction system, the reaction temperature, and the reaction time. When the laccase amount was 9 g, the pH was 4, the reaction temperature was 50 °C, and the reaction time was 3 h; error bars represent standard deviations from twelve replicates)

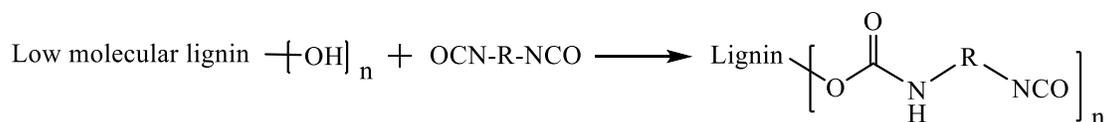


Fig. 10. The reaction of isocyanate with lignin

Study on Gluing Mechanism

SEM analysis

As shown in Figs. 11, untreated lignin particles were relatively smooth, the structure was compact, and the appearance was well maintained. In contrast, the surface of lignin particles treated by laccase was rougher, and granular material appeared, showing

part of the pits. These phenomena were caused by the activation reaction of the laccase with lignin, and the size of the pits and holes were closely related to the degree of activation. Figures 11 also shows that the activated lignin adhesive did not penetrate into the wood ray tissue in the chord and radial directions of the poplar substrate.

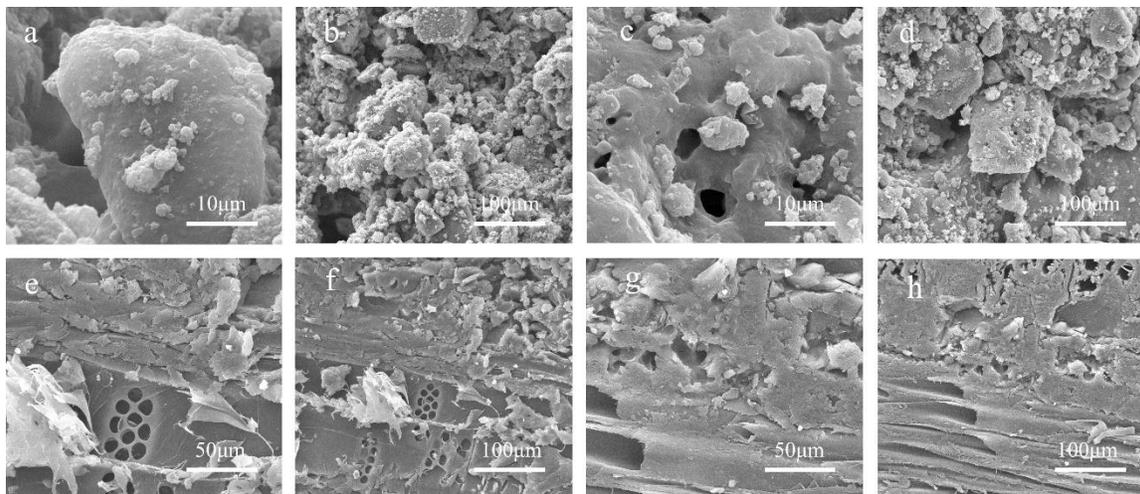


Fig. 11. The SEM micrographs at different magnification of treated lignin (a, b: untreated lignin, c, d: LMS treated lignin) and poplar plywood made by laccase treated cellulose ethanol lignin (e, f: chord-wise, g, h: span-wise). The laccase amount was 9 g, the pH was 4, the reaction temperature was 50 °C, and the reaction time was 3 h.

According to other studies of wood adhesives (*e.g.* PF resin and UF resin), SEM observations usually show the penetration of adhesives in the ray cell, thus achieving the gluing of wood (Bolton *et al.* 1988). In this study, penetration was not observed because the molecular structure and volume of lignin and UF or PF resin adhesive were different. Lignin is polymerized above the glass transition temperature. In the experiments, the failure rate of the activated lignin adhesive was low, indicating the weak mechanical gluing effect, which was consistent with the SEM analysis.

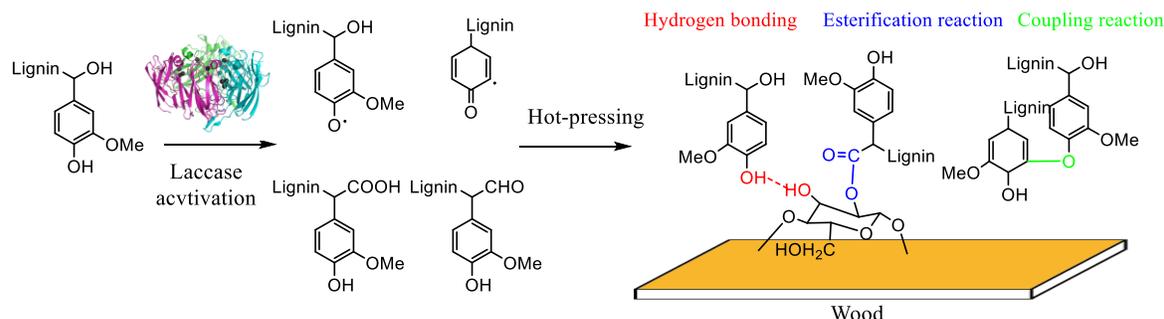


Fig. 12. The curing process of the adhesive

The reactivity of lignin can be improved by demethylating the lignin aromatic rings, and activated lignin can react with molecules bearing nucleophilic groups to form cross-linked complexes. It may also result in partial degradation of lignin and release monomeric subunits, which are also reactive toward other active groups and polymerize in the hot pressing. Based on the FT-IR and NMR analysis, the most likely specific reaction is shown in Fig. 12. After the laccase mediator system treated, parts of C-O bond cleavage, and some

of the phenolic hydroxyl groups were formed. Some hydroxyl groups on the side chain of lignin were oxidized to carbonyl groups by the laccase. During the hot process, the newly formed functional groups partly reacted with the hydroxyl groups on the surface of the wood, and the lignin polycondensation reaction occurred at the same time. In these reactions, the hydrogen bonding and esterification reaction between the activated lignin and wood surface, and the lignin coupling reaction were the mainly three reactions. Theoretically, the hydrogen bonding possibly dominated the bonding system, which was reflected by the poor wet shear strength.

CONCLUSIONS

1. The amount of laccase added and the pH value of the reaction system have a significant effect on the bonding strength, and the addition of Tween-80 and PMDI can improve the wet strength of the lignin adhesive specimen effectively.
2. During laccase treatment of lignin, the shedding of methoxy group and the breakage of β -O-4 structure increased the active functional groups of lignin molecules. The reduced molecular weight promoted the application of lignin in the field of wood adhesives.
3. The hydrogen bonding was the main joint form for the lignin-based wood adhesive.

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