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Catalytic polymerization of lignin model compounds using laccase and mediators

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Biotechnology and environmental effects concept make laccase important to biological characteristics during the processing of wood-based panels. Reactions performed by laccase are complex. The products generated in different phases affect the properties of wooden materials in different ways. The similarity and otherness between laccase system, laccase artificial mediator system and laccase natural mediator system in the same reaction conditions were researched in this paper. Lignin model compound was taken as a research object which was set in processing of wood-based panels. The results show that products polymerize by ether bonds in the laccase system and overlong reaction time causes benzene degradation following unsaturated olefins. However, products polymerize by hydrogen bonds and/or unsaturated bonds in the laccase mediator system and overlong reaction time causes benzene degradation following unsaturated carboxylic acids. Different bonding modes and products affect the physical and mechanical properties of wood-based panels. Moreover, natural mediator relative to artificial mediator has low cost, low toxicity and low catalytic efficiency.

Key words: laccase, mediator, guaiacol, ABTS, vanillin.

INTRODUCTION

Laccase was discovered in the 19th century in nature. Laccase plays multiple roles in synthesis and degradation (Yoshida 1883; Mayer and Staples 2002). Under the sponsorship of environmental friendly biotechnology, the biological properties of laccase have attracted more attention in chitosan, lignin and so on (Sharma and Kuhad 2008; Tetsch et al. 2006). Moreover, biotechnology and environmental effects concept make laccase important to biological characteristics during the processing of wood-based panels. In nature, the most abundant organic matter is cellulose, followed by lignin. Lignin is an aromatic high-molecular compound in the xylary tissues, and plays a role in sticking fibers (Li Jian and Luan Shujie 1993). Volatile organic compounds (VOC) in wood-based panels could be reduced through aromatic alcohol and phenol catalyzed by laccase (Thurston 1994; Reshma et al 2007). The oxidation-reduction potential of laccase is lower. Laccase can partly oxidize phenolic lignin, but not non-phenolic lignin. In order to get away from the restriction, the natural growth process of lignin was simulated and the results showed that some small molecules could effectively strengthen the catalysis of laccase to non-phenolic lignin. The system which contained small molecules and laccase was called laccase mediator system (LMS) (Kawai et al. 1987; Bruce and Palfreyman 1998). For now, there are artificial mediators (ABTS, HBT, NHA, etc.) and natural mediators (vanillin, syringaldehyde, sinapic acid, acetosyringone, etc.). Their catalytic mechanisms and catalyzing efficiencies are various (Moldeset al.2008; Tukayi et al. 2010; Kumarasamy et al. 2010; Atika et al. 2007). Lignin is a kind of complex phenolic polymer which consists of p-coumaric alcohol, coniferyl alcohol, 5-hydroxyl coniferyl alcohol and sinapic acid. Lignin model compound was selected as a research object for qualitative and quantitative analysis. The main structure units of lignin are guaiacyl (G) units, syringe (S) units and hydroxyphenyl (H) units. G units are widespread in hardwood, softwood and herb. The model compounds of G units have guaiacol, 4-methyl guaiacol, 4-vinyl guaiacol, 4-ethyl guaiacol, eugenol, etc. Catalytic mechanisms and catalyzing efficiencies of LMS to non-phenolic lignin have been researched by some scholars, but catalytic mechanisms and catalyzing efficiencies of LMS to phenolic lignin, especially for natural mediators had not been researched (Bourbonnais et al. 1998; Fabbrini et al. 2002; Baiocco et al. 2003). In this paper, guaiacol, ABTS and vanillin were taken as lignin model compound, artificial mediator and natural mediator, respectively, and were set in processing of

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wood-based panels. The reactions of guaiacol and products during the different phases were analyzed. Moreover, the effects of products in the different phases on the properties of wood-based panels were analyzed based on binding mechanism.

EXPERIMENTAL PROCEDURES

Chemicals

Laccase was kindly supplied by Wuhan Yuancheng Technology Development Co., Ltd. (China). The laccase (4000 units/g) was stored at -10^{o}C for further use. Guaiacol (2-metoxyphenol) was purchased from Sinophurm Chemical Reagent Co., Ltd. ABTS (2, 2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonate)) was supplied by Sigma-Aldrich Co. Vanillin (3-methoxy-4-hydroxybenzaldehyde) was supplied by Adamas Reagent Co. All other chemicals were of highest purity of analytical grade from commercial vendors.

LMS treatment of guaiacol

The guaiacol was dissolved in 0.1M acetate buffer (pH 4.5) with final concentration of 10 mmol/L as a target substrate. ABTS and vanillin were dissolved in the target substrate at 5% consistency (by mass of guaiacol). The solution was magnetically stirred at 45^{o}C in O_2 atmosphere (continuous bubbling) to ensure a sufficient supply of oxygen for the enzyme reaction. The enzymatic treatment was performed using 20 U/ml of laccase relative to the liquid volume for 60 min, 240 min, 600 min and 720 min. At pre-determined intervals, the reaction vials were removed and the reaction was stopped immediately by preserving at -10 ^{o}C and avoiding introducing impurities. As control, solutions were treated under the same conditions but without mediators.

UV analysis

To identify the products and reaction rate of guaiacol transformation, especially the effects of mediators, the reaction mixture was assayed by UV-Visible spectrophotometry (unit type: TU-1900 PC). Due to the overlap of characteristic absorption wavelength between guaiacol, ABTS and vanillin, the multi-composition measurement was performed according to additivity of absorbance and Lambert-Beer Law. To measure molar absorption coefficients, standard solutions of guaiacol, ABTS and vanillin were prepared as described in 2.2. Test samples were diluted at 1:50 (v/v), and detection wavelength was from 200 nm to 600 nm. Regulations were analyzed on the basis of absorbancy and Lambert-Beer Law.

FTIR analysis

The changes of molecular structures were qualitatively characterized by using infrared spectroscopy (unit type: MAGNA-IR560 E.S.P). The liquid sample was pre-processed by liquid membrane. All spectra were measured at a resolution of 4cm^{-1}, and 40 scans per sample were recorded. Sampling gain was 1.0. The spectra were baseline corrected and peak heights of the IR bands were measured using OMNIC software.

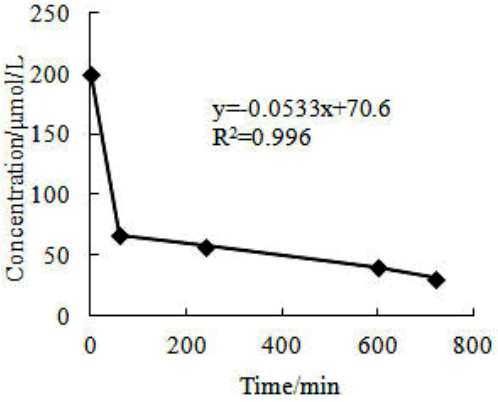

Fig. 1. Concentration of guaiacol treated by laccase with time.

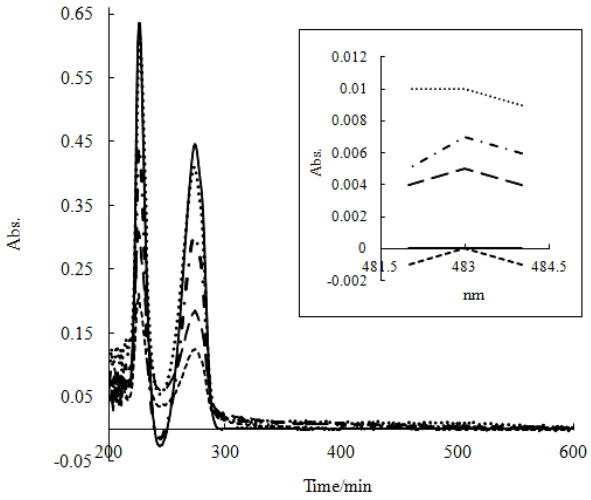

Fig. 2. UV spectra of guaiacol treated by laccase with time.

RESULTS AND ANALYSIS

Laccase treatment of guaiacol

The experiment was conducted as described in 2.2 without mediators. The color of the solution was from purple to colorless. The changes of guaiacol concentration are shown in Figure 1 on the basis of Lambert-Beer Law. The guaiacol concentration was sharp down within 60 min, followed by linear decrease. In Figure 2, the absorbancy of B belt and E belt decreased with the extension of reaction time.

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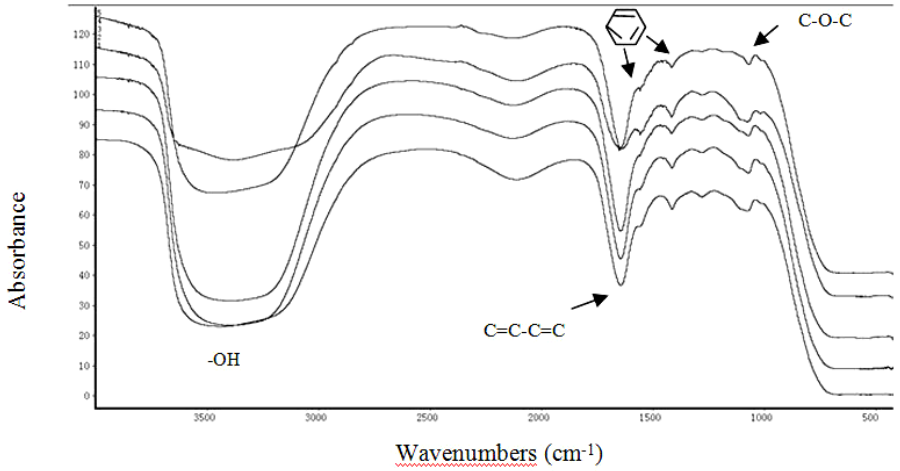

Fig. 3. FTIR spectra of guaiacol treated by laccase with time.

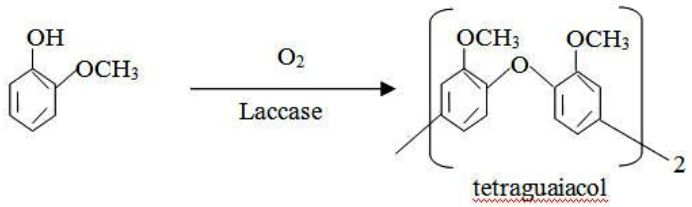

Fig. 4. Reaction process of guaiacol treated by guaiacol.

The main product was tetraguaiacol (Figure 4). The reaction mechanism included generation of phenoxy radicals by the action of laccase and oxygen, and creation of tetraguaiacol by cross-linking reaction. The characteristic absorption wavelength of tetraguaiacol was 484 nm which would degrade with time. Figure 2 also shows that the concentration of the product was max in 60 min, and gradually decreased to 0 in 720 min. However, there was no absorbency of the degradation product which declared that product was a saturated hydrocarbon or a conjugated alkene. Figure 3 shows that the peak intensity of hydroxyl significantly decreased, and peak intensity of ether strengthened in 60 min. This proved that guaiacol was catalyzed into phenoxy radical, and then cross-bonding took place between phenoxy radicals. With time, the peak intensity of ether decreased and the peak intensity of C=C increased. This further specified that the benzene ring was cracked and generated conjugated alkenes.

Laccase-ABTS system treatment of guaiacol

The color of the solution was aubergine in the initial stage, and gradually disappeared with time. UV absorption wavelengths of ABTS were 228 nm and 340 nm. UV absorption wavelengths of guaiacol were 226 nm and 274 nm. The concentration of guaiacol decreased gradually with the reaction time (Figures 5 and 6). The degradation rate of guaiacol was slower than that without mediator in the first 60 min. Then the degradation rate linearly diminished, but quicker than without mediator. Moreover, there were new absorption peaks at 303 nm and 488 nm which declared that new compounds were generated. On the basis of mechanism analysis and experienced wavelength calculation, 303 nm was the featured wavelength of 2-methoxy-para-benzoquinone, and 488 nm was the featured wavelength of 3,3'-dimethoxy-4,4'-biphenoquinone (Figure 8). There were exceptional values at 720 min in Figure 6 which showed that a new compound was created and its absorption band overlapped with E belts of ABTS and guaiacol. This is due to cracking of 2-methoxy-para-benzoquinone into 2-methoxy-butene diacid (Figure 8), verified by the IR spectrum (Figure 7). In the first 60 min, the peak intensity of hydroxyl decreased, meaning that guaiacol was catalyzed into phenoxy radical. The peak intensity of benzene ring decreased, meaning that benzene ring was degraded. The peak intensity of ABTS decreased, meaning that ABTS reacted with laccase and oxygen. The characteristic absorption peaks at 1645 cm^{-1} and 1663 cm^{-1} were C=O of 3,3'-dimethoxy-4,4'-biphenoquinone and

2-methoxy-para-benzoquinone, respectively. With time, the peak intensity of ABTS gradually increased. In 240 min, guaiacol was transformed into 2-methoxy-benzenediol and 3,3'-dimethoxy-4,4'-dioxydiphenyl which strengthened the peak intensity of hydroxyl. Furthermore, 2-methoxy-benzenediol and 3,3'-dimethoxy-4,4'-dioxydiphenyl generated during prior period were transformed into 3,3'-dimethoxy-4,4'-biphenoquinone and 2-methoxy-para-benzoquinone, so the peak intensity of 1645 cm^{-1} and 1663 cm^{-1} increased and the peak intensity of benzene ring continually decreased. In 600 min, the formation reactions of 3,3'-dimethoxy-4,4'-biphenoquinone and 2-methoxy-para-benzoquinone were dominant with the decrease of hydroxyl and benzene ring. Some 2-methoxy-para-benzoquinone was cracked into 2-methoxy-butene diacid in 720 min. During the whole reaction, the peak intensity (1104 cm^{-1}) of the intermediate product was constant after 60 min which illustrated that reaction speed was constant.

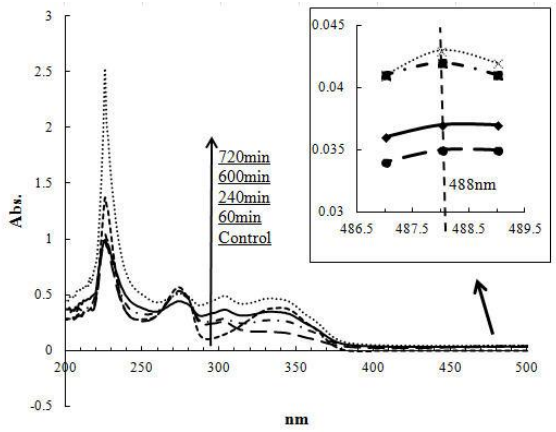

Fig. 5. Absorption of guaiacol treated by laccase-ABTS system with time.

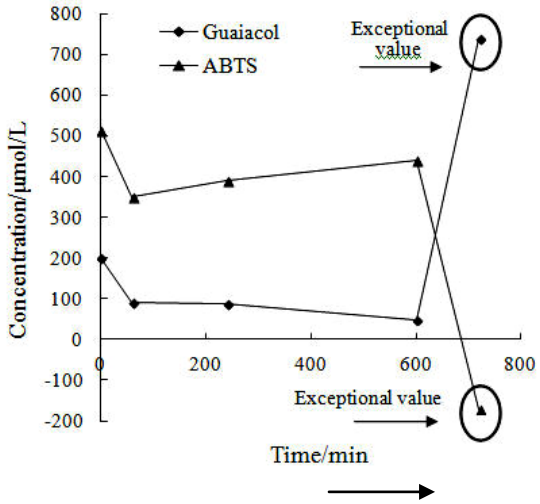

Fig. 6. Concentration of guaiacol and ABTS with time.

Laccase-vanillin system treatment of guaiacol

The color of the solution was aubergine in the initial stage, and gradually disappeared with time. UV absorption wavelengths of vanillin were 226 nm, 279 nm and 309 nm. UV absorption wavelengths of guaiacol were 226 nm and 274 nm. Due to the overlap of substrate and products and the unknown product concentration, only qualitative analysis was performed. It is shown in Figure 9 that vanillin was transformed into intermediate under the effect of laccase and oxygen on the basis of transfer reaction of hydrogen atom. Then, guaiacol was transformed into phenoxy radical by intermediate, and intermediate was reverted to vanillin by absorbing a hydrogen atom. After that, the phenoxy radical was transformed into 2-methoxy-benzenediol and 3,3'-dimethoxy-4,4'-dioxydiphenyl. Then 2-methoxy-benzenediol and 3,3'-dimethoxy-4,4'-dioxydiphenyl were transformed into 2-methoxy-para-benzoquinone and 3,3'-dimethoxy-4,4'-biphenoquinone, respectively. UV absorption wavelengths of 2-methoxy-benzenediol were 267 nm and 306 nm. UV absorption wavelength of 2-methoxy-para-benzoquinone was 303 nm. UV absorption wavelength of 3,3'-dimethoxy-4,4'-biphenoquinone was 488 nm. With time, 2-methoxy-para-benzoquinone was cracked into 2-methoxy-butene diacid. UV absorption wavelength of 2-methoxy-butene diacid was 223 nm. Before 60 min, the reaction of vanillin and laccase was dominant. With time, vanillin reverted, followed by strong absorption of products, verified by the infrared spectrum. There existed a competitive relation between the reaction of -OH and the reaction of C=O all the while. The peak intensity did not change from 60 min to 600 min which showed that the reactions of –OH and C=O were in equilibrium. However, the peak intensities of –OH and benzene ring increased at 1645 cm^{-1} in 720 min. This means that the formation reactions of 2-methoxy-benzenediol and 3,3'-dimethoxy-4,4'-dioxydiphenyl were dominant. After 720 min, the enhancement at 1104 cm^{-1} revealed that 2-methoxy-benzenediol and 3,3'-dimethoxy-4,4'-dioxydiphenyl were generated during this phase. During the whole reaction, the quantities of 2-methoxy-para-benzoquinone and 3,3'-dimethoxy-4,4'-biphenoquinone were small.

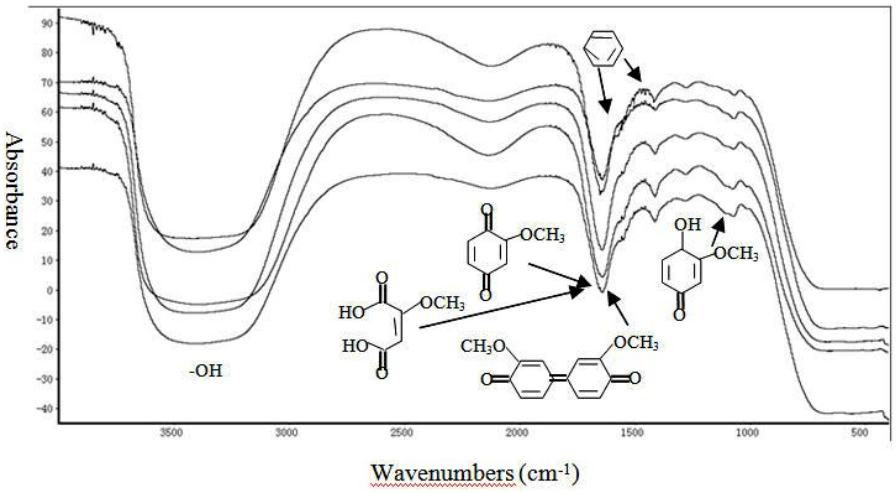

Fig. 7. FTIR spectrum of guaiacol treated by laccase-ABTS system with time.

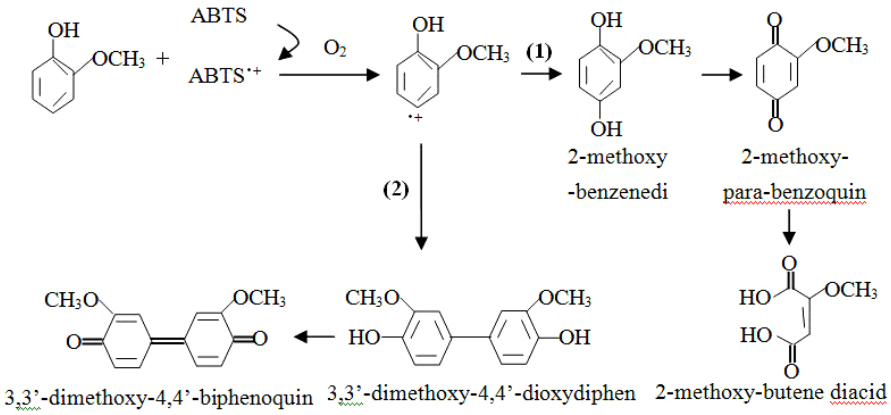

Fig. 8. Reaction process of guaiacol treated by laccase-ABTS system.

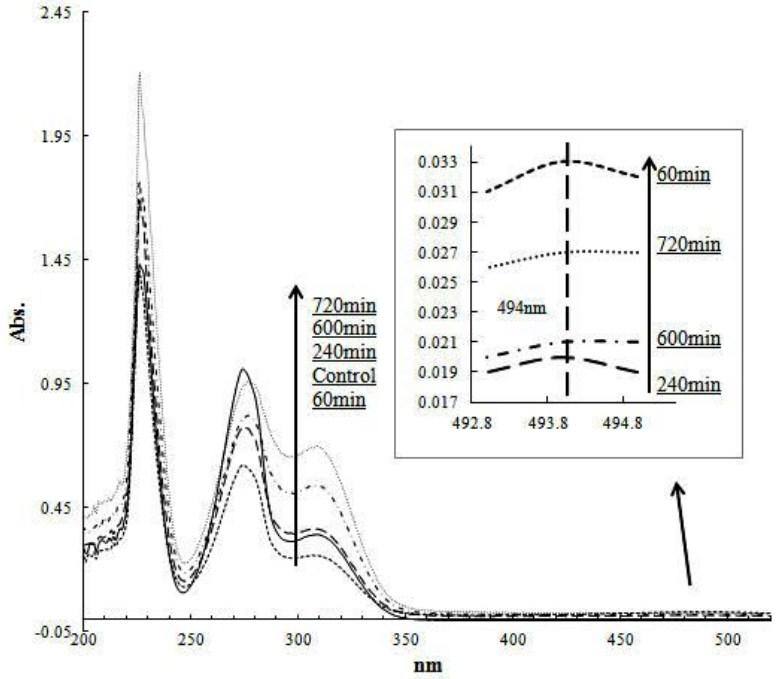

Fig. 9. Absorption of guaiacol treated by laccase-vanillin system with time.

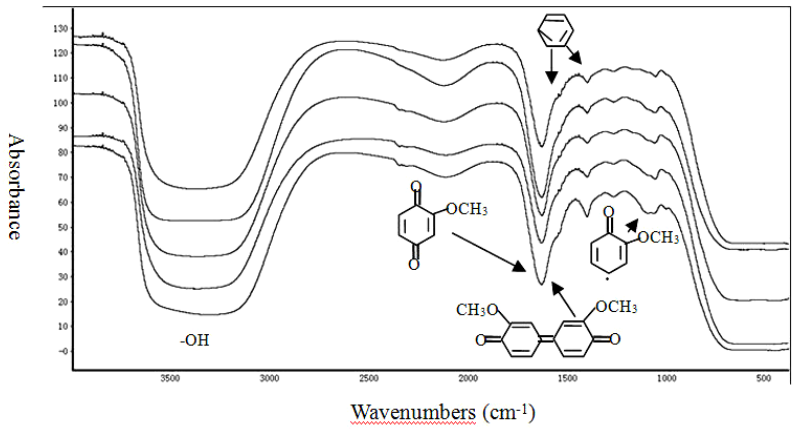

Fig. 10. FTIR spectrum of guaiacol treated by laccase-vanillin system with time

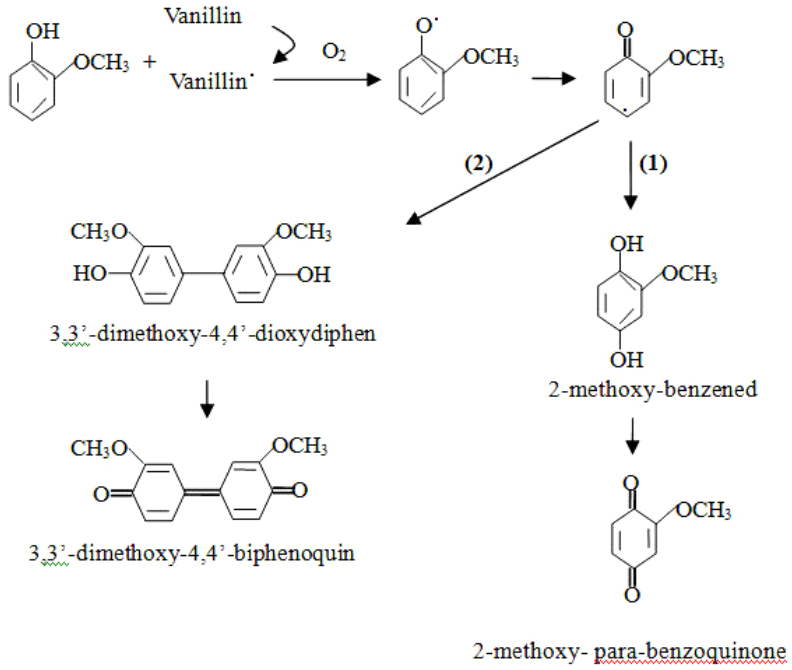

Fig. 11. Reaction process of guaiacol treated by laccase-vanillin system.

Comparative analysis of the different systems

In the laccase system, guaiacol was directly transformed into phenoxy radical, followed by polymerization with ether bond. The product was cracked into unsaturated alkene with time because of the unstability of ether bonds. In the laccase-ABTS system, ABTS reacted preferentially under the action of laccase and oxygen. Then, guaiacol was transformed into 2-methoxy-benzenediol, 2-methoxy-para-benzo-quinone, 3,3'-dimethoxy-4,4'-dioxydiphenyl, 3,3'-dimethoxy-4,4'-biphenoquinone and 2-methoxy-butene diacid by transfer of electron. The main product and the ratio of products were different during the different phases. In the laccase-vanillin system, vanillin reacted preferentially under the action of laccase and oxygen. Then, guaiacol was transformed into 2-methoxy-benzenediol, 2-methoxy-para-benzo-quinone, 3,3'-dimethoxy-4,4'-dioxydiphenyl, 3,3'-dimethoxy-4,4'-biphenoquinone and 2-methoxy-butene diacid by transfer of hydrogen atom. The main product and the ratio of products were different during the different phases. This showed that the reaction mechanisms of laccase-ABTS system and laccase-vanillin system were different with the same products. Furthermore, the reaction rate of the laccase-vanillin system was slower than that of the laccase-ABTS system.

Comparing the laccase system with th laccase

mediator system, the products were different. The main reaction proceeded by ether bond in the laccase system. However, guaiacol was transformed into quinine, acid and so on in the laccase mediator system. The color of the solution changed from purple to aubergine due to quinine which was the main factor to cause the yellow color.

CONCLUSIONS

To ensure the quality of wood-based panels treated by a laccase system, three conditions must be observed: (1) the surface density of lignin must be sufficient in the raw materials; (2) the process time must be kept within 600 min to prevent lignin degradation; (3) a proper amount of water-resistance agents should be added to overcome the instability of the ether bond.

For the laccase-ABTS system applied in processing of wood-based panels, the quality of wood-based panels was discrepant depending on quinone, phenol, acid and ketone. In the first 60 min, phenoxy radicals were the main product which had no observable effect on the quality of wood-based panels. During the phase from 60 min to 240 min, phenol was the main product which could enhance the dry strength by hydrogen bonds, but had no effect on the wet strength. During the phase from 240 min to 600 min, quinone and ketone were the main products which could enhance both dry strength and wet strength by unsaturated bonds. However, elastic modulus of wood-based panels decreased because of lignin degradation from 600 min to 720 min.

The products of laccase-vanillin system and laccase-ABTS system were similar, but the production rates were different. From 0 min to 600 min, formation and transformation of intermediates was slow. During the phase from 600 min to 720 min, phenol was the main product with small amounts of quinone and ketone. If the reaction continued, a mass of quinone and acid would be generated. This showed that catalytic efficiency of vanillin was significantly lower than that of ABTS at the same concentration. However, vanillin had advantages as regards economic cost and toxic properties.

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КАТАЛИТИЧНА ПОЛИМЕРИЗАЦИЯ НА ЛИГНИНОВИ МОДЕЛНИ СЪЕДИНЕНИЯ С ИЗПОЛЗВАНЕ НА ЛАКАЗА И МЕДИАТОРИ

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(Резюме)

Концепцията на биотехнологиите и опазването на околната среда дава възможност за пълно използване на лаказата за биологично изработване на панели на дървесна снова. Продуктите, генерирани в различни фази от процеситевлияят различно на свойствата на дървесните материали. Разгледани са подобието и разликите в изкуствените и естествените медиаторни системи с лаказапри еднакви реакционни условия. Като обект на изследване за получаването на панели на дървена основа са взети моделни лигнинови съединения. Резултатите показват, че продуктите полимеризират чрез етерни връзки и при продължителна реакция се стига до разлагане на бензен с образуване на олефини. От друга страна продуктите на полимеризацията чрез водородни връзки и/или ненаситени връзки в медиаторната система на лаказата води до ненаситеникарбоксилни киселини. Различните типове химични връзки и продукти влияят различно на физичните и механичните свойства на панелите. В сравнение с изкуствените медиатори, естествените имат ниска цена, ниска токсичност и ниска каталитична активност.