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ESR Study in Reactive Oxygen Species Free Radical Production of *Pinus kesiya* var. *langbianensis* Heartwood Treated with Laccase

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Abstract. Enzymatic oxidation of lignin phenolic hydroxyl groups can enhance the level of autoadhesion between wood fibers or particles depending upon the bonding mechanism of wood-based materials without synthetic adhesives such as urea and phenol formaldehyde. The adhesive effect is caused by an increased number of reactive oxygen groups at the fiber surface. The parameters of laccase-treated wood fibers play vital roles in generating reactive oxygen species (ROS) free radicals. Laccase I (white-rot fungi) and laccase II (*Aspergillus* sp.) are used to catalyze the oxidation of heartwood powder of *Pinus kesiya* var. *langbianensis* in suspension under different pH values, temperatures, treatment times and different laccase concentrations. Electron spin resonance spectroscopy and spin trapping technique were used to detect the ROS free radicals generated in the laccase-treated heartwood powder and it was found that in it the concentration of ROS free radicals was higher than that in the control. Analysis of variance indicates that there was a significant difference between the ROS concentration values of laccase-treated heartwood powder under different pH values, treatment temperatures and times, and laccase dosages for both laccase I and II. Furthermore, the concentration of ROS free radicals generated by laccase I is higher than that generated by laccase II. It was found that the optimum conditions for generation of ROS in laccase-treated heartwood powder by the two kinds of laccase are pH 3.0; treatment temperature, 50 °C; treatment time, 2 h; enzyme concentration, 20 units/g of wood powder.

1 Introduction

Laccase is one of few enzymes that has been a subject of study since the end of the last century. Laccase enzymes are glycoproteins found in nature in white-rot fungi, trees and other higher plants, bacteria and insects [1, 2]. It can be used for oxidation of a broad range of aromatic and amino compounds. Laccase is a type of copper-containing polyphenol oxidase and subsequently was demonstrated as an oxidreductase enzyme [3]. These multicopper oxidases are able to catalyze the one-

electron oxidation of phenols and polyphenols, such as lignin, to phenoxy radicals by molecular oxygen while oxygen is reduced to water [4]. It can be applied in fiberboard manufacturing as a partial or complete substitute for formaldehyde or cyanide glue [5]. As a natural polymer, wood will produce free radicals after it is attacked by some exoteric functions. Some kinds of free radicals exist on the exterior wood at room temperature and they are from lignin, cellulose and hemicellulose. The free radical concentration in lignin is the highest among above-mentioned three main components of wood. These free radicals play an important role in the wood processing and protection. The mechanical properties of wood-based panels just treated with laccase without a bonding agent are higher than that of the control without laccase treatment [6–9]. However, few studies were carried out on the change of reactive oxygen species (ROS) concentration in woods. The purpose of this work is to find the optimum parameters for the processing of laccase-treated wood powder which can be used in industrialized manufacture in the future.

2 Materials and Methods

2.1 Materials

Chemicals. *N-tert*-butyl-α-phenylnitrone (PBN) purchased from Sigma Chemical Co. is used as a spin trap, ethyl acetate is used to extract ROS-PBN spin adduct. Other chemicals purchased in China are of analytical grade.

Wood Powders. Wood fiber of *Pinus kesiya* var. *langbianensis* was obtained by Yunnan Jinggu Forestry Co., Ltd., China. Wood powder was made of heartwood with 60–80 meshes.

Enzyme. Enzyme activity was performed in units. One unit is the amount of activity which under standard conditions oxidizes 1 μmol of 2,2-azino-bis-3-ethylbenzo-thiazoline-6-sulfonic acid per minute in sodium tartaric or tartaric acid buffer at pH 4.0 and 30 °C. A Shinadsu UV-2501 ultraviolet and visible spectrophotometer with a measurement wavelength of 420 nm was used.

White-rot fungi laccase was obtained from the Institute of Microbiology, Chinese Academy of Sciences (laccase I, enzyme activity of 20 units/ml) and *Aspergillus* laccase was bought from Novozymes (China) Investment Co., Ltd., Beijing (laccase II, enzyme activity of 280 units/ml). The pH values of buffer mixed with both acetic acid and sodium acetate are 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0.

2.2 Methods

ROS is a general designation of a kind of free radical. It is very difficult to detect it in water solution. The method by Cao et al. for detecting ROS free radicals [10] is used in this work. PBN is used as a spin trap, ROS are detected by electron spin resonance (ESR) from the ROS-PBN spin adduct extracted by ethyl acetate at room temperature.

Wood Powder Treatment. Treatment of the wood powder is made at 5% consistency in an aqueous suspension. Temperature and pH of the suspension are 26 °C and 3.0, respectively. To ensure a sufficient supply of oxygen for the enzyme reaction, the water must be saturated with dissolved atmospheric air at the beginning of each experiment.

Enzyme Treatment. Two kinds of laccase are applied in each experiment. Treatment of the wood powder is set at 5% consistency in an aqueous suspension. Laccase is added at a dosage of 20 units/g of fiber dry substance, and 50 µl PBN (0.04 M) is added, too. The pH values of buffer are 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0. The suspension is treated in water bath at 26 °C for 2 h in order to find the optimum pH value for laccase-treated wood powder. Then equal amounts of laccase and PBN are added into the suspension. pH value of the suspension adjusted by buffer is 3.0, and treatment temperatures are 30, 40, 50, 60, 70 and 80 °C. Finally the same amounts of laccase and PBN are used in this step. pH and temperature of the suspension are 3.0 and 50 °C, treatment times are 1, 2 and 3 h. The suspension is treated at the following conditions: pH 3.0; treatment temperature, 50 °C; treatment time, 2 h; laccase dosages, 10, 20 and 30 units/g, respectively.

ROS Free Radical Measurement. After treatment the suspension was placed into an ice pile in order to immediately stop the reaction between laccase and wood powder. Ethyl acetate is added into the suspension in order to extract a ROS-PBN adduct.

The ROS free radical activity of laccase-treated heartwood powder was measured at room temperature with an X-band ESR spectrometer (Bruker ER200D-SRC). The ESR conditions are as follows: X-band; modulation frequency, 100 kHz; modulation amplitude, 3.2 G; microwave power, 20 mW; central magnetic field, 3385 G; scanning range, 400 G; scanning time, 200 s. The central magnetic field was determined according to the method described by Cao et al. [10]. Three replications under the same conditions are needed. The mean height of three peaks in each signal was taken as the ROS relative concentration value (mm).

3 Results

3.1 ESR Spectra of ROS Concentration of Untreated and Laccase-Treated Heartwood

Three typical ESR spectra of control and laccase-treated wood powder are shown in Fig. 1. The ESR spectrum of heartwood in Fig. 1a shows the kind of classic phenoxy radicals without hyperfine structure due to the heterogenous chemical structure of fibers. This spectrum is consistent with the conclusion of Li [11]. However, ESR spectra in Fig. 1b and c are completely different from that in Fig. 1a because they both have a g-value of 2.005 and a_N of 15.0 G, which is consistent with a triplet of the PBN-ROS signal at $g = 2.005$ with $a_N = 15.0$ G [10].

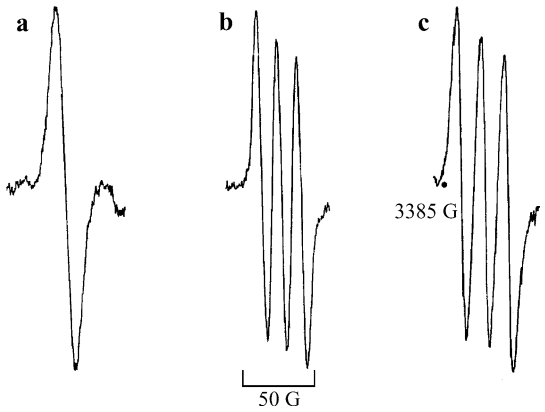

Fig. 1. ESR spectra of ROS in untreated wood (**a**) and wood treated (pH 3.0, 50 °C, 2 h) with laccase I (**b**) and laccase II (**c**).

3.2 Effect of Different pH Values on ROS Concentration in Wood Treated with Laccase

No ROS signal had been detected by ESR at pH 2.0. In the range of pH values from 2.5 to 6.0 (Fig. 2) ROS concentrations of wood powder treated with both laccase I and II significantly fluctuate with changing pH value ($P < 0.01$). The maximum values of ROS concentrations are obtained at pH 3.0 for both laccase I and II. Then the signals decrease with increasing pH until 7.0, indicating that pH 3.0 is the optimum to obtain the signal from laccase-treated wood powder in this experiment.

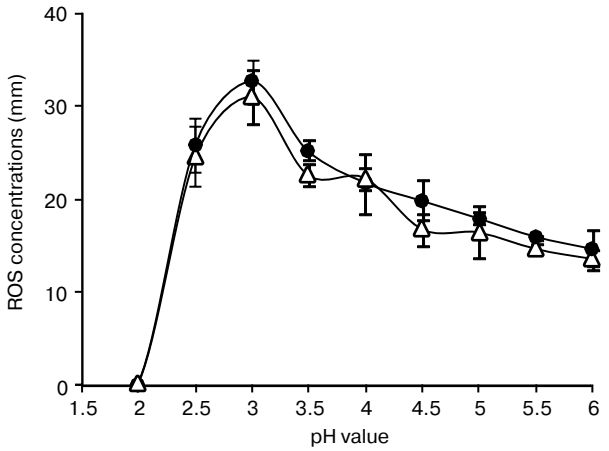

Fig. 2. Changes in the ROS concentration at different pH values: laccase I (△) and laccase II (●).

3.3 Effect of Different Temperatures on ROS Concentration in Wood Treated with Laccase

The changes of the ROS concentration generated from the two kinds of laccase-treated heartwoods at different temperatures are shown in Fig. 3. It can be found that the ROS concentration synchronously increases with the temperature increased from 30 to 50 °C and then decreases at temperatures from 50 to 80 °C. Under the same temperature treatment the effect of laccase I is significantly better than that of laccase II ($P < 0.01$). The optimum temperature for the generation of ROS in laccase-treated wood is about 50 °C in this experiment.

3.4 Effect of Different Laccase Concentrations on ROS Generation in Wood Treated with Laccase

According to our previous research we are sure that laccase dosage is an important factor in processing laccase-treated heartwood powder. When laccase I dosages of 10, 20 and 30 units/g are applied in different experiments, the corresponding ROS concentration values of laccase-I-treated wood powder are reached at 182.0, 332.2 and 137.8 mm, respectively. On the other hand, ROS concentration values of laccase-II-treated heartwood powder are 96.3, 197.8, and 153.2 mm for dosages of 10, 20, and 30 units/g, respectively. Calculating the above data we can conclude that the effect of laccase-I-treated wood powder is better than that of laccase-II-treated wood powder, and the maximum ROS concentration values appear at 20 units/g for both laccase I and II. Analysis of variance (ANOVA) indicates a significant difference between the laccase II dosage and ROS concentration values at $P = 0.01$. However, there is no significant difference between the laccase I dosage and ROS concentration values at $P = 0.01$. For laccase I and II, the optimum dosages are both 20 units/g of wood powder in this experiment.

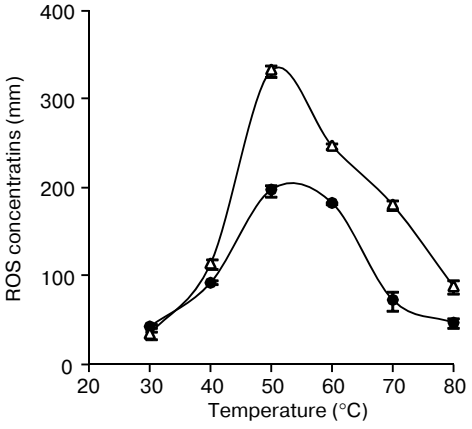

Fig. 3. Changes in the ROS concentration at different temperatures: laccase I (△) and laccase II (●).

4 Discussion

Laccase (EC 1.10.3.2), copper-containing polyphenol oxidase, is chosen for bonding fibers or particles because it can catalyze the one-electron oxidation of phenolic hydroxyl groups while reducing oxygen, yielding phenoxy radicals and water [5]. Petri et al. [4, 12] suggest that a positive correlativity exists between the internal bonding (IB) strength value of fiberboard and the concentration of free radicals with the amount of oxygen cost during the reaction process. ROS free radicals play a vital role in processing and manufacturing wood-based materials to enhance the level of autoadhesion between wood fibers or particles without synthetic adhesives, such as urea and phenol formaldehyde. Claus et al. [13] consider that the bonding effect is caused by the catalytic effect of enzyme only. ESR spectroscopy of beech wood fibers shows that a considerable amount of the laccase-generated radicals is stabilized in the lignin polymer. The enzyme action generates stable free radicals in the lignin, and the bonding mechanism appears to be associated with reactions of these free radicals. Lignin is believed to be an active component in enzymatic bonding [7]. In vivo the monomers are polymerized by a laccase-initiated radical coupling process. Oxidation of lignin monomers by laccase produces a multiplicity of different radical species which participate in the polymerization and form the lignin polymer. In this paper, ESR spectroscopy of *Pinus kesiya* var. *langbianensis* heartwood powder treated by laccase shows that diverse amounts of ROS free radicals are generated under different reaction conditions. It implies that the parameters of laccase-treated heartwood are important for generating ROS free radicals.

The process parameters, such as pH value, treatment temperature and time, and laccase dosage have important effects on the amount of ROS free radicals in laccase-treated heartwood powder of *Pinus kesiya* var. *langbianensis*. ESR measurements show a higher number of ROS free radicals in the laccase-treated samples compared with untreated control. The laccase treatment causes a significant improvement of IB values of laccase-treated fiberboards (LTF) compared with the control, and similar conclusions are also obtained by Claus et al. [7] and Zhu et al. [9, 14]. Above all, researchers point out that different activation and hot press temperatures in producing process can ultimately lead to diverse IB values of LTF. Exceeding laccase-treated time is likely to lessen the amount of ROS free radicals so that finally it may reduce IB values of LTF. Zhu et al. [9] consider that the capacity of laccase-catalyzed amount of lignin is limited in a certain range.

The ANOVA analysis indicates that there is a significant difference between the ROS concentration values of heartwood powder treated by laccase I and both pH value and treatment temperature, except for the treatment time and laccase dosage at $P = 0.01$. However, there is a significant difference between the ROS concentration values of heartwood powder treated by laccase II and the pH values, treatment temperature and time, and laccase dosage at $P = 0.01$. In general, the ROS concentration value of the laccase-I-treated heartwood is higher than that of the laccase-II-treated heartwood. The different effects of laccase I and II show that diverse laccases derived from different fungi have a different ability of catalyzed oxidation when they are used to treat the same substrates under the same conditions. Therefore, different parameters of laccase treatment are applied in manu-

facturing of fiberboards due to different kinds of laccase so that to yield new and more environmentally friendly wood-based panels in the future.

5 Conclusions

Laccase-catalyzed oxidation of wood powder can yield some ROS free radicals. The parameters of pH value, treatment temperature and time, and laccase dosage are important in the processing of laccase-treated heartwood powder of *Pinus kesiya* var. *langbianensis* for the generation of ROS free radicals. The optimum parameters for both laccase-I- and laccase-II-treated heartwood are pH 3.0, 50 °C, 2 h and 20 units/g of wood powder. Laccase I and laccase II derived from different fungi have different effects on the heartwood. Their catalyzed oxidation capacity is different when they are used for treating the same substrates under identical treatment conditions. Furthermore, the effect of laccase I is stronger than that of laccase II.

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