Effect of Common Metal Ions and Anions on Laccase Catalysis of Guaiacol and Lignocellulosic Fiber

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The effects of 12 common metal ionic compounds on the laccase catalytic activity in reactions using guaiacol as the substrate was determined using spectrophotometry. Furthermore, the influence of several metal ionic compounds on the generation of reactive oxygen species (ROS) by oxidation of lignin in jute fiber under laccase catalysis was studied by electron paramagnetic resonance (EPR) spectroscopy using *N-tert*-butyl-alpha-phenylnitrone (PBN) as the spin-trapping agent. Common metal cations, such as K^+, Na^+, Mg^{2+}, Ca^{2+}, and Cu^{2+} and the anion SO_4^{2-} had almost no effect on laccase activity during the initial stage of the catalytic reactions. High concentrations of the Mn^{2+} ion exhibited weak inhibition of laccase; Ag^+ and NO_3^- showed a moderate inhibitory effect on laccase activity during the initial stage of the catalytic reactions. Fe^{2+} had no direct effect on the binding of laccase to its substrate, but strongly retarded the progress of the catalytic reaction by reducing the intermediate free radicals. The ions Cl^-, Fe^{3+}, and Ag^+ exhibited either strong inhibitory effects on the catalysis of the substrate or a destructive effect on the structure of laccase itself. Furthermore, the results showed that an appropriate concentration of Cu^{2+} helped to promote the thermal stability of laccase during the enzymatic reaction. This study could help researchers to avoid the use of inhibitory exogenous metal ions and anions in the application of laccase and to maximize the value of laccase.

Keywords: Laccase; Guaiacol; Jute fiber; Metal ion; Anion; Electron paramagnetic resonance

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INTRODUCTION

The copper-containing polyphenol oxidase, namely laccase, catalyzes the oxidation of multiple substrates such as phenol and its derivatives. In addition, some non-phenolic substrates, such as aromatic amines and carboxylic acids, and their derivatives can also be catalytically oxidized by laccase (Bourbonnais and Paice 1990; Eggert *et al*. 1997; Polak and Jarosz-Wilkolazka 2012). As a cost-effective, industrial ideal green catalyst, laccase is widely applied in various fields of industry (Asgher *et al*. 2016, 2017a; Bilal *et al*. 2017a,b). Lignin contains a phenolic hydroxyl group, making it a suitable substrate for laccase oxidation. Laccase can oxidize the hydroxyl group of lignin to generate active free radicals, thereby initiating the polymerization or degradation of lignin (Witayakran and Ragauskas 2009). Consequently, laccase is widely applied in wood fiber research. The pretreatment of wood with laccase can reduce the amount of adhesive used during the processing of fiberboard. Thus, laccase is useful for the production of environmentally friendly wood-based panels with high hardness and low formaldehyde release (Nasir *et al*. 2013; Kirsch

et al. 2017). In the paper-making industry, laccase is used for the bio-bleaching of paper pulp through laccase-mediator systems or a combination of laccase and other enzymes (Fillat *et al*. 2010; Singh *et al*. 2015). In the textile processing industry, laccase has been used for the pretreatment of bast fiber to partially remove hydrophobic lignin impurities to improve its wettability (Sharma *et al.* 2005; Karaduman *et al.* 2013). In addition, laccase causes indigo dyes on fabric surfaces to lighten, and hence it is generally used for cotton products such as denim – a process that is called denim bio-washing (Montazer and Maryan 2008). It can also be used for the functional modification of bast fiber by catalyzing the graft copolymerization of functional monomers onto the lignin of the fiber, which improves its hydrophilic, hydrophobic, anti-bacterial, and/or anti-oxidative properties, *etc*. (Silva *et al*. 2011; Chen *et al.* 2012; Dong *et al*. 2014). Furthermore, laccase can also be used in the dyeing (Bai *et al*. 2016; Jia *et a*l. 2017) and functional finishing (Montazer *et al*. 2009; Hossain *et al*. 2010; Fu *et al*. 2015) of protein-based fibers during textile processing.

The application of laccase for the functional modification of lignocellulosic fibers has attracted much attention in recent years (Thakur *et al*. 2015; Greimel *et al*. 2017). In general, exogenous ions, such as Na^{+}, Ca^{2+}, Mg^{2+}, and Fe^{2+}, as well as Cl^{-} and SO_4^{2-} ions, are commonly used in this type of modification through the use of additives and auxiliaries. Studies have shown that the presence of some exogenous ions has a noticeable impact on laccase activity and it greatly affects the efficiency of laccase in practical applications. Therefore, research on the influence of exogenous ions on the applications of laccase to lignin or lignified fiber is necessary. It is the way to identify the common ions that can be used to promote the catalytic activity of laccase. However, studies on these ions have focused mainly on the effect of exogenous metal cations on laccase activity during the fermentation process, and consequently the effect of foreign ions on the applications of laccase during fiber processing is unknown (Couto *et al.* 2005; Lorenzo *et al.* 2006; Murugesan *et al.* 2009). Moreover, comparatively little is known about the influence of anions on laccase activity (Morpurgo *et al*. 1974; Winkler *et al*. 1982; Kiiskinen *et al.* 2002). Furthermore, it should be noted that most studies concerning the effects of exogenous ions on laccase activity were not done systematically, the range of ions studied to date is not comprehensive, and the influence of co-existing anions has been largely neglected (Asgher *et al.* 2017b). In addition, it is not sufficient to study the effect of exogenous ions on laccase catalytic efficiency by activity detection alone.

For the laccase-catalyzed bioprocessing of lignin or other model compounds, the influence of exogenous ions on enzymatic activity and reactions as an unavoidable factor needs to be studied more deeply. Guaiacol, as one of the simplest model compounds of lignin, was to be used as a substrate for the determination of laccase activity in this article. The influence of common ions on the reaction undergone by guaiacol under laccase catalysis was systematically studied using UV-Vis spectrophotometry. Furthermore, laccase can catalyze the oxidation of substrates, such as lignin, to generate reactive oxygen species (ROS). Accordingly, the effect of ions on laccase activity can also be determined in terms of the kinds of ROS free radicals produced. Although this method has the potential to provide a more comprehensive insight into the influence of exogenous ions on the catalytic activity of laccase, it has not yet been explored. In this study, jute, which contains an abundant amount of lignin, was used as the substrate, and *N*-*tert*-butyl-alpha-phenylnitrone (PBN) was used to capture the ROS produced in the process. The ROS-PBN adducts were successfully detected using electron spin resonance (ESR) spectroscopy to reveal the overall effect of common metallic compounds on the catalytic oxidation of lignin

from jute by laccase (Capani *et al*. 2001; Cao *et al*. 2005; Zhou *et al*. 2009). This approach can provide more accurate guidance for the actual application of laccase.

EXPERIMENTAL

Materials

The *N-tert*-Butyl-alpha-phenylnitrone was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA), the guaiacol was supplied by TCI (Shanghai, China), and the other analytical reagents were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Jute fibers were obtained from Changshu Ao Cun Long Tai Weaving Co., Ltd. (Jiangsu, China). The fibers were ground into powders and passed through a 60-mesh screen for subsequent use. Laccase from *Trametes versicolor* (biological reagent) with an activity of 17,525 U/g was provided by Sigma-Aldrich Corporation (St. Louis, MO, USA). One unit of laccase activity is defined as the amount of enzyme that oxidizes 1 μmol of 2,2-azino-bis-3-ethyl-benzo-thiazoline-6-sulfonic acid (ABTS) per minute under specific reaction conditions. A UV-2802S spectrophotometer (UNICO Instruments, Shanghai, China) was used to measure the laccase activity.

Methods

Determination of relative activity and inhibitory rate of laccase

An amount of 0.5 mL of a laccase solution was added to 9.5 mL of guaiacol solution (5 mM) with a specific concentration of exogenous ions. The reaction was conducted at 25 °C and a pH of 4.0 (adjusted with 50 mM HAc-NaAc buffer solution). A UV-Vis spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan) was employed to detect the variation of the absorbance due to the substrate at 465 nm at the beginning of the reaction. The rate of reaction was calculated (V_n). The salt-free guaiacol-laccase reaction system was used as a control, and the rate of reaction was denoted as V_0, and then the relative enzyme activity (%) ($100 \times V_n/V_0$) and the inhibitory rate of laccase (%) ($100 \times (V_0 - V_n) / V_0$) were calculated.

Detection of thermal stability of laccase

Laccase solution (9.5 mL) was mixed with 15 mM exogenous ions (1.5 mM solutions of $FeSO_4$ and $FeCl_3$) and the mixture was incubated in a 50 °C water bath for 1 h. Then, 0.5 mL guaiacol solution (95 mM) was added to the solution to start the reaction. A salt-free reaction system was used as a control. The residual enzyme activity was determined as relative activity.

Laccase-catalyzed jute production of ROS free radicals

First, jute powder samples (0.045 g) were placed into 2.5-mL centrifuge tubes. Next, PBN solution (50 μL, final concentration of 10 mM), a certain concentration of laccase solution (200 μL), and different dosages of metal ionic compounds (final concentration of 0 mM to 40 mM) were added into each tube sequentially. Then, buffer solutions with a pH of 4.0 were added to the tubes, ensuring that each reaction system reached a total volume of 500 μL. The suspensions were then mixed by shaking and incubated in a 50 °C water bath for 1 h. The reactions were stopped by placing the tubes in an ice bath. Lastly, ethyl acetate (300 μL) was then added to each tube to extract the PBN-ROS adduct. The organic layer was removed into a 1.5-mL centrifuge tube for subsequent

use in EPR determination of PBN-ROS spin adducts (PBN-ROS complexes are stable in ethyl acetate).

Determination of ROS levels using EPR spectrometry

The measurement of the ROS levels was performed using an A-300 EPR spectrometer (Bruker, Karlsruhe, Germany) at room temperature. The conditions for EPR detection were as follows: X-band, 100 kHz modulation frequency with 4.07 G modulation amplitude; microwave power, 20 mW; center field, 3,512 G; sweep width, 200 G; receiver gain, 2.0×10^5; and resulting sweep time, 240 s. The peak area of the three-line ultra-fine characteristic peaks in each ESR signal was taken as the relative intensity of the ROS free radical (Zhou *et al*. 2014).

Statistical analysis

Experiments were run in triplicates, and the data presented were expressed as mean ± standard deviation (SD), except for the data in Figs. 5 and 6, which were expressed as mean. Statistical differences were determined using ANOVA variance. Difference was considered statistically significant at *$p \leq 0.05$, **$p \leq 0.01$ and ***$p \leq 0.001$.

RESULTS AND DISCUSSION

Effect of Cl^-, NO_3^-, and SO_4^{2-} on Laccase Activity

The sodium ion (Na^+) was used as the co-existing cation to prepare guaiacol-salt solutions with different concentrations of Cl^-, NO_3^-, and SO_4^{2-} ions. Approximately 0.5 mL of the laccase solution was added to start the reaction. Then, the relative activity of laccase was determined (Fig. 1).

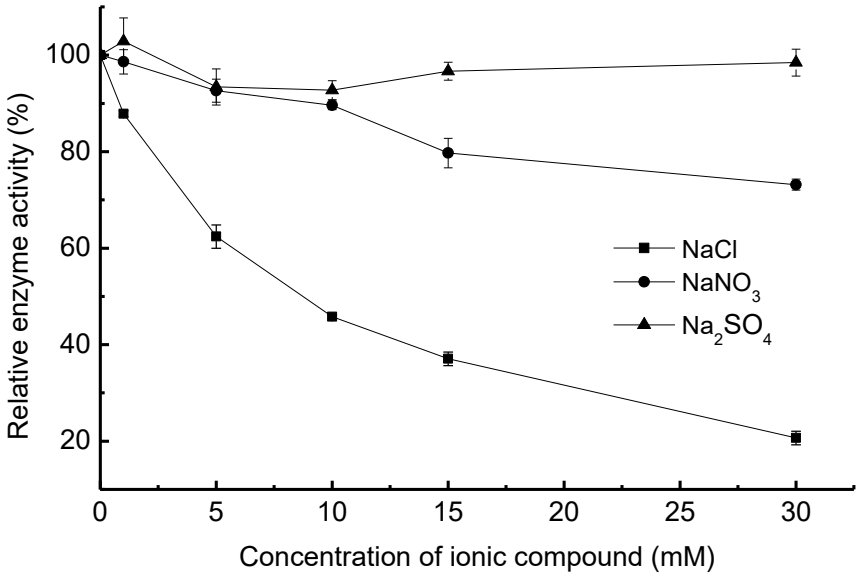

Fig. 1. Effect of Cl^-, NO_3^-, and SO_4^{2-} ions on laccase activity

As shown in Fig. 1, the Cl^-, NO_3^-, and SO_4^{2-} ions exhibited different inhibitory effects on laccase activity. The laccase activity indicated less variation with increased SO_4^{2-} concentration when the Na^+ ion was used as the co-existing cation. When the SO_4^{2-}

concentration reached 15 mM, the inhibitory rate of laccase was maintained at approximately 3%. The NO_3^- ion had a relatively mild inhibitory effect on laccase activity, with an inhibition rate of 26.8% when its concentration reached 30 mM. The Cl^- ion revealed a stronger inhibitory effect compared with those of SO_4^{2-} and NO_3^- ions, which was consistent with previous reports (Chaudhry *et al*. 2014; Pan *et al*. 2014). With an increase in the Cl^- ion concentration, the laccase activity rapidly decreased, and the inhibition rate of 79.3% was attained when the concentration of Cl^- reached 30 mM.

Effect of Common Metal Cations on Laccase Activity

Metal cations have varying degrees of impact on the activities of most enzymes. To further study the effect of common metal ions at various concentrations on laccase activity, a series of tests were performed, and the results are shown in Figs. 2a and 2b.

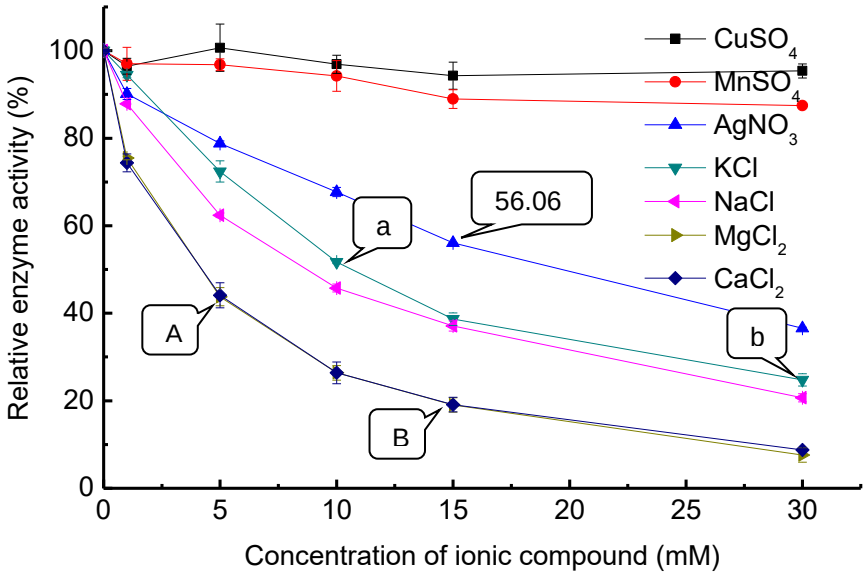

Fig. 2a. Effect of various concentrations of metal ionic compounds on laccase activity

Figure 2a shows that the effects of the K^+ and Na^+ ion concentrations on laccase were similar when the Cl^- ion was used as the co-existing anion. Based on the effect of anions described in Fig. 1, it can be deduced that the effect of K^+ and Na^+ ions on laccase activity was relatively weak.

Furthermore, the Cl^- ion concentrations that corresponded to Na^+ concentrations of 10 mM and 30 mM (a, b) were the same as those that corresponded to concentrations of Mg^{2+} and Ca^{2+} of 5 mM and 15 mM (A, B), respectively. The relative activities were also similar at "A" and "a", and at "B" and "b." This observation indicated that the effects of Mg^{2+} and Ca^{2+} ions on laccase produced by *Trametes versicolor* were relatively weak and similar to that of Na^+. This further confirmed that the inhibitory effect presented by the compounds of the above metal ions with co-existing Cl^- was mainly due to the Cl^- ion.

In addition, when SO_4^{2-} is the co-existing anion, the laccase activity for catalyzing the oxidation of guaiacol showed minimal variation with increased Cu^{2+} concentration. The Mn^{2+} ion exhibited a slight inhibitory effect on laccase, with an inhibition rate of approximately 12% when its concentration was greater than 15 mM.

As shown in Fig. 2a, the observed inhibition of laccase activity caused by $AgNO_3$ was attributed to the joint action of Ag^+ and NO_3^- ions. This finding was based on experiments that the effect of the NO_3^- ion alone on laccase activity; though the inhibition caused by Ag^+ ions on laccase was moderate.

The inhibition of laccase by NO_3^- can be attributed to competitive inhibition, which was completed instantaneously. The Ag^+ did not bind with the free enzyme, but instead it bound to the enzyme-substrate compound during the laccase-catalyzed process (Tu *et al*. 1999a).

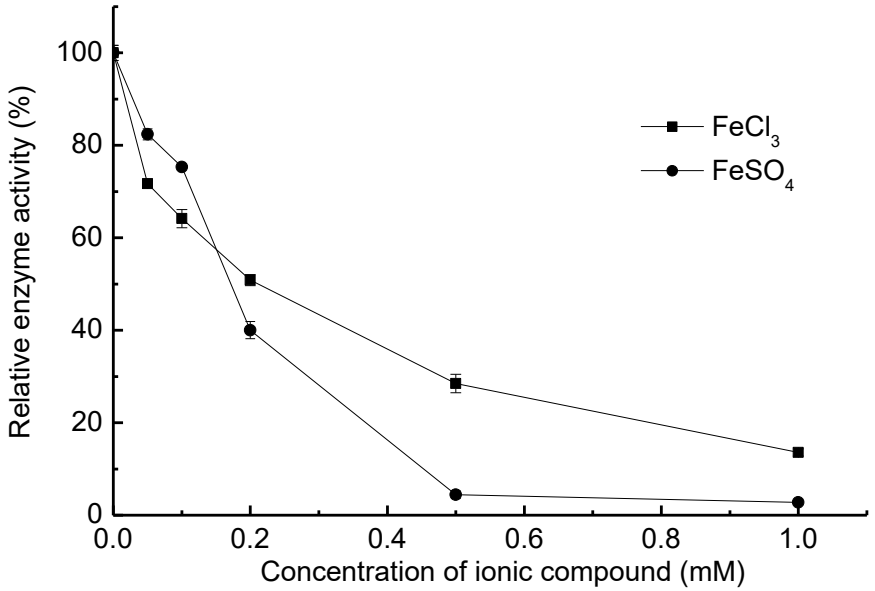

Fig. 2b. Effect of $FeSO_4$ and $FeCl_3$ at various concentrations on laccase activity

As shown in Fig. 2b, the inhibition of laccase activity was rapidly enhanced with an increase in $FeSO_4$ or $FeCl_3$ concentration, which showed that the laccase activity was strongly inhibited by trace amounts of $FeSO_4$ or $FeCl_3$. The inhibition rate was 60.0% when the concentration of $FeSO_4$ was 0.2 mM and the inhibition reached a 98% level when the concentration of $FeSO_4$ was increased to 1 mM. The inhibition induced by the SO_4^{2-} ion at this concentration can be ignored considering the results in Fig. 1. Thus, it can be inferred that the Fe^{2+} ion showed strong inhibition of laccase activity initially, and that the inhibition was rapidly enhanced with an increase in Fe^{2+} concentration. This may have been due to the intermediate being reduced continuously, thereby resulting in strong inhibition of laccase activity (Tu *et al*. 1999b).

In addition, the experimental data in Fig. 1 indicated that the effect of $FeCl_3$ on laccase was due to the joint actions of Fe^{3+} and Cl^- ions. When the $FeCl_3$ concentration was only 1 mM, its inhibition rate was 87%. Considering this together with the results described in Fig. 1, the suppression caused by the Cl^- ion was less than 30%. Therefore, it can be speculated that the inhibition caused by Fe^{3+} exceeded 55%. This means that Fe^{3+} also had a noticeable impact on the laccase activity during the initial stage of the enzymatic reaction, and that the inhibition rate increased with increased $FeCl_3$ dosage.

Effect of Common Ions on the Catalytic Properties of Laccase

Effect of common ions on the thermal stability of laccase

This study of laccase thermal stability focused on several ionic compounds that showed strong effects on laccase activity under the optimal process conditions (Kudanga *et al*. 2008; Silva *et al*. 2011), and the specific results are shown in Fig. 3.

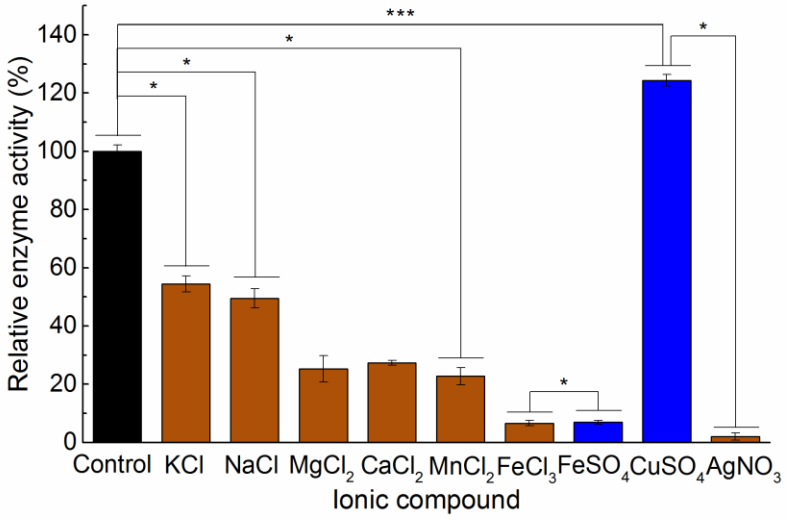

Fig. 3. Effect of common ions on thermal stability of laccase; the concentration of $FeSO_4$ and $FeCl_3$ was 1.5 mM, while that of the other ions was 15 mM, *p≤0.05, **p ≤ 0.01 and ***p ≤ 0.001

In cases where the Cl^- ion was used as the co-existing anion, compared with the control, the influence of K^+, Na^+, Mn^{2+} (*p≤ 0.05), Mg^{2+} and Ca^{2+} cations on the thermal stability of laccase was similar to their inhibitory effects on initial laccase activity, as described in Figs. 2a and 3.

It should be noted that the addition of 15 mM Cu^{2+} greatly improved the laccase thermal stability by 24% (***p ≤ 0.001), although it had almost no effect on its activity. Previous studies (Wang *et al.* 2003; Zhang *et al.* 2009) have indicated that the effect of exogenous Cu^{2+} on laccase is two-fold: (a) it could combine with free ω-carboxylic anions on the acidic amino acid residues around the active sites of laccase molecules and inhibit its catalytic activity; and (b) it could affect the conformation of laccase by interacting with the atoms or groups near the enzyme surface, facilitating the electron transfer in the enzyme molecules, which was manifested as activation. In the study, at a pH of 4.0 and 50 °C, molecular thermal motion was intensified and thus the effect of path-(a) was weakened and path-(b) played a dominant role. This path contributed considerably to the improvement of laccase thermal stability.

In the current study, the effect of adding $AgNO_3$ on the catalytic properties of laccase was opposite to that of adding $CuSO_4$ (*p≤ 0.05). And as shown in Fig. 3, the relative laccase activity was almost lost (only 2.0%). This observation contradicted the 56.1% inhibition extent seen in Fig. 2(a), which may have been because of the inhibitory effect of Ag^+ on laccase was similar to that of Hg^{2+} in the absence of any substrate, as described in previous reports (Tu *et al.* 2000; Krajewska *et al*. 2004). Due to its large ionic radius, the Ag^+ ion took a relatively long time to enter the enzyme molecule and it progressively disturbed the structure of laccase. High temperature can accelerate this

process and decrease the laccase stability as well, which explained why the results from this study indicated the denaturation and inactivation of laccase under incubation at 50 °C for 1 h. In the presence of a substrate, Ag^+ mainly participates in the binding of the enzyme-substrate compound, rather than acting on the structure of laccase itself. Thus, these results indicated that the inhibition of laccase activity by the Ag^+ ion was relatively mild during the enzymatic process.

The effect of $FeSO_4$ (1.5 mM) on the thermal stability of laccase (relative activity 6.72%) was similar to that of $FeCl_3$ (relative activity 6.58%) (*$p \leq 0.05$), and unexpectedly weaker than the effect of 0.5 mM $FeSO_4$ on laccase activity (relative activity 4.42%). The weakening of laccase inhibition upon incubation at 50 °C for 1 h may have been because of Fe^{2+} in the laccase solution being gradually oxidized to Fe^{3+} at higher temperatures.

Effect of common ions on the generation of ROS free radicals in laccase-activated jute fiber

Though metal ionic compounds exhibited considerable impact on the activity and thermal stability of laccase, their influence on the whole catalytic process is still unknown. Therefore, the effects of six metal ionic compounds on the treatment of jute fiber with laccase were investigated by EPR spectroscopy and the spin-trapping method. The specific results are shown in Figs. 4a and 4b.

As shown in Fig. 4a, the ionic compounds KCl, NaCl, $CaCl_2$, $FeSO_4$ and $AgNO_3$, extensively inhibited the laccase catalysis. Such inhibitory effects of KCl, NaCl, and $CaCl_2$ were rapidly enhanced by increasing their respective concentrations. When their concentrations exceeded 5 mM, the decline of the EPR signal intensity was alleviated. Taken together with the influence of metal ionic compounds on laccase activity, it can be inferred that during the actual catalytic process undertaken by laccase, the use of Fe^{2+}, Ag^+, Cl^-, and NO_3^- ions should be avoided to ensure effective catalytic activity.

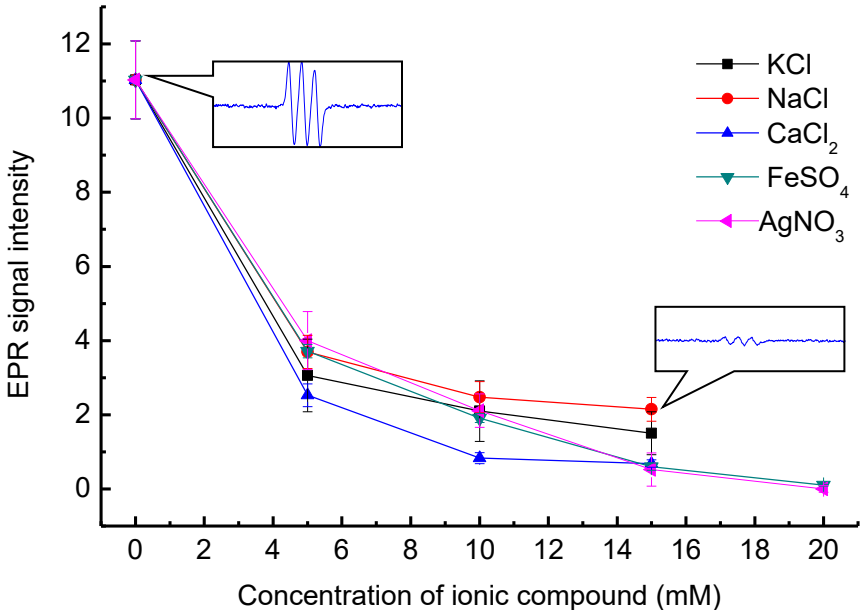

Fig. 4a. Signal intensity as a function of different concentrations of ionic compounds in laccase-activated jute fiber

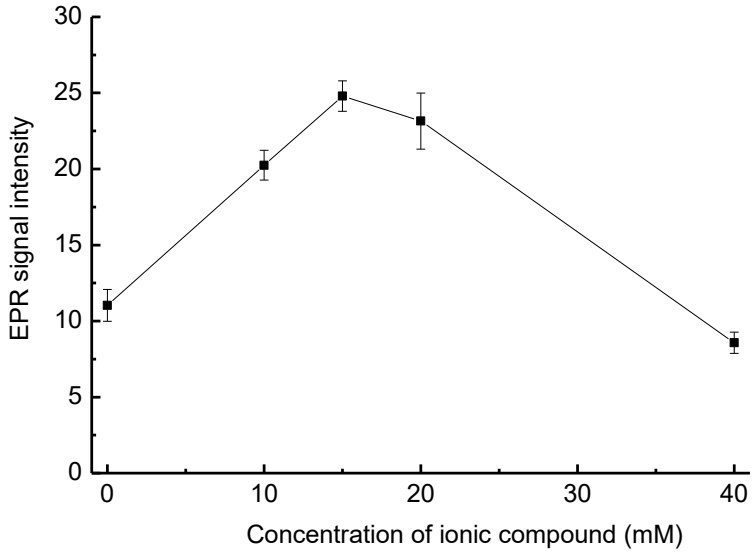

Fig. 4b. Signal intensity as a function of $CuSO_4$ concentration in laccase-activated jute

As shown in Fig. 4b, Cu^{2+} had a hermetic effect on the generation of ROS free radicals in laccase-activated jute, stimulating it at low doses but inhibiting it at high doses. A small amount of copper ions clearly favored the enzymatic process, and the EPR signal intensity reached a peak value at 15 mM of Cu^{2+} ion; however, any further increase was unfavorable for the enzymatic reaction, and the Cu^{2+} ion showed an inhibitory effect on the enzymatic reaction at concentrations above 40 mM.

Although the inhibition effect of exogenous Cu^{2+} on laccase was weak at pH 4 and 50 °C, the combined probability of interactions with substrates and active sites was gradually enhanced with the increase in exogenous Cu^{2+} dosage. When the Cu^{2+} concentration was less than 15 mM, the degree of promotion exceeded that of inhibition with increased Cu^{2+} concentration. This manifested as an overall increase in activation and enhancement of EPR signal intensity. However, at concentrations higher than 15 mM, the degree of promotion was lower than that of inhibition. This led to a weakening of net activation, and the EPR signal intensity gradually declined with increased Cu^{2+} concentration. At concentrations that exceeded 40 mM, the inhibitory effect of exogenous Cu^{2+} exceeded its promoting effect, which resulted in net suppression, and the EPR signal intensity became lower than that of the control sample, which was consistent with previous reports (Alcalde 2007).

Effect of $FeSO_4$ and $AgNO_3$ on Laccase Catalysis

The influence of $FeSO_4$ and $AgNO_3$ compounds on the initial activity and the thermal stability of laccase have been previously confirmed, but their influence on the overall process of laccase catalysis is still unknown. The variation of absorbance values in the laccase-guaiacol reaction system with various concentrations of $FeSO_4$ or $AgNO_3$ solutions was determined at different time intervals. As shown in Fig. 5, the increase in absorbance at 465 nm in the reaction medium containing $AgNO_3$ over time was found to be the opposite of the effect for Fe^{2+}. With time, the increase in the absorbance value tended to slow down and the inhibition of $AgNO_3$ on laccase activity was enhanced, which indicated that the suppression of laccase catalysis by Ag^+ exhibited properties of anticompetitive inhibition (Tu *et al*. 1999).

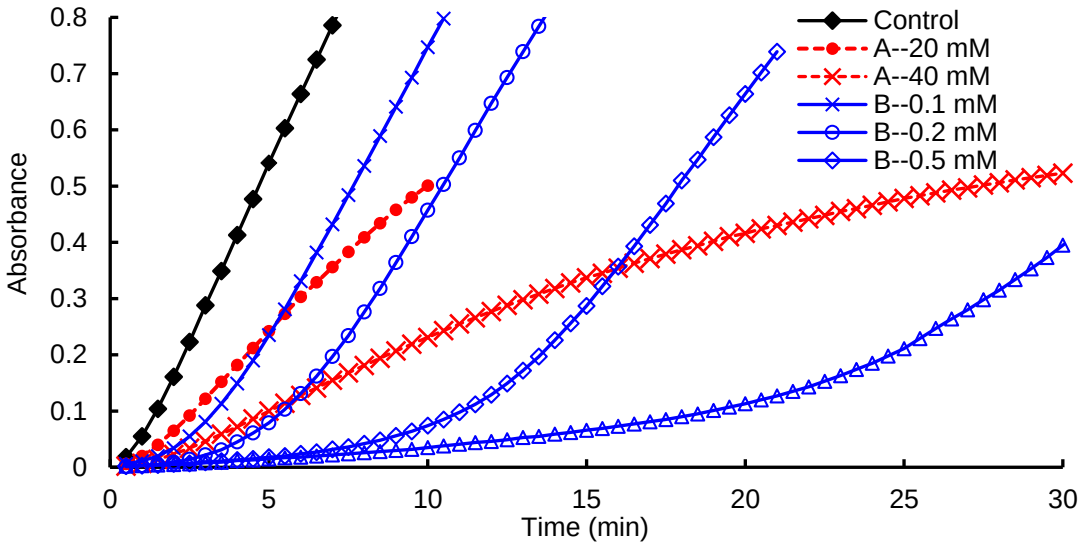

Fig. 5. Effect of $FeSO_4$ and $AgNO_3$ at various concentrations on the laccase catalytic process (A: $AgNO_3$; B: $FeSO_4$)

However, the absorbance in the reaction system containing $FeSO_4$ slowly increased during the initial stage of the reaction. After some time, the enzymatic reaction gradually recovered. However, with an increase in $FeSO_4$ concentration, the recovery time of laccase activity became longer. The recovery time was 2 min with 0.1 mM $FeSO_4$ in the reaction system and increased to 14 min when the concentration of $FeSO_4$ was 0.5 mM. When the concentration of $FeSO_4$ was high at 1.0 mM, the recovery time reached 25 min. This suppression was mainly due to Fe^{2+}. A plot of absorbance *versus* time revealed that the inhibition of laccase activity by Fe^{2+} fits the category of 'intense transient inhibition.' It should be noted that the effect slowed down the progress of the enzymatic reaction, but did not completely prevent it. Furthermore, the recovery of laccase activity gradually declined with increased Fe^{2+} concentration, as shown in Table 1. This may have been due to the reducing effect of Fe^{2+}, which not only delayed the progress of the enzymatic reaction, but also restrained the laccase activity by the oxidization of Fe^{2+} to Fe^{3+}.

Table 1. Comparison of Laccase Activity in the Reaction System Containing Various Concentrations of $FeSO_4$ before and after Recovery

Concentration of $FeSO_4$ (mM)	Relative enzyme activity (%)	
	Initial stage of catalysis	After the recovery of activity
0.1	75.33±4.75	81.48±2.34 (after 5 min)
0.2	40.03±2.78	73.19±0.96 (after 8 min)
0.5	4.42±0.01	59.61±0.82 (after 15 min)
1.0	2.76±0.02	28.58±0.37 (after 25 min)

Effect of Delayed Addition of Fe^{2+} on Laccase Catalysis

Further study on the effect of Fe^{2+} on the enzymatic process was conducted by delaying the addition of $FeSO_4$. The results are shown in Fig. 6.

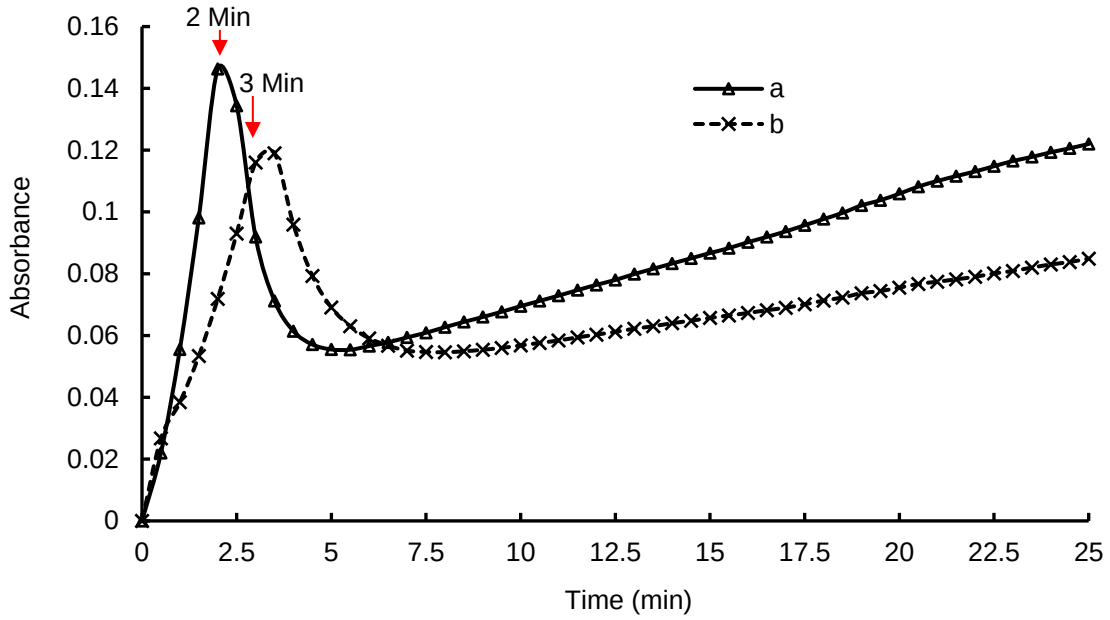

Fig. 6. Influence of delayed addition of Fe^{2+} on absorbance during laccase catalysis; a: The final concentration of laccase was 0.06 U/mL, and $FeSO_4$ was added with a delay of 2 min; b: The final concentration of laccase was 0.03 U/mL, and $FeSO_4$ was added with a delay of 3 min.

Figure 6 shows clearly that the absorbance at 465 nm gradually increased with time, and that the enzymatic reaction proceeded normally before the Fe^{2+} was added. When the same amount of $FeSO_4$ was added to different reaction systems at 2 min (a) and 3 min (b), the absorbance of the reaction system did not continue to increase, but rapidly decreased, which indicated that the enzymatic activity of laccase was severely inhibited. After about 3 min to 4 min, the enzymatic reaction restarted. This indicated that the addition of Fe^{2+} led to the restoration of reactive intermediates to the initial state due to the reducing effect of Fe^{2+} (Tu *et al*. 1999). When enough Fe^{2+} ion was added, the reaction instantly generated new intermediates, but the existing intermediates were also reduced, which explained the observed constant decrease in absorbance. The effect of Fe^{2+} on the intermediates was eliminated and the enzymatic process gradually resumed when all the added Fe^{2+} was converted to Fe^{3+}. However, the restored laccase activity was lower than that without the addition of $FeSO_4$ because the Fe^{3+} converted from the added Fe^{2+} also possessed a strong inhibitory effect on laccase activity.

The generation of ROS free radicals in laccase-activated jute fiber was further studied to verify the effect of the delayed addition of Fe^{2+} on laccase catalysis. The result is shown in Fig. 7.

As shown in Fig. 7, the EPR signal intensity showed hardly any change in the first 2 min after $FeSO_4$ addition. Thereafter, the signal intensity increased gradually. This may have been due to the presence of PBN, which instantly combined with generated ROSs and formed a relatively stable complex (Zhou *et al*. 2014). The addition of Fe^{2+} could rapidly reduce the new ROSs that formed but was ineffective against the stable ROS-PBN adducts. This may explain why almost no change was observed in the EPR signal within a certain period after Fe^{2+} addition. However, when the added Fe^{2+} was completely consumed, the ROSs generated by jute could be re-trapped by PBN, resulting in a continual increase in the EPR signal intensity.

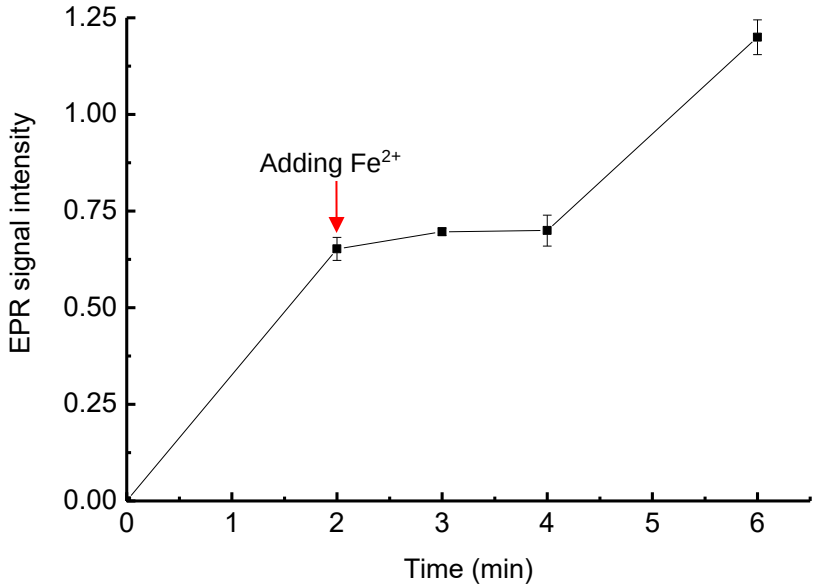

Fig. 7. Effect of Fe^{2+} on laccase catalysis of jute fiber and generation of free radicals (added with a delay of 2 min)

CONCLUSIONS

1. In this study, the effects of several metal ionic compounds on laccase activity, thermal stability, and the generation of ROS radicals in laccase-activated jute fiber were investigated, and the results showed that Cl^- and cations, such as Fe^{3+}, Fe^{2+} and Ag^+ exhibited strong inhibition of laccase activity at 25 °C and pH 4. In addition, most of the compounds containing these ions exhibited clear inhibition of the generation of ROSs in laccase-activated jute, indicating appropriate copper ions favored the enzymatic process. It is recommended to avoid contact with metal cations, such as Fe^{2+}, Fe^{3+}, and Ag^+, and anions such as Cl^- and NO_3^- during laccase-catalyzed oxidation of substrates. However, an appropriate amount of exogenous Cu^{2+} could be added to facilitate the enzymatic reaction.
2. Laccase can catalyze the oxidation of varied substrates and presents great application potential for material modification and processes. As exogenous metal ionic compound is one of the most complex and unavoidable factors in the laccase catalytic oxidation, this study provides detailed guidance for their correct usages.

ACKNOWLEDGMENTS

This work was financially supported by the National Natural Science Foundation of China (51603087, 21674043), the Program for Changjiang Scholars and Innovative Research Teams in Universities (IRT_15R26), Key Research & Development Plan of Jiangsu Province (BE2016208), Fundamental Research Funds for the Central Universities (JUSRP51717A), and the Nantong Science and Technology Project of China (GY12015027, MS12015036).

REFERENCES CITED

Alcalde, M. (2007). "Laccases: Biological functions, molecular structure and industrial applications," in: *Industrial Enzymes*, J. Polaina, A.P. MacCabe (eds.), Springer Netherlands, BER. 461-476. DOI: 10.1007/1-4020-5377-0_26

Asgher, M., Noreen, S., and Bilal, M. (2017a). "Enhancement of catalytic, reusability, and long-term stability features of *Trametes versicolor IBL-04* laccase immobilized on different polymers," *Int. J. Biol. Macromol.* 95, 54-62. DOI: 10.1016/j.ijbiomac.2016.11.012

Asgher, M., Noreen, S., and Bilal, M. (2017b). "Enhancing catalytic functionality of *Trametes versicolor* IBL-04 laccase by immobilization on chitosan microspheres," *Chem. Eng. Res. Des.* 119, 1-11. DOI: 10.1016/j.cherd.2016.12.011

Asgher, M., Wahab, A., Bilal, M., and Iqbal, H. (2016). "Lignocellulose degradation and production of lignin modifying enzymes by *Schizophyllum commune* IBL-06 in solid-statefermentation," *Biocatal. Agric. Biotechnol.* 6, 195-201. DOI: 10.1016/j.bcab.2016.04.003

Bai, R., Yu, Y., Wang, Q., Yuan, J., and Fan, X. (2016). "Effect of laccase on dyeing properties of polyphenol-based natural dye for wool fabric," *Fiber. Polymer.* 17(10), 1613-1620. DOI: 10.1007/s12221-016-5598-5

Bilal, M., Asgher, M., Iqbal, H., Hu, H., and Zhang. X. (2017a). "Delignification and fruit juice clarification properties of alginate-chitosan-immobilized ligninolytic cocktail," *LWT - Food Sci. Technol.* 80, 348-354. DOI: 10.1016/j.lwt.2017.02.040

Bilal, M., Asgher, M., Parra-Saldivar, R., Hu, H. Wang, W., Zhang, X., and Iqba, H. (2017b). "Immobilized ligninolytic enzymes: An innovative and environmental responsive technology to tackle dye-based industrial pollutants – A review," *Sci. Total Environ.* 576, 646-659. DOI: 10.1016/j.scitotenv.2016.10.137

Bourbonnais, R., and Paice, M. G. (1990). "Oxidation of non-phenolic substrates," *FEBS. Lett.* 267(1), 99-102. DOI: 10.1016/0014-5793(90)80298-W

Cao, Y., Guo, P., Xu, Y., and Zhao, B. (2005). "Simultaneous detection of NO and ROS by ESR in biological systems," *Methods Enzymol.* 396(Part E), 77-83. DOI: 10.1016/S0076-6879(05)96008-4

Capani, F., Loidl, C. F., Aguirre, F., Piehl, L., Facorro, G., Hager, A., Paoli, T. D., Farach, H., and Pecci-Saavedra, J. (2001). "Changes in reactive oxygen species (ROS) production in rat brain during global perinatal asphyxia: An ESR study," *Brain Res.* 914(1-2), 204-207. DOI: 10.1016/S0006-8993(01)02781-0

Chaudhry, M. T., Zohaib, M., Rauf, N., Tahir, S. S., and Parvez, S. (2014). "Biosorption characteristics of *Aspergillus fumigatus* for the decolorization of triphenylmethane dye acid violet 49," *Appl. Microbiol. Biotechnol.* 98(7), 3133-3141. DOI: 10.1007/s00253-013-5306-y

Chen, Y. M., Wan, J. Q., Ma, Y. W., Tang, B., Han, W. J., and Ragauskas, A. J. (2012). "Modification of old corrugated container pulp with laccase and laccase–mediator system," *Bioresour. Technol.* 110(2), 297-301. DOI: 10.1016/j.biortech.2011.12.120

Couto, S. R., Sanromán, M., and Gübitz, G. M. (2005). "Influence of redox mediators and metal ions on synthetic acid dye decolourization by crude laccase from *Trametes hirsuta*," *Chemosphere* 58(58), 417-422. DOI: 10.1016/j.chemosphere.2004.09.033

Dong, A. X., Yuan, J. G., Wang, Q., and Fan, X. R. (2014). "Modification of jute fabric via laccase/t-BHP-mediated graft polymerization with acrylamide," *J. Appl. Polym. Sci.* 131(12), 469-474. DOI: 10.1002/app.40387

Eggert, C., Temp, U., and Eriksson, K. E. L. (1997). "Laccase is essential for lignin degradation by the white-rot fungus *Pycnoporus cinnabarinus*," *FEBS Lett.* 407(1), 89-92. DOI: 10.1016/S0014-5793(97)00301-3

Fillat, A., Colom, J. F., and Vidal, T. (2010). "A new approach to the biobleaching of flax pulp with laccase using natural mediators," *Bioresource Technol.* 101(11), 4104-4110. DOI: 10.1016/j.biortech.2010.01.057

Fu, J., Su, J., Wang P., Yu, Y., Wang, Q., and Cavaco-Paulo, A. (2015). "Enzymatic processing of protein-based fibers," *Appl. Microbiol. Biotechnol*. 99(24), 10387-10397. DOI: 10.1007/s00253-015-6970-x

Greimel, K. J., Kudanga,T., Nousiainen, P., Sipilä, J., Acero, E. H., Nyanhongo, G. S., and Guebitz, G. M. (2017). "Two distinct enzymatic approaches for coupling fatty acids onto lignocellulosic materials," *Process Biochem.* DOI: 10.1016/j.procbio.2017.01.008

Hossain, Kh. M. G., González, M. D., Monmany, J. M. D., and Tzanov, T. (2010). "Effects of alkyl chain lengths of gallates upon enzymatic wool functionalisation," *J. Mol. Catal. B-Enzym.* 67(3-4), 231-235. DOI: 10.1016/j.molcatb.2010.08.011

Jia, W., Wang, Q., Fan, X., Dong, A., Yua, Y., and Wang, P. (2017). "Laccase-mediated in situ oxidation of dopa for bio-inspired coloration of silk fabric," *RSC Adv.* 7, 12977-12983. DOI: 10.1039/C6RA25533G

Karaduman, Y., Gokcan, D., and Onal, L. (2013). "Effect of enzymatic pretreatment on the mechanical properties of jute fiber-reinforced polyester composites," *J. Compos. Mater.* 47(10), 1293-1302. DOI: 10.1177/0021998312446826

Kiiskinen, L. L., Viikari, L., and Kruus, K. (2002). "Purification and characterisation of a novel laccase from the ascomycete *Melanocarpus albomyces*," *Appl. Microbiol. Biotechnol.* 59(2), 198-204. DOI: 10.1007/s00253-002-1012-x

Kirsch, A., Reuter, P., Kharazipour, A., and Euring, M. (2017). "Comparision of different Raw densities and board thicknesses in the production of enzymatically bonded fiberboards," *J. Mater. Sci. Res.* 6(2), 69-78. DOI: 10.5539/jmsr.v6n2p69

Krajewska, B., Zaborska, W., and Chudy, M. (2004). "Multistep analysis of Hg^{2+} ion inhibition of jack bean urease," *J. Inorg. Biochem.* 98(6), 1160-1168. DOI: 10.1016/j.jinorgbio.2004.03.014

Kudanga, T., Prasetyo, E. N., Sipilä, J., Nousiainen, P., Widsten, P., Kandelbauer, A., Nyanhongo, G. S., and Guebitz, G. (2008). "Laccase-mediated wood surface functionalization," *Eng. Life Sci.* 8(3), 297-302. DOI: 10.1002/elsc.200800011

Lorenzo, M., Moldes, D., and Sanromán, M. A. (2006). "Effect of heavy metals on the production of several laccase isoenzymes by *Trametes versicolor* and on their ability to decolourise dyes," *Chemosphere* 63(6), 912-917. DOI: 10.1016/j.chemosphere.2005.09.046

Montazer, M., Dadashian, F., Hemmatinejad, N., and Farhoudi, K. (2009). "Treatment of wool with laccase and dyeing with madder," *Appl. Biochem. Biotech.* 158(3), 685-693. DOI: 10.1007/s12010-008-8403-0

Montazer, M., and Sadeghian Maryan, A. (2008). "Application of laccases with cellulases on denim for clean effluent and repeatable biowashing," *J. Appl. Polym. Sci.* 110(5), 3121-3129. DOI: 10.1002/app.28920

Morpurgo, L., Rotilio, G., Agrò, A. F., and Mondoví, B. (1974). "Anion binding to *Rhus vernicifera* laccase," *BBA-Protein Struct.* 336(336), 324-328. DOI: 10.1016/0005-2795(74)90411-5

Murugesan, K., Kim, Y. M., Jeon, J. R., and Chang, Y. S. (2009). "Effect of metal ions on reactive dye decolorization by laccase from *Ganoderma lucidum*," *J. Hazard. Mater.* 168(1), 523-529. DOI: 10.1016/j.jhazmat.2009.02.075

Nasir, M., Gupta, A., Beg, M. D. H., Chua, G. K., Jawaid, M., Kumar, A., and Khan, T. A. (2013). "Fabricating eco-friendly binderless fiberboard from laccase-treated rubber wood fiber," *BioResources* 8(3), 3599-3608. DOI: 10.15376/biores.8.3.3599-3608

Pan, T., Sun, Y. J., Wang, X. L., Shi, T., and Zhao, Y. L. (2014). "Influence of counteranions on catalytic ability of immobilized laccase in Cu-alginate matrices: Inhibition of chloride and activation of acetate," *Chinese Chem. Lett.* 25(7), 983-988. DOI: 10.1016/j.cclet.2014.05.045

Polak, J., and Jarosz-Wilkolazka, A. (2012). "Fungal laccases as green catalysts for dye synthesis," *Process Biochem.* 47(9), 1295-1307. DOI: 10.1016/j.procbio.2012.05.006

Sharma, H. S. S., Whiteside, L., and Kernaghan, K. (2005). "Enzymatic treatment of flax fibre at the roving stage for production of wet-spun yarn," *Enzyme Microb. Technol.* 37(4), 386-394. DOI: 10.1016/j.enzmictec.2004.10.007

Silva, C., Matamá, T., Kim, S. Y., Padrão, J., Prasetyo, E. N., Kudanga, T., Nyanhongo, G. S., Guebitz, G. M., Casal, M., and Cavaco-Paulo, A. (2011). "Antimicrobial and antioxidant linen via laccase-assisted grafting," *React. Funct. Polym.* 71(7), 713-720. DOI: 10.1016/j.reactfunctpolym.2011.03.011

Singh, G., Kaur, K., Puri, S., and Sharma, P. (2015). "Critical factors affecting laccase-mediated biobleaching of pulp in paper industry," *Appl. Microbiol. Biotechnol.* 99(1), 155-164. DOI: 10.1007/s00253-014-6219-0

Thakur, K., Kalia, S., Sharma , N., and Pathania, D. (2015). "Laccase-mediated biografting of p-coumaric acid for development of antibacterial and hydrophobic properties in coconut fibers," *J. Mol. Catal. B: Enzym.* 122, 289-295. DOI: 10.1016/j.molcatb.2015.10.002

Tu, C. Q., Liang, H., and Wang, G. H. (1999 a). "Inhibition of the oxidoreductase activity of *Rhus vernicifera* laccase by Ag^{+} ion," *Guangxi Sciences* 6(2), 109-110, 114. DOI: 10.3969/j.issn.1005-9164.1999.02.010

Tu, C. Q., Liang, H., and Wang, G. H. (1999 b). "Catalyzed oxidation by *Rhus vernicifera* laccase (XIII) - Effects of Fe^{2+} ion on the catalytic oxidation by laccase," *Chem. J. Chinese U* 20(2), 243-247. DOI: 10.3321/j.issn:0251-0790.1999.02.013

Tu, C. Q., Liang, H., and Wang. G. H. (2000). "Study on the interaction of *Rhus vernicifera* Laccase with Hg^{2+} Ion," *Spectrosc. Spect. Anal.* 20(4), 572-574. DOI: 10.3321/j.issn:1000-0593.2000.04.033

Wang, X. W., Zhan, H. Y., and He, W. (2003). "The effect of Cu(II) on laccase activity," *Paper and Paper Making* 0(4), 43-44. DOI: 10.3969/j.issn.1001-6309.2003.04.017

Winkler, M. E., Spira, D. J., LuBien, C. D., Thamann, T. J., and Solomon, E. I. (1982). "Anion binding to oxidized type 2 depleted and native laccase: A spectroscopically effective model for exogenous ligand binding to the type 3 - type 2 active site," *Biochem. Biophys. Res. Commun.* 107(2), 727-734. DOI: 10.1016/0006-291X(82)91551-0

Witayakran, S., and Ragauskas, A. J. (2009). "Synthetic applications of laccase in green chemistry," *Adv. Synth. Catal.* 351(9), 1187-1209. DOI: 10.1002/adsc.200800775

Zhang, Y., Shen, G. Q., Yu, Y. S., and Zhou, H. L. (2009). "The hormetic effect of cadmium on the activity of antioxidant enzymes in the earthworm *Eisenia fetida*," *Environ. Pollut.* 157(11), 3064-3068. DOI: 10.1016/j.envpol.2009.05.039

Zhou, C. X., Dong, A. X., Wang, Q., Fan, X. R., and Cao, Y. L. (2014). "EPR technology spectroscopic investigation of generation of reactive oxygen species free radicals in laccase-activated jute fiber," *Appl. Magn. Reson.* 45(5), 505-515. DOI: 10.1007/s00723-014-0534-6

Zhou, G. W., Li, J. N., Chen, Y. S., Zhao, B. L, Cao, Y. J, Duan, X. F., and Cao, Y. L. (2009). "Determination of reactive oxygen species generated in laccase catalyzed oxidation of wood fibers from Chinese fir (*Cunninghamia lanceolata*) by electron spin resonance spectrometry," *Bioresour. Technol.* 100(1), 505-508. DOI: 10.1016/j.biortech.2008.06.010

Article submitted: January 5, 2017; Peer review completed: April 6, 2017; Revised article received and accepted: May 21, 2017; Published: May 31, 2017.
DOI: 10.15376/biores.12.3.5102-5117