Chemical Modification of Laccase from *Aspergillus oryzae* and its Application in OCC Pulp

Yangmei Chen,[a,b] Jinquan Wan,[c] Qitang Wu,[a] and Yongwen Ma [c]

Laccase activity and stability were improved and modified through the interaction between laccase amino acid residues (*e.g.*, amino groups (-NH_2) and carboxyl groups (-COOH)) and the chemical reagents thiourea dioxide (TDO) and L-phenylalanine methyl ester hydrochloride (L-PME). Compared with the control sample, the activity of the laccase modified with these chemical reagents was increased by 209% and 50%, respectively. The stability of the laccase modified with L-PME increased by 56.9%. However, the laccase modified with TDO only improved slightly. It was clear that reagent L-PME was more efficacious than TDO. The paper formed with addition of L-PME-modified laccase exhibited better performance in terms of tensile strength, bursting strength, and tear strength, which increased by 13%, 10%, 9%, respectively, compared with the paper formed with unmodified laccase.

Keywords: Laccase; Modification; Thiourea dioxide; L- Phenylalanine methyl ester hydrochloride; OCC pulp

Contact information: a: Key Laboratory of Soil Environment and Waste Reuse in Agriculture of Guangdong Higher Education Institutes, College of Natural Resources and Environment, South China Agricultural University, Guangzhou 510642, P.R. China; b: The Key Lab of Pollution Control and Ecosystem Restoration in Industry Clusters, Ministry of Education, South China University of Technology, Guangzhou Higher Education Mega Centre, Guangzhou 510006, PR China; c: College of Environment and Energy, South China University of Technology, Guangzhou 510006, P.R. China;
** Corresponding author: chenyangmei_2001@sina.com*

INTRODUCTION

The swelling ability, fiber strength, and paper strength properties of recycled plant fiber decrease to varying degrees compared with virgin plant fiber (Chen *et al.* 2010b). These detrimental losses eventually prevent their utilization in papermaking. To increase the papermaking potential and add value to recycled plant fiber, it is necessary to modify the recycled plant fiber through various methods. The laccase-mediator system is an ideal biological modification of recycled fiber because of its milder treatment conditions, minimal damage to fiber, and environmentally friendly process (Chen *et al.* 2010a).

Laccase is a multicopper oxidase that catalyses the unspecific oxidation of phenolic compounds and, in the presence of oxidative mediators, also non-phenolic and high redox-potential compounds, which are widely present in plants, fungi, and bacteria (Baldrian 2006; Morozova *et al.* 2007; Giardina *et al.* 2010). Laccase oxidizes a wide range of substrates; thus, it has diverse potential biotechnological applications (Hildén *et al.* 2009). Laccase is a 'green' catalyst because it reacts with air and produces water as the only by-product (Riva 2006). In addition, laccase has unique properties that are not present in chemical reagents, resulting in its good application potential in the food industry, chemical analysis, medicine, environmental protection, paper industry, *etc*.

Laccase can become unstable and is easily inactivated in high temperature and other extreme conditions (pH, salt concentration, the presence of foreign materials, *etc.*), which limits its industrial application (Forde *et al.* 2010). Hence, laccase must be modified to improve its properties.

Changing enzyme properties by chemical modification has been explored since 1966, when a thiolsubtilisin was created by chemical transformation (CH_2OH to CH_2SH) of the active site (Neet and Koshland 1966; Polgar and Bender 1966). Li *et al.* (2007) studied the catalytic property and stability of laccase modified with dextran; conjugation with dextran strengthened the molecular structure of laccase and, consequently, enhanced its catalytic capability at a high temperature and in the presence of an organic solvent. Hua *et al.* (2007) estimated the stability of the modified laccase through phthalic anhydride. The modified laccase had noticeably improved thermal stability; the half-life of enzyme activity at 55 °C was extended from 192.5 min to 532.4 min. Xiong *et al.* (2011) compared the pH stability and thermal stability of native laccase and laccase modified by succinic anhydride. The chemical modification with succinic anhydride did not change the optimum temperature for catalysis, but it caused the optimum pH of the catalysts to shift from 4.5 to 5.5. Moreover, the enzymatic activity increased by 60%. Compared with native laccase, the modified laccase exhibited remarkably higher pH stability and thermal stability. However, the above modification methods include complicated steps, harsh reaction conditions, and higher cost of processing. In addition, while the stability of laccase activity was modified to some extent, the nominal activity of the modified laccase was lower than that of unmodified native laccase.

Thus, there are noteworthy limitations to the general use of laccase in many industrial processes, especially in the modification of the recycled plant fiber. However, linking certain compounds to the side chains of particular amino acid residues of an enzyme, or to a terminal amino acid residue of an enzyme, can modify the enzyme properties with respect to stability and/or activity (Siddiqui and Cavicchioli 2003).

The goal of this study was to improve laccase properties including stability and activity by linking a compound or a portion of the compound to the amino groups ($-NH_2$) or carboxyl groups (-COOH) of its amino acid residues. Furthermore, the physical properties of the recycled plant fiber treated by the modified laccase were evaluated.

EXPERIMENTAL

Materials

Laccase was purchased from Sukahan Bio-technology Co. Ltd. (Weifang, China). L-Phenylalanine methyl ester hydrochloride (L-PME) and 1-ethyl-3-(3-dimethyllaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Shanghai Yuanye Bio-technology Co. Ltd. (Shanghai, China). Thiourea dioxide (TDO) was purchased from Aladdin Industrial Corporation (Shanghai, China).

Dimethyl sulfoxide (DMSO) was purchased from Yonghua, Jiangsu Fine Chemicals Co., Ltd. (Suzhou, China). 2,2'-azin-obis-(3-ethylbenzyl thiozoline-6-sulfonate) (ABTS) was purchased from Guangzhou Qiyun Bio-technology Co., Ltd. (Guangzhou, China), and histidine was purchsed from Guangzhou Feibo Bio-technology Co., Ltd. (Guangzhou, China). A commercial sample of old corrugated containers (OCC) pulp was secured from a Dongguan paper mill, China.

Chemical Modification of Carboxyl Groups of Laccase

Laccase (10 mL, 10 mg/mL), L-PME (4 mL, 1 mg/mL), and EDC (5 mg) were mixed thoroughly by stirring. The resulting mixture was incubated at various temperatures (from 4 °C to 60 °C) for different reaction times (from 0 to 24 h). The laccase and L-PME solutions were prepared at pH 5.8 using 0.06 mol/mL NaH_2PO_4/ Na_2HPO_4 buffer. After treatment, the mixture was dialyzed in 0.2 mol/mL Na_2HPO_4/citric acid buffer (pH 5.0) at room temperature for 24 h to remove the excess reagents. The modified laccase was stored at < 0 °C prior to use and analysis.

Chemical Modification of Amino Groups of Laccase

Laccase (10 mL, 10 mg/mL), TDO (10 mL, 1 mg/mL), and DMSO (50 mg) were mixed by stirring. The resulting mixtures were incubated at different temperatures (from 4 °C to 60 °C) at various reaction times (from 0 to 24 h). The laccase and TDO solutions were prepared in 0.06 mol/mL NaH_2PO_4/ $Na_{2H}PO_4$ buffer (pH 7.8). After treatment, the mixture was dialyzed in 0.2 mol/mL Na_2HPO_4/citric acid buffer (pH 5.0) at room temperature for 24 h to remove the excess reagents. The modified laccase was stored at < 0 °C prior to use and analysis.

Laccase Activity Assay

Laccase activity was determined by the oxidation of ABTS compound (Sealey and Ragauskas 1998). The assay mixture contained 1.50 mL of 0.50 mM ABTS and 1.50 mL of 0.10 M sodium acetate buffer solution (pH 4.8) with varying amounts of laccase (0 µL to 50 µL). This mixture was immediately analyzed using a S3100 UV/Vis spectrophotometer (λ = 420 nm, $\varepsilon 420 = 3.6 \times 10^4$ mol/L cm^{-1}) and then re-analyzed every 30 s for the next 3 min.

Laccase Stability Assay

To assess whether the modification improved laccase stability, continuous thermoinactivation of unmodified and modified laccase was performed at T_{50} (72 °C in each case). Samples were removed at a certain time interval, transferred to a microtitre plate held on ice, and following re-warming to room temperature, assayed by the standard protocol (Sealey and Ragauskas 1998). Each point represented was assayed in triplicate with a standard error of 10%. All experiments were performed eight to ten times.

Pulp Treatment

Native or modified laccase and 1% (w/w) of histidine relative to the dry pulp were added with stirring to a 5% consistency aqueous suspension of OCC pulp and buffered to pH 7 using 0.1 M sodium bicarbonate solution. The pulp slurry was magnetically stirred at room temperature under O_2 atmosphere (continuous bubbling) for 1.5 h. After treatment, the pulp sample was filtered, washed with deionized water until the filtrate was colorless, and air-dried.

Paper Testing

Handsheets were prepared according to TAPPI T205 sp-12 (2011) and conditioned for at least 24 h at 23 °C and 50% relative humidity before physical testing. The tensile strength, bursting strength, and tearing strength were measured according to TAPPI T494 om-13 (2011), T 810 om-11 (2011), and T 414 om-12 (2011), respectively. Each of the strength property results was reported as the average of four measurements.

RESULTS AND DISCUSSION

Effect of L-PME and TDO Amount on the Activity of Modified Laccase

Figure 1 shows the activity of modified laccase with different amounts of L-PME and TDO reagents. For tests using L-PME, the reaction conditions were: laccase dose of 10 mL, reaction time of 24 h, 4 °C, and pH 5.8. For TDO tests, the reaction conditions were: laccase dose of 10 mL, reaction time of 24 h, 4 °C, and pH 5.8. The activity of laccase increased first and then decreased with the increasing amounts of L-PME or TDO. When the amounts of L-PME and TDO were 4 mg and 4.5 mg, respectively (*i.e.*, 4% and 4.5% relative to dry laccase), compared with the control, the activity of modified laccase reached its maximum, showing improvements by 209% and 50%, respectively. This effect was attributed to the reaction between the terminal amino acid groups (-COOH and -NH_2) of laccase and L-PME and TDO, resulting in L-PME and TDO grafted into the side chain of laccase. Employing a higher amount of L-PME and TDO was not beneficial to laccase activity.

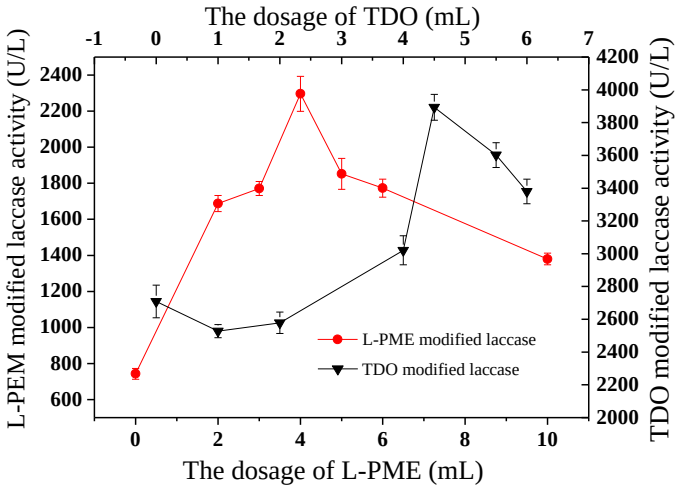

Fig. 1. Activity of laccase modified with different amounts of L-PME and TDO

Modified Laccase Activity under Different Reaction Times

Reaction time was also followed for the L-PME and TDO treatment process, as shown in Fig. 2. Shorter reaction time can lead to incomplete reactions, and higher reaction time can lead to decreased laccase activity and wasted energy (Wang *et al.* 2005). For L-PME tests, the reaction conditions were: L-PME dose of 4 mL, laccase dose of 10 mL, 4 °C, and pH 5.0. For TDO tests, the reaction conditions were: TDO dose of 4.5 mL, laccase dose of 10 mL, 4 °C, and pH 9.0. The activities of both modified laccases first increased then decreased with increasing reaction time. The L-PME- and TDO-modified laccases reached their maximum activity when the reaction time was 8 h and 13 h, respectively, and the activity decreased above these values. This was due to the pH change in the reaction system over time. The L-PME-modified laccase system changed from pH 5 to 4.4 when the reaction time was 12 h, and the TDO-modified laccase system changed from pH 9 to 5.38 when the reaction time was 16 h. The pH changes affected the reaction of between laccase and the reagents and also affected the laccase activity.

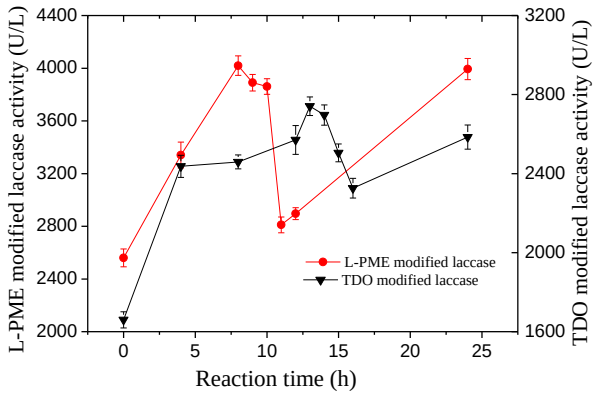

Fig. 2. The activities of laccase treated with L-PME and TDO reagents at different reaction time

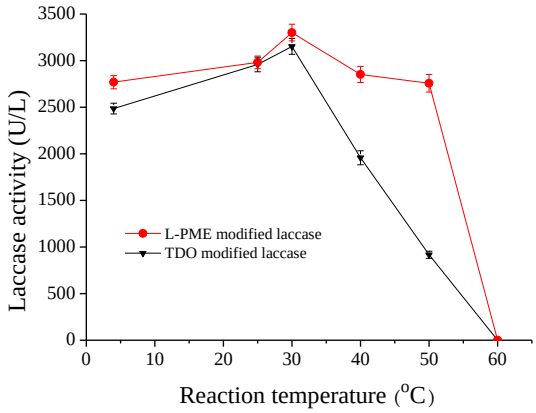

Fig. 3. The activity of laccase treated with L-PME and TDO reagents at different reaction temperatures

Activities of the Modified Laccase at Various Reaction Temperature

The effect of reaction temperature on the activities of laccase treated with L-PME or TDO reagents is shown in Fig. 3. For L-PME tests, the reaction conditions were: L-PME dose of 4 mL, laccase dose of 10 mL, reaction time of 24 h, and pH 5.0. For TDO tests, the reaction conditions used were: TDO dose of 4.5 mL, laccase dose of 10 mL, reaction time of 24 h, and pH 9.0. The activity of both modified laccases increased first and then decreased with increasing reaction temperature, reaching a maximum value at 30 °C and then sharply declining. However, the decline of the activity of L-PME-modified laccase was lower than that of TDO-modified laccase above 30 °C. Thus, the L-PME-modified laccase showed better heat-resistance or thermal stability. When the reaction temperature was 60 °C, both modified laccases had almost zero activity. This was because laccase activity was inhibited when the reaction temperature was too low or high. Therefore, 30 °C was chosen as the most practical temperature for the modification of laccase with L-PME or TDO. This was inconsistent with the results obtained by Forde *et al.* (2010). In this previous study, the best reaction temperature for the modification of laccase with ethylene-glycol-N-hydroxy succinimide (EGNHS) and glutaraldehyde or citraconic anhydride was room temperature. This was most likely due to the fundamental differences in laccase materials and the reagents used for enzyme treatment.

Activity of the Modified Laccase at Different pH

The activity of modified laccases at different pH values is shown in Table 1. For L-PME tests, the reaction conditions were: L-PME dose of 4 mL, laccase dose of 10 mL, reaction time of 24 h, and 4 °C. For TDO tests, the reaction conditions were: TDO dose of 4.5 mL, laccase dose of 10 mL, reation time of 13 h, and 4 °C.

Table 1. Activities of the Control Sample, L-PME Modified-Laccase, and TDO-Modified Laccase with Different pH Values

Control	pH	3.06	4.04	5.00	6.00	7.03	7.91	8.96
	Activity (U/L) ± SD	32.41 ± 1.02	2069 ± 37	2560 ± 55	3236 ± 62	3328 ± 48	3111 ± 85	1660 ± 32
L-PME modified laccase	pH	3.53	4.52	5.53	6.59	7.55	9.09	9.49
	Activity (U/L) ± SD	2342 ± 98	4005 ± 102	4500 ± 75	3930 ± 95	2285 ± 48	2921 ± 68	1590 ± 76
TDO modified laccase	pH	4.03	5.02	6.02	7.00	8.06	9.05	10.02
	Activity (U/L) ± SD	2051 ± 79	2736 ± 90	2653 ± 87	2255 ± 103	1176 ± 42	479 ± 12	449 ± 10

SD = Estimated standard deviation of the mean

The laccase modified with L-PME had better acid and alkali resistance than the control sample. When the pH was 4.04 and 8.96, the unmodified laccase activity was 2069 U/L and 1660 U/L, respectively. However, the activity of the modified-laccase with L-PME was 2342 U/L and 2921 U/L, respectively, when the pH was at 3.53 and 9.09. The acid resistance of the laccase modified with TDO was similar to the control sample. However, the alkali resistance of the modified laccase with TDO was slightly less than that of the control sample. When the pH was at 4.03 and 9.05, the activities of the modified laccase was 2051 U/L and 479 U/L, respectively. Therefore, the optimal pH ranges of the unmodified laccase, the laccase modified with L-PME, and the laccase modified with TDO were 4 to 9, 3.5 to 9.5, and 4 to 8, respectively. It was remarkable that the L-PME-modified laccase exhibited a broader active pH range. This result was different from data obtained by other authors. For example, Hua *et al.* (2007) reported that the optimum pH for activity improvement of laccase through phthalic anhydride modification was 4.5 to 8.4. Xiong *et al.* (2011) demonstrated that the optimum pH of activity improvement of laccase from *Aspergillus oryzae* using phthalic anhydride, succinic anhydride, and maleic anhydride modification was 4.0 to 7.5. These results were due to the different modification groups incorporated in laccase. In this study, the carboxyl groups (-COOH) of amino acid residues of laccase were modified and/or linked by L-PME. However, amino groups ($-NH_2$) of amino acid residues of laccase were modified and/or linked by other compounds in other studies (Hua *et al.* 2007; Xiong *et al.* 2011).

Stability of Laccase

Laccase inactivation during catalysis is a great obstacle that limits its industrial application, which can be resolved by producing laccases with better pH and thermal stability through chemical modification (Liu *et al.* 2010). To establish the inactivation of laccase at high temperatures, the laccase from all the preparations were incubated for a different duration at 72 °C, and the laccase activities were determined (Figs. 4 and 5). The laccase activity decreased with increasing holding time at 72 °C, *i.e.*, the laccase was inactive at 72 °C. When it was treated with L-PME, the half-life of the modified laccase was about 16.05 min, which was increased by 56.9% compared with the control. When the laccase was treated with TDO, the half-life of the modified laccase was 7.6 min, which was increased by 1.3% compared with the control. The stability of the laccase treated by L-PME was noticeably improved, but that of the laccase treated by TDO was only improved slightly. Thus, the laccase modified with L-PME was more thermostable than the control. The thermal stability experiment also revealed that the modified laccase was superior to the native laccase. This observation was related to the structure of laccase and the modification reagent employed. Laccase stability is closely related to hydrogen bond formation, salt bridges, surface charge distribution of the protein, the protein stack effect, and the amino acid composition. The thermal stability could be caused by the sugar content, which was greater in modified laccase than in the control. However, the impurities in the crude extract may facilitate the heat inactivation of enzyme (Zouari *et al.* 1987). Therefore, when L-PME was added to the laccase solution, the structure and other properties of laccase were more stable in higher temperature. TDO easily decomposes at high temperature, and as the temperature increases, the decomposition increases, resulting in the decreased activity of the modified laccase at higher temperature.

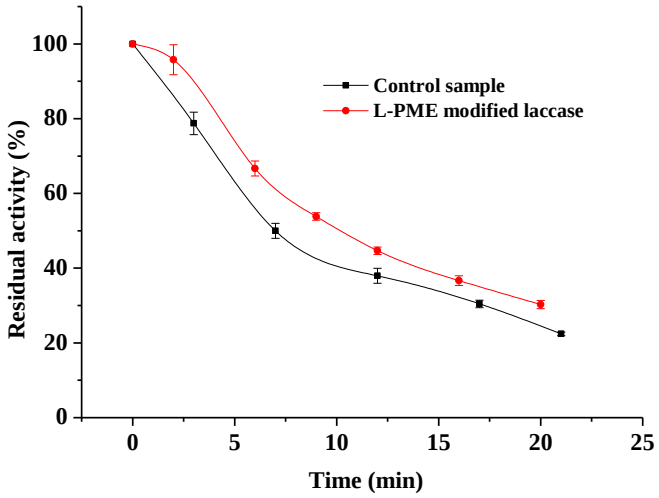

Fig. 4. Thermal-inactivation at 72 °C of the untreated laccase (control sample), and the laccase chemically modified with L-PME. All experiments were performed in triplicate in 0.06 mol/mL NaH_2PO_4/ Na_2HPO_4 buffer at pH 5.8 with a standard error of 10% or less between replicates. For both enzymes, the level of laccase activity at 0 min at room temperature was taken as 100%.

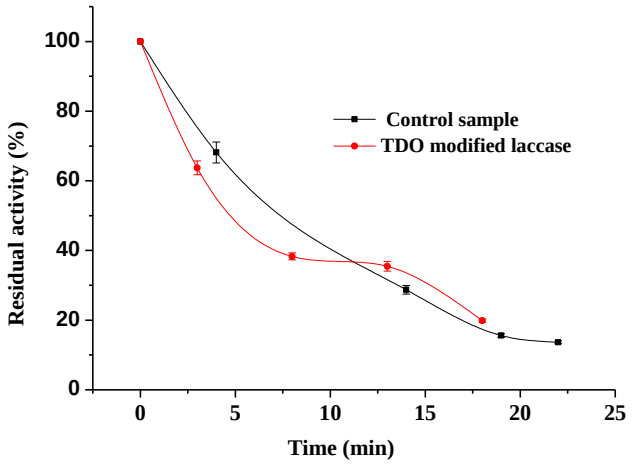

Fig. 5. Thermal inactivation at 72 °C of the untreated laccase (control sample) an d the laccase chemically modified with TDO. All experiments were performed in triplicate in 0.06 mol/mL NaH_2PO_4/ Na_2HPO_4 buffer at pH 7.8 with a standard error of 10% or less between replicates. For both enzymes, the level of laccase activity at 0 min, at room temperature was taken as 100%.

Improvement of Strength Properties of OCC Pulp Treated by Modified Laccase

Table 2 shows the tensile index, burst index, and tear index of OCC pulp treated with native laccase at pH 5.8; they were improved by 23%, 2%, and 30%, respectively, compared with the control sample. The tensile index, burst index, and tear index of OCC pulp treated by the L-PME-modified laccase were improved by 13%, 10%, and 9%, respectively, compared with the native laccase. In addition, the tensile index, burst index, and tear index of OCC pulp treated by the native laccase at pH 7.8 were improved by 28%, 33% and 10%, respectively, compared with the control. The corresponding properties of OCC pulp treated by the TDO-modified laccase were improved by 5%, 7%, and 6%, respectively, compared with the native laccase. The L-PME-modified laccase was prepared using pH 5.8 phosphate buffer; TDO-modified laccase was prepared using pH 7.8 phosphate buffer.

Table 2. Strength Properties of the Control OCC Pulp, Pulp Treated with Native Laccase, and Pulp Treated with Modified Laccases

Samples	Tensile Index (N·m/g) ± SD	Burst Index (kPa·m²/g) ± SD	Tear Index (mN·m²/g) ± SD
Control (pH 5.8)	28.2 ± 1.2	2.04 ± 0.02	8.48 ± 0.15
Native laccase (pH 5.8)	34.7 ± 0.9	2.08 ± 0.03	11.1 ± 0.08
L-PME-modified laccase (pH 5.8)	39.2 ± 1.0	2.29 ± 0.02	12.0 ± 0.12
Control (pH 7.8)	25.3 ± 0.6	1.45 ± 0.04	6.69 ± 0.09
Native laccase (pH 7.8)	32.3 ± 0.5	1.93 ± 0.05	7.37 ± 0.13
TDO-modified laccase (pH 7.8)	34.0 ± 0.3	2.06 ± 0.07	7.81 ± 0.11

SD = Estimated standard deviation of the mean

The improvement of physical properties of OCC pulp treated by laccase-mediator system is due to the formation of phenolic oxygen radicals on the surface of pulp fiber treated by laccase, resulting in the formation of small molecules that cross-link with the fiber. Phenols act as a bridge between fibers, thereby enhancing the physical properties of the paper (Chen *et al*. 2010a). The laccase grafted by L-PME and TDO reagents easily reacted with the substrates, leading to the enhancement of the phenolic oxygen radical generation of the surface of pulp fiber treated by chemically modified laccase. Therefore, the strength properties of the OCC pulp treated by the modified-laccase/histidine system were better than that of the native laccase/histidine system.

CONCLUSIONS

1. The activities of both modified laccases obviously increased after L-PME and TDO treatments. The improvement in the activity of L-PME treated laccase was better than that of TDO-treated laccase.
2. The stability of L-PME treated laccase remarkably increased compared with the control sample. However, the stability of TDO treated laccase changed only slightly.
3. The strength properties were the maximum when the OCC pulp was treated by the L-PME treated laccase/histidine system.
4. The methods using L-PME and TDO modified-laccases were feasible, and the activity and stability of laccase were greatly improved.

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