Purification and Characterization of White Laccase from the White-rot Fungus *Panus conchatus*

Pandeng Zhou,[a,b] Chaoying Fu,[a] Shiyu Fu,[a,*] and Huaiyu Zhan [a]

Laccase is a kind of polyphenol oxidase having potential in applications for pulp bleaching, waste water treatment in mills, and removal of phenols in the food industry. The normal laccase from fungus or bacterial contains four copper atoms per protein molecular, imparting a blue color. Here it is reported that a white laccase is produced by a white rot fungus *Panus conchatus* from its solid-state fermentation. The activity center of this laccase is Cu_2FeZn, which lacks the typical type-1 blue copper color. The polyacrylamide gel electrophoresis of purified laccase showed a main polypeptide with a molecular weight of about 60 kDa. Laccase substrate 2,6-dimethoxylphenol and others, such as syringaldazine, o-tolidine, and ABTS, were readily oxidized, among which the K_m for syringaldazine was the highest. The isoelectric point of this enzyme was 3.6 and it was stable at temperatures below 45 °C over a wide range of pH (4-12).

Keywords: White-rot fungus; Panus conchatus; White laccase; Activity center; Copper; Iron; Zinc

*Contact information: a: State Key Laboratory of Pulp and Paper Engineering, South China University of Technology, Guangzhou 510640; b: College of Chemical and Environmental Engineering, Hunan City University, Yiyang Hunan 413000, China; *Corresponding author: shyfu@scut.edu.cn*

INTRODUCTION

In recent years, laccase has provoked considerable interest in a wide range of applications such as delignification and pulp bleaching, water or soil bioremediation, dye decolorization, and chemo-enzymatic synthesis (Martínez *et al*. 2009; Faraco *et al*. 2009; Sadhasivam *et al*. 2009; Mikolasch and Schauer 2009). The characteristics and functions of laccase depend on both the fungal species and the cultural conditions (Giardina *et al*. 1999). Most fungal laccase varieties belong to the family of blue copper phenol oxidases, containing four copper atoms per molecule distributed among three different copper binding sites (Cole *et al*. 1990; Giardina *et al*. 1999; Fraterrigo *et al*. 1999). The type-1 (or blue copper) site is responsible for the blue color of laccase and has a maximum absorbance at 605 nm in the visible spectrum (Giardina *et al*. 1999). The type-2 copper site exhibits no signal in UV-visible spectrum, but has signals in ESR spectra (Cole *et al*. 1990; Fraterrigo *et al*. 1999). The type-3 site is a double-copper center, corresponding to a UV absorbance near 320 nm (Giardina *et al*. 1999; Fraterrigo *et al*. 1999). However, yellow laccase from *Panus tigrinus* or *Pleurotus ostreatus* has no maximum in the blue region of the absorption spectrum; this can be explained by the fact that the copper is in its reductive (Cu^+) valence state (Leontievsky *et al*. 1997; Pozdnyakova *et al*. 2004). A white laccase isoenzyme (POXA1) from *Pleurotus ostreatus* has only one copper, but two zinc and one iron atom per molecule (Palmieri *et al*. 1997).

Usually, typical blue laccase can use phenolic compounds or phenolic units in lignin as substrates. Natural or synthetic mediators, such as 3-hydroxyanthranilic acid,

ABTS, HBT, *etc*., are often required for promoting catalytic ability and extending the application of laccase in degrading non-phenolic compounds (Camarero *et al*. 2007; Call and Mücke 1997). A yellow or white laccase may oxidize non-phenolic compounds in the absence of a mediator, thus exhibiting higher redox potential and broader specificity (Leontievsky *et al*. 1997; Xu *et al*. 2001).

In this work, another white laccase producer, *Panus conchatus,* was researched. Compared with the lignocellulolytic enzyme system of the well-known white-rot fungi *Phanerochaete chrysosporium*, the enzymes produced by *P. conchatus* contain high levels of extracellular laccase activity with minimum cellulase activity, which suggests their potential application in the selective delignification of pulp (Hu *et al*. 2001). The major ligninolytic enzymes of this fungus are laccase and manganese peroxidase, with no lignin peroxidase. The crude *P. conchatus* laccase has been successfully used for pulp bleaching in the presence of some mediators in previous studies (Aniwar *et al*. 2003; Mo *et al*. 2006).

The objective of this work was to identify and characterize the white laccase produced by *Panus conchatus*. The laccase was purified and metal constituents in the protein were determined. This work was able to describe members of the laccase family and provide new insight to an oxidative enzyme.

EXPERIMENTAL

Material and Methods

Fungus and culture conditions

A strain of white-rot fungus, *Panus conchatus*, was screened and isolated from decayed wood samples (Hu *et al*. 2001) and was maintained on a malt agar slant at 4 °C in the laboratory.

The fungus was cultured in 30 mL of malt juice medium in a rotating flask at 40 °C for 7 days. Freshly harvested 7-day-old mycelia of fungus were mixed with 100 mL sterilized water and homogenized in a high-speed blender at 10000 rpm for 2 min. Ten mL of homogeneous mycelial suspension was inoculated in a 500 mL Erlenmeyer flask containing 10 grams (o.d. weight) sterilized wet rice straw with a moisture content of 65%. Solid-state fermentation was carried out at 40 °C for 28 days. The laccase activity of the crude cultivation extract was determined every two days.

Laccase assays

Laccase activity was measured by monitoring the oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) at 420 nm (ε_{420nm} = 36,000 $M^{-1} \cdot cm^{-1}$). Measurements were performed in triplicate for each sample analysis. One international unit of enzyme activity is defined as the amount of enzyme required to oxidize 1 µmol substrate in one minute (Bourbonnais and Paice 1990).

Enzyme purification

The crude enzyme was harvested from the fermentation culture of *P. conchatus* after 24 days. Ammonium sulphate precipitation was used as the initial step in enzyme purification. Solid ammonium sulphate was added to the crude enzyme to 30% saturation under constant stirring at 4 °C for 3 h. The precipitate was removed by centrifuging at 8000 g for 5 min at 4 °C. Supplemental ammonium sulphate was added into the

supernatant to 80% saturation. The precipitated proteins were collected by centrifuging and were re-dissolved in deionized water. Buffer exchange and further concentration against 50 mM NaH_2PO_4 buffer (pH 5.0) were performed in an Amicon stirred ultrafiltration cell (Amicon, Beverly, MA) with an YM-10 membrane (molecular weight cut-off 10,000 Da). Then, the enzyme concentrate was loaded on a DEAE-cellulose DE-32 column, which was pre-equilibrated with 200 mL phosphate buffer (50 mM, pH 5.0), and was eluted with a linear gradient of 0-0.5 M NaCl in 400 mL phosphate buffer (50 mM, pH 5.0). Fractions containing laccase activity were pooled and concentrated by ultrafiltration through an Amicon membrane YM-10. The concentrated protein was finally loaded on a Sephadex G-100 column and eluted with water to obtain purified laccase. Fractions containing laccase were pooled, concentrated, and freeze-dried.

SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 11% polyacrylamide gel as described by Laemmli (1970). Markers with molecular masses ranging from 14.4 to 97.4 kDa (Bio-Rad) were used as standards and were run with each gel. Protein bands were stained with Coomassie brilliant blue G-250. Native polyacrylamide gel electrophoresis (Native-PAGE) was performed following the same method as described above without sodium dodecyl sulfate. Proteins with laccase activity on Native-PAGE gels were visualized by staining with ABTS at pH 4.0. Isoelectric focusing (IEF) was performed on 5% polyacrylamide gels containing ampholytes covering a pH range from 2.5 to 5.5. The anode solution was 1 M phosphoric acid and the cathode solution was 1 M sodium hydroxide.

UV-visible spectroscopy

The UV-visible absorption spectrum of the purified *Panus conchatus* laccase was determined at wavelengths between 200 and 700 nm at room temperature in a phosphate buffer (50 mM, pH 6.0) using a SHIMADZU 2550 spectrophotometer.

Amino acid sequence analysis

The N-terminal amino acid sequence of purified laccase was determined by step-wise Edman degradation as described by Palmieri *et al*. (1997). A Perkin-Elmer applied biosystem 477A protein sequencer was used and was equipped with a 120A HPLC apparatus for on-line phenylthiohydantoin-amino acid analysis. Proteins isolated by SDS-PAGE were electroblotted onto a polyvinylidene difluoride membrane. The excised bands were directly analyzed. The constituents of amino acids were determined using a Hitachi 835-50 amino acid analyzer after hydrolysis of 100 mg laccase in 6 M hydrochloride at 110 °C for 24 h.

Atomic absorption spectroscopy

The zinc, copper, and iron contents in laccase were determined with a Perkin-Elmer 5100 atomic absorption spectrometer equipped with a Zeeman graphite furnace module and an autosampler.

Substrate specificity and stability

The compounds listed in Table 5 were tested as electron-donor substrates of laccase. Substrate specificity was qualitatively studied by measuring the initial velocity of the catalytic reaction at 25 °C. Initial rates were calculated from the linear portion of the

progress curve. The wavelengths used for measuring oxidation kinetics were 420 nm for ABTS (ε = 36 000 $M^{-1}\cdot cm^{-1}$), 590 nm for o-tolidine (ε = 6 340 $M^{-1}\cdot cm^{-1}$), 530 nm for syringaldazine (ε = 65 000 $M^{-1}\cdot cm^{-1}$), and 468 nm for 2,6-dimethoxylphenol (ε = 49 600 $M^{-1}\ cm^{-1}$) (Xiao *et al*. 2004; Di Nardo *et al*. 2004).

The stability was studied by incubating 4.0 U purified laccase in 1,000 mL of buffer under a variety of pH (diluted sulfuric acid 2 to 4, sodium acetate 4 to 6, phosphoric buffer 6 to 9, sodium bicarbonate/sodium hydroxide 10 to 11, potassium chloride/sodium hydroxide 12 to 13), temperature, and incubation time conditions. The relative activity was calculated by normalizing the activity measured at pH 4.0 at room temperature without incubation to 100%.

RESULTS

Production and Purification of Laccase

Laccase and manganese peroxidase are the two ligninolytic enzymes that are extracellularly excreted by *Panus conchatus* in solid-state fermentation (Fig. 1) and submerged fermentation (data not shown). During solid-state fermentation, laccase activity reached a maximum of 33 IU/g straw after 22 days cultivation and maintained a high level of activity for at least 3 days. Meanwhile, manganese peroxidase activity was very slight, and not even detectable after 24 days. No lignin peroxidase activity was detected at any time in the cultures. Therefore, the crude enzyme from solid-state fermentation was harvested after 24 days, which contained only laccase as a ligninolytic enzyme.

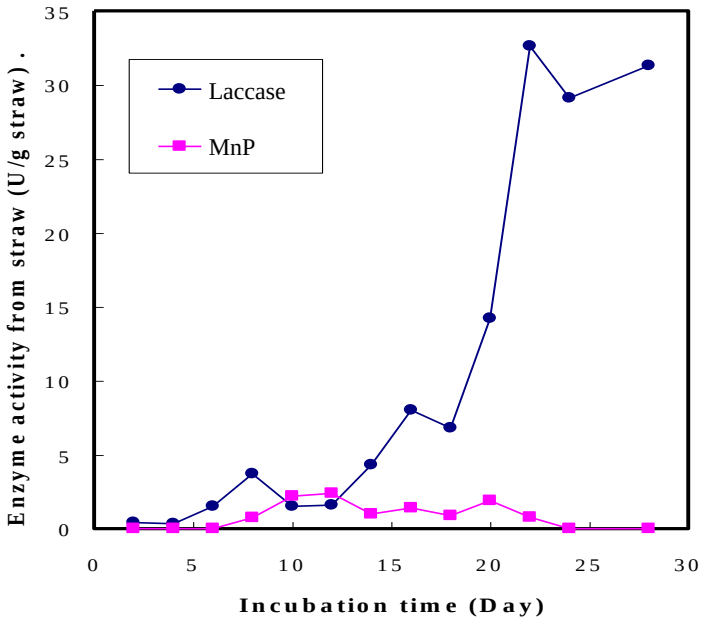

Fig. 1. Profile of ligninolytic enzymes produced by *P. conchatus* during solid-state fermentation (mean of triplet data)

Ammonium sulfate-precipitated enzyme was loaded on a DEAE-cellulose column and eluted with a continuous gradient 0 to 0.5 M NaCl in phosphate. The elution profile is shown in Fig. 2. One peak of laccase activity was eluted from DEAE-cellulose column.

The fractions with high laccase activity were pooled and loaded onto a Sephadex G-100 column. A colorless fraction with laccase activity was obtained and a white laccase protein was obtained after being lyophilized. The separated colorful compounds, probably from the degraded lignin in straw, were retained on the column without collection.

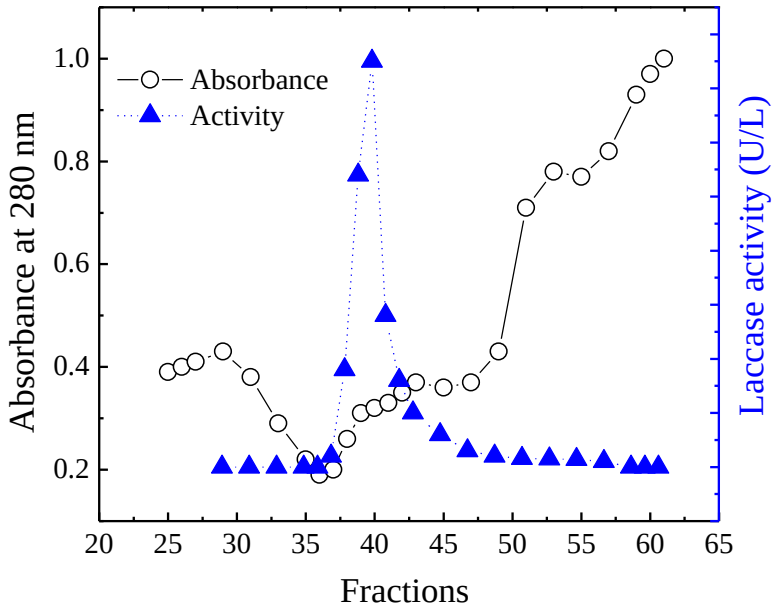

Fig. 2. DEAE-cellulose chromatography of *P. conchatus* crude enzymes

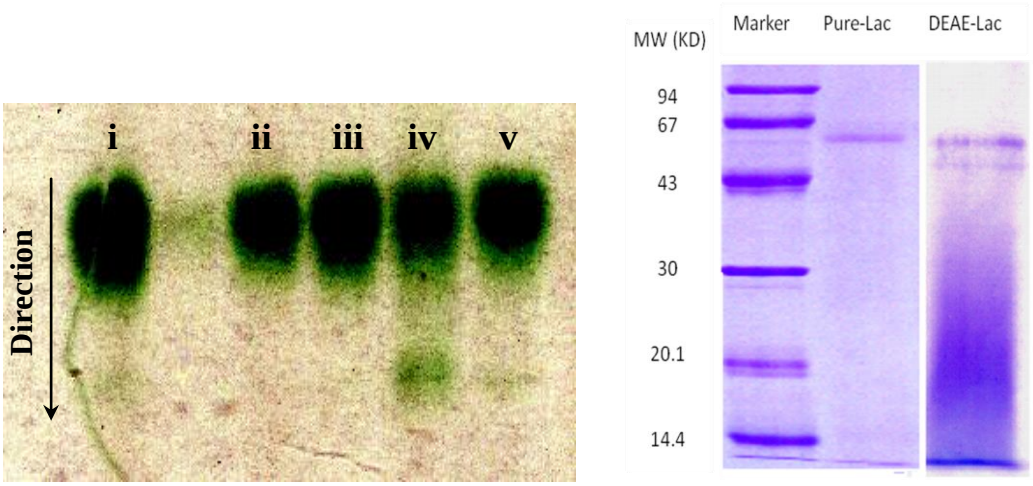

Fig. 3. Electrophoresis images of purified *Panus conchatus* laccase: A) Native-PAGE of crude enzyme (i), enzymes treated by both DEAE-cellulose and Sephadex chromatography (ii and iii, two vials from Sephadex separation), enzymes treated by DEAE-cellulose (iv and v, two vials from DEAE-cellulose separation); B) SDS-PAGE of purified laccase (ii in A) and DEAE-laccase

Either crude or purified enzyme preparations were run on native-PAGE media. The laccase protein bands can be observed by staining with ABTS solution because of the formation of green-colored oxidative products. At the beginning of the reaction with ABTS, the green band was narrow but easy to diffuse, resulting in wide bands in the PAGE gels (Fig. 3a). Figure 3a also shows that there were two laccase isoenzymes in the

crude enzyme preparation (Fig. 3a-i) extracted from solid-state fermentation, as visualized on Native-PAGE gel. One enzyme had higher activity and was purified further. The other had minor activity with no further purification. In partially purified enzyme fractions by DEAE-cellulose chromatography, there were still two isoenzymes with laccase activity, as shown in Figs. 3a-iv and v. When these fractions were concentrated and passed through gel filtration chromatography, the purified protein contained only one laccase isoenzyme and only one protein band on Native-PAGE (Figs. 3a-ii, iii) and SDS-PAGE gel (Fig. 3b). The DEAE purified enzyme (DEAE-Lac in Fig 3 B) has two protein bands also, one of them is strong and another is weak. The crude enzyme have many bands that didn't separate well. The molecular mass of the purified laccase determined by SDS-PAGE analysis was about 60 kDa. The isoelectric point of laccase was 3.6.

Molecular Mass Measured by MALDI-MS

More accurate determination of molecular mass of laccase was carried out using MALDI-mass spectrometry (Fig. 4). The measured molecular mass of purified *P. conchatus* laccase was 56.1 kDa, which is close to that of other white-rot fungi, but less than that of plant laccase such as sycamore. The molecular mass of this laccase is 60 kDa based on SDS-PAGE. In general, the molecular mass of laccases from groups of terrestrial fungi was obtained based on PAGE in the range of 60 to 80 kDa, including laccase from *Armillaria mellea* (80 kDa, Curir *et al*. 1997), *Pycnoporus cinnabarinus* (74 kDa, Eggert *et al.* 1996), *Trametes sanguinea* (62 kDa, Nishizawa *et al.* 1995), *Trametes villosa* (63 kDa, Xu *et al.* 1999), *Schizophyllum commune* (62, 64, 72 kDa, Phillips and Leonard 1976), *Pleurotus ostreatus* (59 kDa, Sannia *et al.* 1986), *Rigidoporus lignosus* (52, 55 kDa, Geiger *et al.* 1986), *Phellinus noxius* (70 kDa, Driouich *et al.* 1992), *Botrytis cinerea* (74 kDa, Slomczynski *et al.* 1995). The laccase from wood, Sycamore (95-100 kDa, Thurston 1994) is bigger than that from fungi.

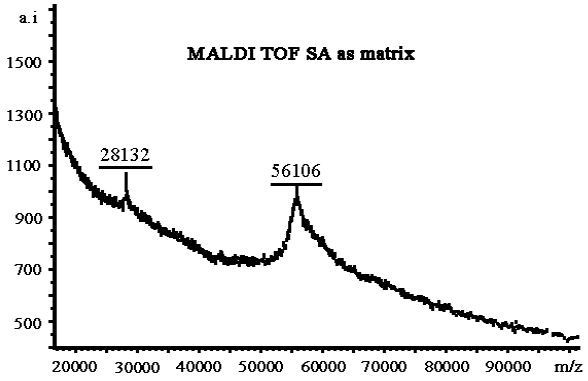

Fig. 4. MALDI TOF spectrum of *Panus conchatus* laccase
∗ The analyte mixed with a saturated sinipinic acid solution.

UV-visible Absorption Characteristics

The UV-visible spectrum of purified laccase shown in Fig. 5 differs from typical blue laccase as widely reported (Giardina *et al*. 1999; Fraterrigo *et al*. 1999). There is a shoulder of absorbance at 320 nm, corresponding to the two-copper center of a type-3 site. However, there was no absorbance peak around 600 nm, which indicates that the *Panus conchatus* laccase does not exhibit an apparent color, that means no type 1 copper.

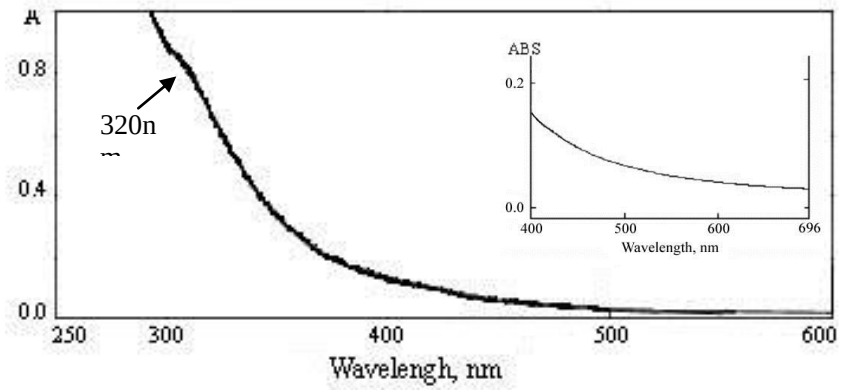

Fig. 5. UV-visible spectra of laccase produced by *Panus conchatus*

Metal Composition

Three kinds of metal elements, copper, iron, and zinc, were found in purified laccase (0.1 mg/mL) as determined by atomic absorption spectroscopy. The content of each element was 2.0 mol/mol copper/protein, 1.0 mol/mol zinc/protein, and 1.0 mol/mol iron/protein. The metal composition suggests that the ratio of copper/zinc/iron is 2:1:1 for the *P. conchatus* laccase. This result is in accordance with the UV-visible spectrum, in which no blue copper in type-1 site appears, since type-1 and 2 sites are taken by zinc and iron. However, the metal composition of *P. conchatus* laccase was different from the earlier reported *Pleurotus ostreatus* laccase isoenzyme, which contained copper, iron, and zinc in a ratio of 1:1:2 (Palmieri *et al*. 1997).

The constitution of metal atoms in laccase may be dependent on the culture conditions. Thurston (1994) proposed that the typical laccases with Cu-I and Cu-II are produced from submerged culture. Iimura *et al*. (1995) got a colorless laccase from solid-state fermentation of *Coriolus versicolor*, and Leonitievsky got a yellow laccase from solid-state culture of fungus (Leonitievsky *et al*. 1997). The reason for this laccase was regarded as the peptide of laccase was combined with degraded lignin derivatives (Mot *et al*. 2012). However, the present laccase produced by *P. conchatus* was colorless in solid-state fermentation.

The same work was repeated in submerged cultivation (not showed here), and the result was the same. The reason is not the reduction of copper in protein by reductant, but rather replacement by other metal atoms. The freeze-dried enzyme is white, so we named it the white laccase.

Table 1. Amino Acids in Purified Laccase from *P. conchatus*

Amino acid	%		Amino acid	%
TRP	1.5		MET	6.6
ASP	7.0		ILE	2.6
THR	4.5		LEU	3.2
SER	2.4		TYR	17.0
GLU	9.4		PHE	5.3
GLY	6.0		LYS	3.7
ALA	8.8		HIS	1.0
CYS	4.2		ARG	2.0
VAL	10.0			

N-terminus Amino Acid Sequences

The constituents of amino acids in purified laccase from *P. conchatus* are listed in Table 1. The content of tyrosine is the highest, contributing 17% of the dry mass. The second most abundant is valine, at levels as high as 10%.

The N-terminus peptide sequences of purified *P. conchatus* laccase are given and compared with the sequences of other known laccases in Table 2. It was found that the N-terminus peptide sequences of laccase from *P. conchatus* were close to that of Basidiomycete species such as *P. cinnabarinus, T. versicolor, Basidiomycete PM, C. hirsutus, C. subvermispora, Phlebia radiata, etc.* (Bourbonnais *et al*. 1995; Eggert *et al*. 1996; Coll *et al*. 1993; Kojima *et al*. 1990; Fukushima and Kirk 1995; Saloheimo *et al*. 1991), but much different from laccase produced by Ascomycete fungi (Perry *et al*. 1993; Germann *et al*. 1988; Williamson 1994).

Table 2. N-terminal Amino Sequences of Laccase from *P. conchatus* and Other Fungi

Microorganism	Reference	N-terminal amino acid sequence
P. conchatus	-	A I G P V T D L H I V N D N I
P. cinnabarinus	Eggert *et al. 1996*	A I G P V A D L T L T N A A V S P D G F S
T. versicolor *I* *II* *III*	Bourbonnais *et al. 1995*	 A I G P V A S L V V A N A P V S P D G F L G I G P V A D L T I T D A A V S P D G F S G I G P V A D L T I T D A E V S P D G L S
Basidiomycete PM1	Coll *et al. 1993*	S I G P V A D L T I S N G A V S P
C. hirsutus	Kojima *et al. 1990*	A I G P T A D L L T I S N A E V S P D G F A
C. subvermispora	Fukushima *et al. 1995*	A I G P V A D L E I T D A F V S P D G P
Phlebia radiata	Saloheimo *et al. 1991*	S I G P V T D F H I V N A A V S P
A. bisporus	Perry *et al. 1993*	D T - K T F N F D L V N T R L A
N. crassa	Germann *et al. 1988*	G G G G G C N S P T N R Q C W S P
Cryptococcus neoformans	Williamson *et al. 1994*	X K T D E S P E A V S D N Y M P K

Substrate Specificity and Stability

Laccase catalyzes the oxidation of a variety of phenolic compounds and aromatic amine substrates and reduces molecular oxygen to water as a co-product. Several substituted phenols and aromatic amines were studied as possible substrates in this work. The relationship between initial reaction rate and initial substrate concentrations

produced typical Michaelis-Menten curves. The parameters K_m and V_{max} for different substrates were calculated and are listed in Table 3. The value of K_m for syringaldazine was the highest.

Table 3. Kinetic Parameters of Catalytic Oxidation by *Panus conchatus* Laccase

Substrate	K_m (mmol/L)$^{-1}$	V_{max} (mol/min)	Relatives, R^2
2, 6- Dimethoxylphenol	0.1011	54.2	0.9995
Syringaldazine	14.11	3180	0.9852
o-Tolidine	0.1415	12.0	0.9883
ABTS	0.0116	188	0.9987

Note: The K_m and V_{max} were calculated according to Cornish-Bowden (1974).

The activity of laccase was stable and could be kept for long periods of time at room temperature. Figure 6a shows that the relative activity of laccase decreased when incubated in higher temperatures (45 to 70 °C). Half of the laccase activity was lost when incubated for 24 h at 45 °C and for only 10 h at 60 °C. Activity was lost very quickly at 70 °C; only 5% activity was left after 4 h incubation. The sensitivity of laccase activity to pH is shown in Fig. 6b. It was found that laccase is stable over a wide range of pH (4 to 12) after being kept at room temperature for 24 h, which suggests potential application in mildly alkali bio-pulping or bio-bleaching.

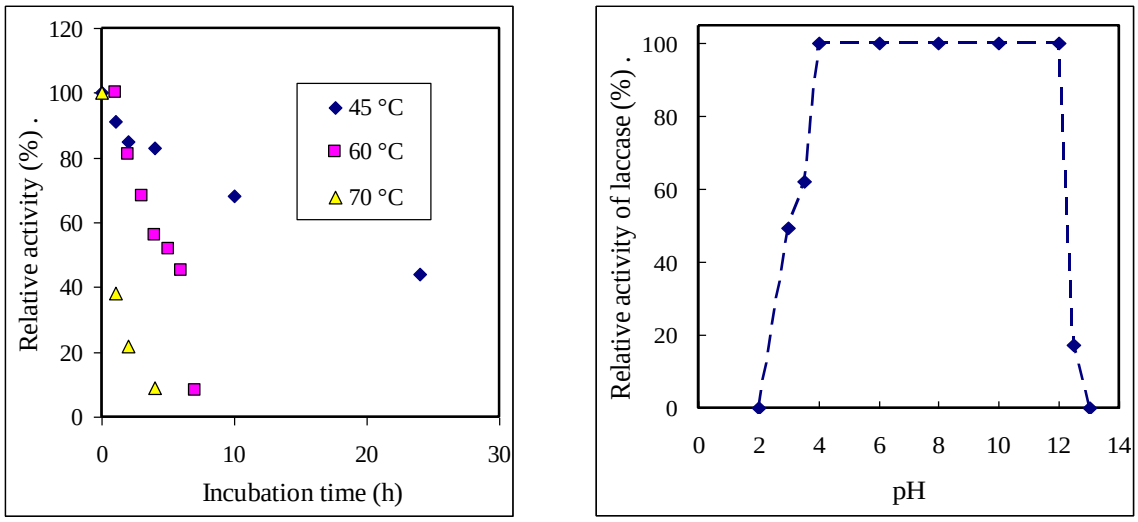

Fig. 6. Stability of *Panus conchatus* laccase under: A) different temperature, B) different pH

CONCLUSIONS

1. The purified enzyme from *Panus conchatus* belongs to the laccase family due to the observations that: (1) the enzyme exhibited a broad pattern of substrate (Table 4), (2) oxygen was used as an oxidative substrate, while no H_2O_2 was required to start the catalytic oxidation, and (3) the determined N-terminal primary structure of the

enzyme exhibited a high degree of similarity with the corresponding sequences of known Basidiomycete laccases (Table 3).

2. The *Panus conchatus* laccase exhibited white rather than the typical blue color, as evidenced by UV-visible spectroscopy and atomic absorption spectroscopy. The ratio of constituents Cu to Zn to Fe was 2:1:1. The two copper atoms constitute the double copper center, which is the same as type-3 (Cu-III) in typical blue laccase. The type-1 and 2 copper atoms were substituted by metal atoms (Zn and Fe). The laccase with 4 coppers per protein has different redox potential from plants and fungi because of the different ligands in the type-1 site. Similarly, the redox potential might be different when type-1 copper is substituted by another metal atom. Further study is now under way to prove this assumption.
3. The laccase produced by *P. conchatus* was colorless. The reason is not the reduction of copper in protein by reductant, rather replacement by other metal atoms.

ACKNOWLEDGMENTS

This research was supported by the National Natural Science Foundation of China (No. 30771689). The authors thank Professor Qizhi Yao for her guidance and constructive comments. The authors thank Mr. Hao Liu for his editorial work.

REFERENCES CITED

Aniwar, A., Fu, S. Y., and Zhan, H. Y. (2003). "Study on biochemical pulping and bleaching of reed," *Trans. Chin. Pulp Paper* 18, 51-55.

Bourbonnais, R., and Paice, M. G. (1990). "Oxidation of non-phenolic substrates: An expanded role for lactase in lignin biodegradation," *FEBS letters* 267, 99-102.

Bourbonnais, R., Paice, M. G., Reid, I. D., Lanthier, P., and Yaguchi, M. (1995). "Lignin oxidation by laccase isozymes from *Trametes versicolor* and role of the mediator 2.2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization," *Appl. Environ. Microbiol.* 61, 1876-1880.

Call, H. P., and Mücke, I. (1997). "History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozym®-process)," *J. Biotechnol.* 53(2), 163-202.

Camarero, S., Ibarra, D., Martínez, Á. T., Romero, J., Gutiérrez, A., and Río, J. C. (2007). "Paper pulp delignification using laccase and natural mediators," *Enzyme Microb. Technol.* 40, 1264-1271.

Cole, J. L., Clark, P. A., and Solomon, E. I. (1990). "Spectroscopic and chemical studies of the laccase trinuclear copper active site: Geometric and electronic structure," *J. Am. Chem. Soc.* 112(26), 9534-9548.

Coll, P. F. M., Fernandez-Abalos, J. M., Villanueva, J. R., Santamaria, R., and Perez, P. (1993). "Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT2971)," *Appl. Environ. Microbiol*. 59(8), 2607-2613.

Cornish-Bowden, A. (1974). "A simple graphical method for determining the inhibition constants of mixed, uncompetitive and non-competitive inhibitors," *Biochemical Journal* 137, 143-144.

Curir, P., Thurston, C. F., D'Aquila, F., Pasini, C., and Marchesini, A. (1997). "Characterization of a laccase secreted by *Armillaria mellea*," *Plant Physiol. Biochem. (Paris)* 35, 147-153.

Di Nardo, C., Cinquegrana, A., Papa, S., Fuggi, A., and Fioretto, A. (2004). "Laccase and peroxidase isoenzymes during leaf litter decomposition of *Quercus ilex* in a Mediterranean ecosystem," *Soil Biol. Biochem.* 36(10), 1539-1544.

Driouich, A., Laine, A. C., Vian, B., and Faye, L. (1992). "Characterization and localization of laccase forms in stem and cell cultures of sycamore," *Plant Journal* 2(1), 13-24.

Eggert, C., Temp, U., Eriksson, K. E. L. (1996). "The ligninolytic system of the white rot fungus *Pycnoporus cinnabarinus*: Purification and characterization of the laccase," *Appl. Environ. Microbiol*. 62(4), 1151-1158.

Faraco, V., Pezzella, C., Miele, A., Giardina, P., and Sannia, G. (2009). "Bio-remediation of colored industrial wastewaters by the white-rot fungi *Phanerochaete chrysosporium* and *Pleurotus ostreatus* and their enzymes," *Biodegradation* 20(2), 209-220.

Fraterrigo, T. L., Miller, C., Reinhammar, B., and McMillin, D. R. (1999). "Which copper is paramagnetic in the type 2/type 3 cluster of laccase?," *J. Biol. Chem.* 4(2), 183-187.

Fukushima, Y., and Kirk, T. K. (1995). "Laccase component of the *Ceriporiopsis subvermispora* lignin-degrading system," *Appl. Environ. Microbiol.* 61(3), 872-876.

Geiger, J. P., Rio, B., Nandris, D., and Nicole, M. (1986). "Laccases of *Rigidoporus lignosus* and *Phellinus noxius*. I. Purification and some physicochemical properties," *Appl. Biochem. Microbiol.* 12, 121-133.

Germann, U. A., Muller, G., Hunziker, P. E., and Lerch, K. (1988). "Characterization of two allelic forms of *Neurospora crassa* laccase: Amino- and carboxyl-terminal processing of a precursor," *J. Biol. Chem.* 263(2), 885-896.

Giardina, P., Palmieri, G., Scaloni, A., Fontanella, B., Faraco, V., Cennamo, G., and Sannia, G. (1999). "Protein and gene structure of a blue laccase from *Pleurotus ostreatus*," *Biochem. J.* 341(3), 655-663.

Hu, P. P., Fu, S. Y., and Yu, H. S. (2001). "Effects of nitrogen nutrient on production of lignocellulolytic enzyme profiles and laccase isoforms by *Panus conchatus* during the solid state fermentation," *J. Cell Sci. Tech.* 9, 1-7.

Iimura, Y., Katayama, Y., Kawai, S., and Morohoshi, N. (1995). "Degradation and solubilization of C13 and C14 side chain labeled synthetic lignin (dehydrogenative polymerization) by laccase III of *Coriolus versicolor*," *Biosci. Biotech. Biochem.* 9, 903-905.

Kojima, Y., Tsukuda, Y., Kawai, Y., Tsukamoto, A., Sugiura, J., Sakaino, M., and Kita, Y. (1990). "Cloning, sequence analysis and expression of ligninolytic phenoloxidase genes of the white rot basidiomycete *Coriolus hirsutus*," *J. Biol. Chem.* 265(25), 15224-15230.

Laemmli, U. K. (1970). "Cleavage of structural proteins during assembly of the head of bacteriophage T4," *Nature* 227, 680-685.

Leontievsky, A., Myasoedova, N., Pozdnyakova, N., and Golovleva, L. (1997). "Yellow laccase of *Panus tigrinus* oxidizes non-phenolic substrates without electron-transfer mediators," *FEBS Letters* 413, 446-448.

Martínez, Á. T., Ruiz-Dueñas, F. J., Martínez, M. J., Río, J. C., and Gutiérrez, A. (2009). "Enzymatic delignification of plant cell wall: From nature to mill," *Curr. Opin. Biotechnol.* 20, 348-357.

Mikolasch, A., and Schauer, F. (2009). "Fungal laccases as tools for the synthesis of new hybrid molecules and biomaterials," *Appl. Microbiol. Biotechnol.* 82(4), 605-624.

Mo, J. L., Fu, S. Y., and Zhan, H. Y. (2006). "Improving laccase activity of *Panus conchatus* by mutagenesis and used for biobleaching," *Trans. Chin. Pulp Paper* 21, 29-33.

Moţ, A. C., Pârvu, M., Damian, G., Irimie, F. D., Darula, Z., Medzihradszky, K. F., Brem, B., and Silaghi-Dumitrescu, R. (2012). "A yellow laccase with blue spectroscopic features from *Sclerotinia sclerotiorum*," *Process Biochemistry* 47, 968-975.

Nishizawa, Y., Nakabayashi, K., and Shinagawa, E. (1995). "Purification and characterization of laccase from white rot fungus *Trametes sanguinea* M85-2," *J. Ferment Bioeng.* 80(1), 91-93.

Palmieri, G., Giardina, P., Bianco, C., Scaloni, A., Capassoi, A., and Sannia, G. (1997). "A novel white laccase from *Pleurotus ostreatus*," *J. Biol. Chem.* 272, 31301-31307.

Perry, C. R., Matcham, S. E., Wood, D. A., and Thurston, C. (1993). "The structure of laccase protein and its synthesis by the commercial mushroom *Agaricus bisporus*," *J. Gen. Microbiol.* 139(1), 171-178.

Phillips, L. E., and Leonard, T. J. (1976). "Benzidine as a substrate for measuring phenoloxidase activity in crude cell-free extracts of *Schizophyllum commune*," *Mycologia* 68(2), 277-285.

Pozdnyakova, N. N., Rodakiewicz-Nowak, J., and Turkovskaya, O. V. (2004). "Catalytic properties of yellow laccase from *Pleurotus ostreatus* D1," *J. Mol. Catal. B: Enzymatic* 30(1), 19-24.

Sadhasivam, S., Savitha, S., and Swaminathan, K. (2009). "Redox-mediated decolorization of recalcitrant textile dyes by *Trichoderma harzianum* WL1 laccase," *World J. Microbiol. Biotechnol.* 25(10), 1733-1741.

Saloheimo, M., Niku-Paavola, M. L., and Knowles, J. K. (1991). "Isolation and structural analysis of the laccase gene from the lignin degrading fungus *Phlebia radiata*," *J. Gen Microbiol.* 137(7), 1537-1544.

Sannia, G., Giardina, P., Luna, M., Rossi, M., and Buonocore, V. (1986). "Laccase from *Pleurotus ostreatus*," *Biotechnol. Lett.* 8(11), 797-800.

Slomczynski, D., Nakas, J. P., and Tanenbaum, S. W. (1995). "Production and characterization of laccase from *Botrytis cinerea* 61-34," *Appl. Environ. Microbiol.* 61(3), 907-912.

Thurston, C. F. (1994). "The structure and function of fungal laccase," *Microbiology* 140(1), 19-26.

Williamson, P. R. (1994). "Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: Identification as a laccase," *J. Bacteriol.* 176(3), 656-664.

Xiao, Y. Z., Chen, Q., Hang, J., Shi, Y. Y., Wu, J., Hong, Y. Z., and Wang, Y. P. (2004). "Selective induction, purification and characterization of a laccase isozyme from the basidiomycete *Trametes* sp. AH28-2," *Mycologia* 96(1), 26-35.

Xu, F., Palmer, A. E., Yaver, D. S., Berka, R. M., Gambetta, G. A., Brown, S. H., and Solomon, E. I. (1999). "Targeted mutations in a *Trametes villosa* laccase, axial perturbations of the T1 copper," J. Biol. Chem. 274(18), 12372-12375.

Xu, F., Deussen, H.-J.W., Lopez B., Lam, L. and Li, K. (2001). Enzymatic and electrochemical oxidation of N-hydroxy compounds: Redox potential, electron-transfer kinetics, and radical stability. Eur. J. Biochem. 268, 4169±4176.

Article submitted: February 26, 2013; Peer review completed: May 26, 2013; Revised version received and accepted: February 10, 2014; Published: February 12, 2014.