Purification and Characterization of Laccase from the White Rot Fungus *Trametes versicolor*

Moon-Jeong Han, Hyoung-Tae Choi and Hong-Gyu Song*

Division of Biological Sciences, Kangwon National University, Chuncheon 200-701, Republic of Korea

Laccase is one of the ligninolytic enzymes of white rot fungus *Trametes versicolor* 951022, a strain first isolated in Korea. This laccase was purified 209-fold from culture fluid with a yield of 6.2% using ethanol precipitation, DEAE-Sepharose, Phenyl-Sepharose, and Sephadex G-100 chromatography. *T. versicolor* 951022 excretes a single monomeric laccase showing a high specific activity of 91,443 U/mg for 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate. The enzyme has a molecular mass of approximately 97 kDa as determined by SDS-PAGE, which is larger than those of other laccases reported. It exhibits high enzyme activity over broad pH and temperature ranges with optimum activity at pH 3.0 and a temperature of 50°C. The K_m value of the enzyme for substrate ABTS is 12.8 µM and its corresponding V_{max} value is 8125.4 U/mg. The specific activity and substrate affinity of this laccase are higher than those of other white rot fungi, therefore, it may be potentially useful for industrial purposes.

Key words: enzyme purification, laccase, *Trametes versicolor*, white rot fungus

White rot fungi can completely mineralize lignin and a wide variety of aromatic compounds. These compounds include xenobiotic pollutants like polycyclic aromatic hydrocarbons. The degradation of these aromatic compounds by white rot fungi depends on the production and secretion of lignin peroxidase, manganese-dependent peroxidase, and laccase. These are the key components of lignin degrading enzyme systems (Xiao *et al.*, 2003). One of these enzymes is laccase (benzenediol:oxygen oxidoreductase; EC. 1.10.3.2), a polyphenol oxidase that contains four coppers, and is able to oxidize its substrates by using molecular oxygen as an electron acceptor (Thurston, 1994). The substrates oxidized by laccase include *ortho*-, *para*-, diphenol, and aromatic compounds containing hydroxyl and amine groups. In addition to lignin degradation, laccase has various functions including participation in pigmentation in fungi (Clutterbuck, 1990), plant pathogenicity (Iyer and Chattoo, 2003), and degradation of many aromatic compounds (Xiao *et al.*, 2003). Because of those various functions and broad substrate specificity it can be utilized in many industrial processes like biopulping, textile dye bleaching, phenolics removal, effluent detoxification, and other processes (Nyanhongo *et al.*, 2002; Shin, 2004). Fungal strains with a high enzymatic activity and substrate affinity are essential for

industrial applications. In this study, laccase of *Trametes versicolor* 951022 was purified and characterized. This laccase, which had first been isolated in Korea, was highly active in degrading polycyclic aromatic hydrocarbons (Han *et al.*, 2004).

Materials and Methods

Chemicals and fungus

DEAE-Sepharose™ Fast Flow, Phenyl Sepharose™ 6 Fast Flow and standard proteins for SDS-PAGE were purchased from GE Healthcare Bioscience (USA). Sephadex G-100 was obtained from Sigma (USA), and all other purified chemicals were obtained from Sigma and Aldrich (USA).

T. versicolor 951022 was originally isolated in Kangwon-do, Korea. The fungal material used in our experiments was obtained from the Mycology Laboratory of Kangnung National University. The fungus was cultivated in the potato dextrose agar (PDA) medium (Difco, USA) and YMG broth medium (yeast extract 0.4%, malt extract 1.0%, glucose 0.4%) on a rotary shaker (130 rpm, 35°C), and was utilized as inoculum.

Enzyme assay

Laccase activity was determined by oxidation of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) as a substrate. The reaction mixture contained 5 mM ABTS in 0.1 M sodium acetate buffer (pH 5.0) and a suit-

* To whom correspondence should be addressed.
(Tel) 82-33-250-8545; (Fax) 82-33-241-4627
(E-mail) hgsong@kangwon.ac.kr

able amount of enzyme. Oxidation of ABTS was followed by an absorbance increase at 420 nm. The enzyme activity was expressed in units defined as the amount of enzyme oxidizing 1 μmol of ABTS min^{-1} (ε_{420} = 36,000 M^{-1} cm^{-1}, Childs and Bardsley, 1975).

Purification of fungal laccase

Presence of laccase activity in *T. versicolor* 951022 was tested by the changes in colony color with the addition of several drops of *o*-tolidine (4,4'-diamino-3,3'-dimethylbiphenyl; Sigma, USA) solution (conc. 1 mg/ml) to fungal colonies. After confirmation of laccase activity, YMG broth medium (500 ml) was inoculated with several agar pieces (10 × 10 mm) cut from an actively growing culture of *T. versicolor* 951022 on PDA. The fungal culture was incubated at 35°C on a rotary shaker (130 rpm) for 3 days, and the culture broth was clarified through filter paper (Whatman No. 3). The aqueous solution was precipitated with 60 ~ 80% ethanol and precipitated proteins were collected by centrifugation at 7,080 g for 30 min. The precipitate was redissolved in 100 ml of buffer A (0.1 M sodium acetate buffer, pH 5.0). The redissolved protein was applied to a DEAE-Sepharose™ Fast Flow column (4.0 cm × 25 cm), which was equilibrated with buffer A. The column was subsequently washed with 785 ml of equilibration buffer, and the enzyme fractions eluted with a linear concentration gradient of 0 ~ 0.5 M NaCl in the same buffer at a flow rate of 30 ml/h. Fractions containing laccase activity were then pooled, concentrated by ultrafiltration (Centriprep YM-10, Millipore, USA), and loaded onto a Phenyl Sepharose™ 6 Fast Flow column (2.8 cm × 3.25 cm) equilibrated with buffer B (20% ammonium sulfate added to buffer A). The enzyme fractions were eluted with a linear reverse gradient of buffer A with 20 ~ 0% ammonium sulfate at a flow rate of 2.0 ml/h. Fractions containing laccase activity were collected, dialyzed using dialysis membrane, concentrated through Centriprep YM-10, and loaded onto a Sephadex G-100 column (2.5 cm × 60 cm). The column was equilibrated and eluted with buffer A at a flow rate of 0.7 ml/min. Fractions containing laccase activity were collected and concentrated.

Characterization of fungal laccase

Homogeneity of the laccase was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis performed on 12.5% polyacrylamide gel. Proteins were stained with a silver staining kit (BioRad, USA), and the molecular mass of denatured laccase was estimated with molecular weight markers (Sigma, USA). The optimum pH for laccase was determined using citrate-phosphate buffer (pH 2.5 ~ 7.0) and phosphate buffer (pH 6.5 ~ 8.0). The effect of pH on stability of the purified laccase was determined after 1 h incubation of laccase in 0.1 M citrate-phosphate buffer (pH 3, 4, 5, 6 and 7) without substrate. The optimum temperature of laccase

was determined between 0°C and 60°C. The effect of temperature on stability of the purified laccase was determined during 4 h incubation of laccase in 0.1 M sodium acetate buffer (pH 3.0) at 0, 15, 30, 40, 50 and 60°C. The effects of pH and temperature were expressed as the relative laccase activity measured by the ABTS assay method described above. Kinetic studies were performed by measuring the initial velocity in 1-ml glass cuvette with 1-cm path length, and the velocities of enzyme-catalyzed reactions were measured at 420 nm for ABTS. Purified laccase for analysis of N-terminal sequence was confirmed by gel permeation chromatography (GPC) (Protein Pak 300SW, 7.5 × 300 mm column, Waters, Japan). The column was equilibrated with 10 mM sodium acetate buffer and was eluted with the same buffer at a flow rate of 1 ml/min. Fractions containing laccase were collected and concentrated. N-terminal amino acid sequence determination was made at the Korea Basic Science Institute using an Applied Biosystems Procise 492 clc protein sequencer (Applied Biosystems, USA).

Results

Purification of laccase from T. versicolor

The presence of laccase activity in *T. versicolor* 951022 was tested by reaction of fungal mycelium with *o*-tolidine. When *o*-tolidine solution was added, the edge of fungal colony showed green to blue color (result not shown), indicating the production of melanin intermediates by laccase (Ross, 1982). Under the culture conditions in this study, laccase was the major ligninolytic enzyme, and activity of lignin peroxidase was not detectable. In liquid culture, a maximum laccase activity was obtained after 3 days of incubation in YMG medium (Fig. 1). *T. versicolor* 951022 produced a high level of laccase without added inducers within a relatively short period of incubation.

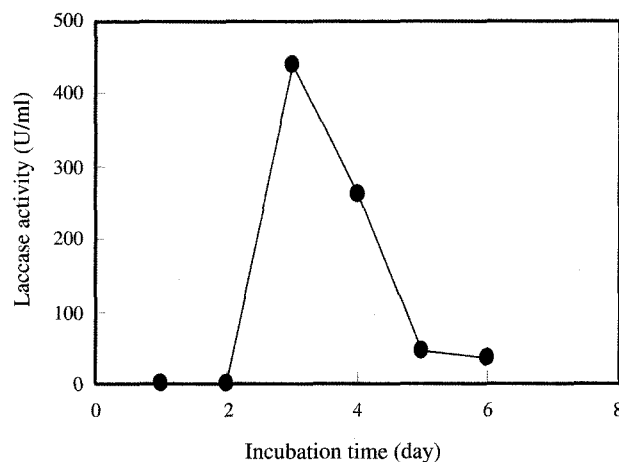The laccase secreted by *T. versicolor* 951022 was puri-

Fig. 1. Time course of laccase production by *T. versicolor* 951022 during growth in YMG medium.

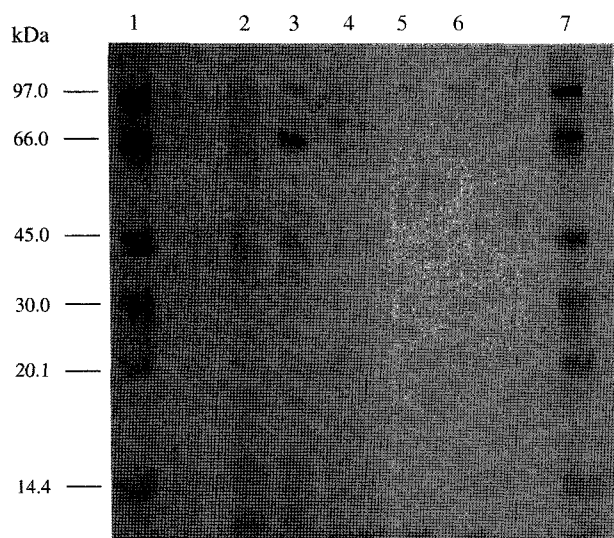fied to homogeneity according to the procedure summarized in Table 1. After size exclusion chromatography

Fig. 2. SDS-polyacrylamide gel electrophoretic analysis of purified laccase from *T. versicolor* 951022. Lane 2, culture supernatant; lane 3, ethanol precipitation; lane 4, pooled fractions from DEAE anion chromatography; lane 5, pooled fractions from phenyl HI-chromatography; lane 6, size exclusion chromatography. Lanes 1 and 7 contain molecular standards: rabbit muscle phosphorylase b (97.0 kDa), bovine serum albumin (66.0 kDa), chicken egg white ovalbumin (45.0 kDa), bovine erythrocyte carbonic anhydrase (30.0 kDa), soybean trypsin inhibitor (20.1 kDa), and α-lactalbumin (14.4 kDa).

with Sephadex G-100 column, the purified laccase protein appeared as a single band in SDS-PAGE analysis (Fig. 2). It did not have any isozyme and the molecular mass of the denatured laccase was estimated to be 97 kDa by SDS-PAGE analysis. *T. versicolor* 951022 appeared to have a laccase of monomeric protein, and the purification was 209-fold with an overall yield of 6.2% (Table 1). *T. versicolor* 951022 showed a very high specific activity of 91,443 U/mg for ABTS as a substrate.

Characteristics of the purified laccase

As shown in Table 1, the laccase from *T. versicolor* 951022 exhibited a high specific activity with ABTS as a substrate. The apparent K_m value of the enzyme for ABTS determined from the Lineweaver-Burk plot was estimated to be 12.8 μM, and the corresponding V_{max} value was 8125.4 U/mg. The first 7 residues from the N-terminal amino acid sequence of the purified laccase was GIGP-VAD (Table 2). The optimum pH of the laccase was 3.0 with ABTS as a substrate in citrate-phosphate buffer (Fig. 3A). However, this laccase showed a high relative activity that was stable over a broad pH range (from 2.5 to 4). The temperature optimum of the laccase was 50°C with ABTS as a substrate in citrate-phosphate buffer of pH 3.0 (Fig. 4A), and its activity was stable over a broad temperature range. This enzyme was very stable at temperatures ranging from 0 to 50°C for a long time, but the activity decreased to about 40% after 1 h at 60°C (Fig. 4B).

Table 1. Purification of laccase from *T. versicolor* 951022*

Purification step	Total protein (mg)	Total enzyme activity (Unit)	Specific activity (U/mg)	Recovery (%)
Cell-free extract	301.5	131,890	437.4	100
$(NH_4)_2SO_4$ precipitation	75.3	73,672	977.9	55.9
DEAE-Sepharose	3.1	16,539	5,267.3	12.5
Phenyl-Sepharose	0.3	15,447	51,488.5	11.7
Sephadex G-100	0.09	8,230	91,443.3	6.2

*Laccase activity was determined with 5 mM ABTS as a substrate.

Table 2. N-Terminal amino acid sequences of the laccases of *T. versicolor* 951022 and some other white rot fungi

| Microorganisms	N-terminal amino acid sequences	Reference
Trametes versicolor 951022	G I G P V A D	this work
Trametes versicolor ATCC 20869 laccase II	G I G P V A D	Bourbonnais *et al.* (1995)
Trametes versicolor ATCC 20869 laccase I	A I G P V A S	Bourbonnais *et al.* (1995)
Trametes villosa I	A I G P V A D	Yaver *et al.* (1996)
Basidiomycete PM1	S I G P V A D	Coll *et al.* (1993)
Phlebia radiata	S I G P V T D	Saloheimo *et al.* (1991)
Coriolus hirsutus	G I C T K A N	Shin and Lee, (2000)
Pleurotus ostreatus POXA1	A I G P T G D	Palmieri *et al.* (1997)
Phellinus ribis	A I V S T P L	Min *et al.* (2001)
Agaricus bisporus	D T X K T F N	Perry *et al.* (1993)
Neurospora crassa	G G G G G C N	German *et al.* (1988)
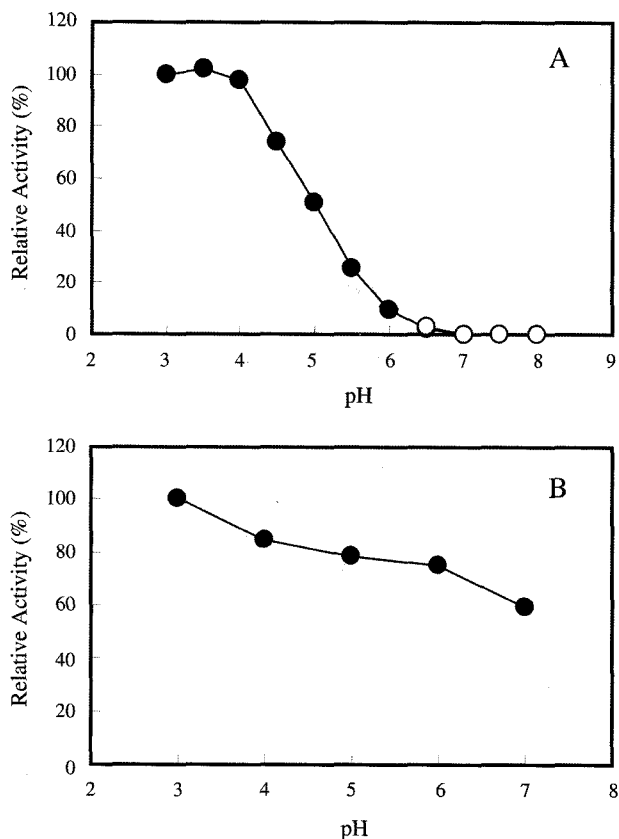
Fig. 3. Effect of pH on activity (A) and stability (B) of the purified laccase of *T. versicolor* 951022. Effect of pH on the laccase was measured in citrate-phosphate buffer (pH 2.5 ~ 7.0; ●), phosphate buffer (pH 6.5 ~ 8.0; ○). Stability of enzyme activity was determined after 1 h incubation at pH 3, 4, 5, 6 and 7.

Fig. 4. Effect of temperature on activity (A) and stability (B) of the purified laccase of *T. versicolor* 951022. Effect of temperature on the laccase was measured between 0 and 60°C. Stability of enzyme activity was determined at 0°C (●), 15°C (○), 30°C (▲), 40°C (△), 50°C (■) and 60°C (□) after preincubation for 0 ~ 4 h.

Discussion

T. versicolor 951022 secreted a laccase, which was confirmed by reaction of colony with *o*-tolidine solution. A large amount of laccase was produced during 3-4 days of incubation in liquid culture, but there was no detectable activity of lignin peroxidase. *Trichophyton rubrum* LKY-7 also showed the same pattern of enzyme production (Jung *et al.*, 2002). Laccase production can be increased by inducers such as lignin preparations, various aromatic compounds, and even some xenobiotics of low molecular weight (Nyanhongo *et al.*, 2002). In contrast, the potential inducers of laccase tested did not increase the laccase activity of the lignin-degrading basidiomycete PM1 (Coll *et al.*, 1993). In fact, a large amount of laccase could be produced without any inducers in the culture of *T. versicolor* (Bourbonnais *et al.*, 1995). *T. versicolor* 951022 also produced a high level of laccase without added inducers within a relatively short period of incubation. Other strains of *T. versicolor* showed a maximum activity at 10 days of incubation (Sack *et al.*, 1997) and laccase production reached its maximum value at 17 days in the
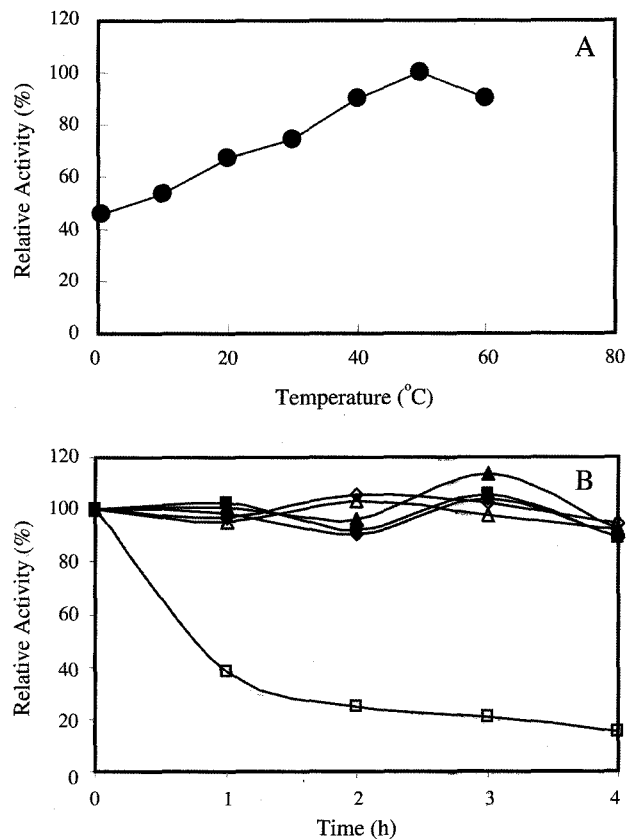
culture of *T. trogii* (Levin *et al.*, 2003). Iyer and Chattoo (2003) reported a maximum activity of laccase from *Magnaporthe grisea* at 24 h of incubation, but the total activity was not as high as that seen in this study.

The laccase secreted by *T. versicolor* 951022 was purified to homogeneity (Table 1), and the purified laccase protein appeared to be a monomeric protein similar to other fungal laccases (Garzillo *et al.*, 1998; Shin and Lee, 2000; Xiao *et al.*, 2003). It did not have any isozyme and the molecular mass of denatured laccase was estimated to be 97 kDa by SDS-PAGE analysis (Fig. 2). The molecular mass of this laccase is larger than other laccases from the same or related species of *Trametes* or other fungi, which range 61 ~ 81 kDa (Yaver *et al.*, 1996; Palmieri *et al.*, 1997; Garzillo *et al.*, 1998; Périé e *et al.*, 1998; Shin and Lee, 2000; Min *et al.*, 2001; Jung *et al.*, 2002; Iyer and Chattoo, 2003; Xiao *et al.*, 2003). The high molecular mass of the protein might lower the recovery rate of this enzyme, especially at the final stage of purification. Many laccases contain some carbohydrate (Fukushima and Kirk, 1995; Eggert *et al.*, 1996), and the high molecular weight of laccase in this study may be partly due to the presence

of carbohydrate. Measurement of carbohydrate content should be carried out in a further study. *T. versicolor* 951022 has a very high specific activity of 91,443 U/mg with ABTS as a substrate, although specific activity can be different with the substrate used and the conditions of assay. Furthermore, units of enzyme activity in each study may be defined differently. In spite of the differences in the conditions of each enzyme assay, the specific activity in this study is much higher than those in other studies using the same substrate ABTS, which showed activities between 152 ~ 610 U/mg (Eggert *et al.*, 1996; Yaver *et al.*, 1996; Muñoz *et al.*, 1997; Garzillo *et al.*, 1998; Shin and Lee, 2000; Jung *et al.*, 2002; Xiao *et al.*, 2003).

When kinetic parameters of *T. versicolor* 951022 laccase were examined, K_m value of the enzyme for ABTS was 12.8 μM and its corresponding V_{max} value was 8125.4 U/mg. When the same ABTS was used as a substrate in kinetic studies, K_m values of other laccases were higher. The laccase in *Trametes* sp. strain AH28-2 had a K_m value of 25 μM for ABTS (Xiao *et al.*, 2003). Laccases L1 and L2 in *Ceriporiopsis subvermispora* showed K_m values of 30 and 20 μM, respectively (Fukushima and Kirk, 1995). A K_m value of 45 μM was observed in the laccase from *Trichophyton rubrum* LKY-7 (Jung *et al.*, 2002) and 56.7 μM was the K_m for ABTS in *Coriolus hirsutus* (Shin and Lee, 2000). Some other laccases showed even higher K_m values (Palmieri *et al.*, 1997; Min *et al.*, 2001). Compared to those laccases, this laccase had very low K_m value indicating a high substrate affinity. The V_{max} value of this enzyme was similar to that of the laccase from *Coriolus hirsutus* (Lee and Shin, 1999).

The N-terminal amino acid sequence for the first 7 residues was GIGPVAD (Table 2). This is the same as the laccase II from other strains of *T. versicolor* (Bourbonnais *et al.*, 1995). This sequence is similar to that of the laccase I from *T. versicolor* (Bourbonnais *et al.*, 1995) and laccases from *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996) and *Phlebia radiata* (Saloheimo *et al.*, 1991). Some other laccases have quite different N-terminal amino acid sequences (Perry *et al.*, 1993; Muñoz *et al.*, 1997; German *et al.*, 1988; Shin and Lee, 2000). Although the first 7 residues of the acid sequence are same as in the laccase II from *T. versicolor* in the study by Bourbonnais *et al.* (1995), the laccase in this study is different from theirs. Their laccase II had a molecular mass of 70 kDa while ours weighed 97 kDa. Unlike their 2 isozymes, *T. versicolor* 951022 produced just one laccase. The longer amino acid sequence is a key characteristics for the differentiation of those laccases from different strains of *T. versicolor*.

The optimum pH of the laccase from *T. versicolor* 951022 was 3.0 with ABTS as a substrate in citrate-phosphate buffer (Fig. 3A). Although pH optima may depend on the substrate used (Fukushima and Kirk, 1995), most laccases in other fungi also have an optimum pH of

around 3 with ABTS as a substrate (Palmieri *et al.*, 1997; Garzillo *et al.*, 1998; Périé *et al.*, 1998; Shin and Lee, 2000; Jung *et al.*, 2002). The activity of many laccases decrease rapidly beyond optimum pH (Min *et al.*, 2001; Jung *et al.*, 2002; Iyer and Chattoo, 2003), but this laccase showed a high relative activity over a broad pH range from 2.5 to 4. The laccase of *Ceriporiopsis subvermispora* also showed a similar pattern in its pH optimum curve (Fukushima and Kirk, 1995). The laccase of *Trichophyton rubrum* LKY-7 had a pH optimum of 3, but was more stable at pH 6 (Jung *et al.*, 2002). Stability of the laccase in this study was highest at its optimum pH of 3.0, however (Fig. 3B). Stability of laccase at non-optimal pH has been reported in other studies (Min *et al.*, 2001). Stability of the laccase of *T. versicolor* 951022 is quite high over a broad pH range. This could be a very useful characteristic for various industrial applications.

The temperature optimum of the laccase was 50°C with ABTS as a substrate in citrate-phosphate buffer of pH 3.0 (Fig. 4A). Temperature ranges for high enzyme activity are narrow in many laccases (Shin and Lee, 2000; Min *et al.*, 2001; Iyer and Chattoo, 2003). However, this laccase showed a high relative activity and high stability over a broad temperature range (Fig. 4B). Optimum temperature of laccases I from *Pleurotus eryngii* was 65°C and that of laccase II from the same organism was 55°C. However, stability at those temperatures was somewhat low and their half-lives were less than 30 min at 55°C (Muñoz *et al.*, 1997). The laccase in this study was more stable than other laccases at higher temperature (Périé *et al.*, 1998; Shin and Lee, 2000; Jung *et al.*, 2002; Iyer and Chattoo, 2003). However, it was less stable at high temperatures than those from *Trametes* sp. strain AH28-2 (Xiao *et al.*, 2003), *Pycnoporus cinnabarinus* (Eggert *et al.*, 1996), and basidiomycete PM1 (Coll *et al.*, 1993). This may be due to differences in pH in the reaction which were higher than pH 3 in those reports. It has been reported that the stability of laccase at a higher temperature increased under neutral pH conditions (Min *et al.*, 2001; Jung *et al.*, 2002). The substrate used in enzyme assays may affect thermal stability, since ABTS shows a lower affinity than the DMP used in those studies (Min *et al.*, 2001).

The results obtained in this study show that the laccase of *T. versicolor* 951022 has many desirable characteristics as a catalytic agent in terms of specific activity, substrate affinity, and stability in relation to pH and temperature. More studies on substrate specificity, inhibition pattern, inducers, and many other factors are necessary to assess its industrial applications.

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References

Bourbonnais, R., M.G. Paice, I.D. Reid, P. Lanthier, and M. Yaguchi. 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.

Childs, R. and W. Bardsley. 1975. The steady-state kinetics of peroxidase with 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) as chromogen. *Biochem. J.* 145, 93-103.

Clutterbuck, A.J. 1990. Absence of laccase from yellow-spored mutants of *Aspergillus nidulans. J. Gen. Microbiol.* 136, 1731-1738.

Coll, P., J. Fernández-Abalos, J. Villanueva, R. Santamaría, and P. Perez. 1993. Purification and characterization of a phenoloxidase (laccase) from the lignin-degrading basidiomycete PM1 (CECT 2971). *Appl. Environ. Microbiol.* 59, 2607-2613.

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

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

Garzillo, A., M. Colao, C. Caruso, C. Caporale, D. Celletti, and V. Buonocore. 1998. Laccase from the white-rot fungus *Trametes trogii. Appl. Microbiol. Biotechnol.* 49, 545-551.

German, U., G. Müller, P. Hunziker, and K. Lerch. 1998. Characterization of two allelic forms of *Neurospora crassa* laccase. *J. Biol. Chem.* 263, 885-896.

Han, M.-J., H.-T. Choi, and H.-G. Song. 2004. Degradation of phenanthrene by *Trametes versicolor* and its laccase. *J. Microbiol.* 42, 94-98.

Iyer, G. and B. Chattoo. 2003. Purification and characterization of laccase from the rice blast fungus, *Magnaporthe grisea. FEMS Microbiol. Lett.* 227, 121-126.

Jung, H., F. Xu, and K. Li. 2002. Purification and characterization of laccase from wood-degrading fungus *Trichophyton rubrum* LKY-7. *Enzyme Microb. Technol.* 30, 161-168.

Lee, Y.-J. and K.-S. Shin. 1999. Purification and properties of laccase of the white-rot basidiomycete *Coriolus hirsutus. J. Microbiol.* 37, 148-153.

Levin, L., A. Viale, and A. Forchiassin. 2003. Degradation of organic pollutants by the white rot basidiomycete *Trametes trogii. Int. Biodet. Biodeg.* 52, 1-5.

Min, K. L., Y. H. Kim, Y. W. Kim, and H. S. Jung, and Y. C. Hah. 2001. Characterization of a novel laccase produced by the

wood-rotting fungus *Phellinus ribis. Arch. Biochem. Biophys.* 392, 279-286.

Muñoz, C., F. Guillén, A. Martínez, and M. Martínez. 1997. Laccase isoenzymes of *Pleurotus eryngii*: Characterization, catalytic properties, and participation in activation of molecular oxygen and Mn^{2+} oxidation. *Appl. Environ. Microbiol.* 63, 2166-2174.

Nyanhongo, G., J. Gomes, G. Gübitz, R. Zvauya, J. Read, and W. Steiner. 2002. Production of laccase by a newly isolated strain of *Trametes modesta. Biores. Technol.* 84, 259-263.

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

Périé, F., G. Reddy, N. Blackburn, and M. Gold. 1998. Purification and characterization of laccases from the white-rot basidiomycete *Dichomitus squalens. Arch. Biochem. Biophys.* 353, 349-355.

Perry, C., M. Smith, C. Britnell, D. Wood, and C. Thurston. 1993. Identification of two laccases genes in the cultivated mushroom *Agaricus bisporus. J. Gen. Microbiol.* 139, 1209-1218.

Ross, I.K. 1982. The role of laccase in carpophore initiation in *Coprinus congregatus. J. Gen. Microbiol.* 128, 2763-2770.

Sack, U., T. Heinze, J. Deck, C. Cerniglia, R. Martens, F. Zadrazil, and W. Fritsche. 1997. Comparison of phenanthrene and pyrene degradation by different wood-decaying fungi. *Appl. Environ. Microbiol.* 63, 3919-3925.

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

Shin, K.S. and Y.J. Lee. 2000. Purification and chracterization of a new member of the laccase family from the white-rot basidiomycete *Coriolus hirsutus. Arch. Biochem. Biophys.* 384, 109-115.

Shin, K.S. 2004. The role of enzymes produced by white-rot fungus *Irpex lacteus* in the decolorization of the textile industry effluent. *J. Microbiol.* 42, 37-41.

Thurston, C.F. 1994. The structure and function of fungal laccase. *Microbiol.* 140, 19-26.

Xiao, Y., X. Tu, J. Wang, M. Zhang, Q. Cheng, W. Zeng, and Y. Shi. 2003. Purification, molecular characterization and reactivity with aromatic compounds of a laccase from basidiomycete *Trametes* sp. strain AH28-2. *Appl. Microbiol. Biotechnol.* 60, 700-707.

Yaver, D., F. Xu, E. Golightly, K. Brown, S. Brown, M. Rey, P. Schneider, T. Halkier, K. Mondorf, and H. Dalboge. 1996. Purification, characterization, molecular cloning, and expression of two laccase genes from the white rot basidiomycete *Trametes villosa. Appl. Environ. Microbiol.* 62, 834-841.