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Purification and characterization of a novel laccase from the mushroom *Pleurotus nebrodensis*

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A novel laccase with a molecular mass of 64 kDa and the N-terminal sequence AIGPDDTINF was isolated from fresh fruiting bodies of the mushroom *Pleurotus nebrodensis*. The purification protocol comprised ion exchange chromatography on DEAE-cellulose, CM-cellulose, and Q-Sepharose, and gel filtration on Superdex 75. The laccase was adsorbed on DEAE-cellulose and Q-Sepharose, but not on CM-cellulose. It demonstrated an optimal temperature of 70°C. The enzyme activity increased steadily over the temperature range 20°C–70°C. There was only a slight reduction in activity at 80°C. However, all activity disappeared following exposure to 100°C for 10 minutes. The enzyme activity changed only slightly over the pH range 3–5, with the optimum at pH 5, but underwent a precipitous decline when the pH was elevated to 6, and was undetectable at pH 8 and pH 9.

Key words: Laccase, *Pleurotus nebrodensis*, purification, characterization

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INTRODUCTION

Laccase (E.C.1.10.3.2, benzenediol:oxygen oxidoreductase) is a copper-protein belonging to the group of blue oxidases (Baldrian, 2006). Laccase catalyzes the oxidation of various phenolic compounds and aromatic amines with molecular oxygen as the electron acceptor (Palmieri *et al.*, 1993). It is widely distributed among plants, insects, bacteria, and fungi, and laccase from fungi, especially from white rot fungi, plays an important role in the natural degradation of lignin (Baldrian, 2006). Fungal laccases demonstrate great applications in biodegradation of lignin, and recently they have also found potential applications in wood pulping, manufacturing of textile dyes, detoxification of polluted water, and as biosensors (Widsten & Kandelbauer, 2008).

Mushrooms have drawn attention of investigators since they constitute a source of important compounds comprising lectins, laccases, proteases, ribonucleases, ribosome inactivating proteins, antibacterial proteins, antifungal proteins, and polysaccharides (Ng, 2004). Many of them belong to white rot fungi, and a number of mushroom laccases have been reported in the literature (Hu *et al.*, 2011; Li *et al.*, 2010; Wang & Ng, 2006a). To date, there are only several publications on *Pleurotus nebrodensis*, most of which are on classification of the mushroom and the taxonomic relationship between *P. nebrodensis* and *P. eryngii* (Urbanelli *et al.*, 2007; Zhang *et al.*, 2006). One study showed that the mushroom is abundant in vitamin B12 and riboflavin, and has a low caloric content and a high gastronomic value (La Guardia *et al.*, 2005). Only one protein, a hemolysin, has been isolated from *P. nebrodensis* (Lv *et al.*, 2009). In view of the importance of laccases, the present study aimed to isolate and characterize a laccase from the mushroom *P. nebrodensis*, and to compare its characteristics with those of other laccases from the genus *Pleurotus*.

MATERIALS AND METHODS

Isolation of laccase. Fresh fruiting bodies (1500 g) of the mushroom *P. nebrodensis* were homogenized in distilled water (4500 ml) using a Waring blender. The homogenate was centrifuged at 12000 rpm for 15 minutes. Tris/HCl buffer (1 M, pH 7.4) was added to the resulting supernatant to a final concentration of 10 mM. The supernatant was then loaded on 5 cm × 20 cm columns of DEAE-cellulose (Sigma, USA) in 10 mM Tris/HCl buffer (pH 7.4). Unadsorbed proteins were collected in the flowthrough fraction, and adsorbed proteins were eluted with the same buffer containing 0.8 M NaCl. Enzyme fraction was further purified on 2.5 cm × 20 cm columns of CM-cellulose (Sigma, USA) in 10 mM NH_4OAc buffer (pH 4.5). After elution of unadsorbed proteins, the same NH_4OAc buffer with 1 M NaCl was used for adsorbed ones. Subsequently, the active fraction was chromatographied on a 2.5 cm × 20 cm column of Q-Sepharose (GE Healthcare, USA). Following elution of unadsorbed proteins with 10 mM Tris/HCl buffer (pH 7.4), the adsorbed proteins were eluted with a linear concentration gradient of NaCl (0–1 M) in the same Tris/HCl buffer. Finally, the laccase fraction was dialyzed, lyophilized, and then chromatographied on a Superdex 75 HR 10/30 column (GE Healthcare) in 0.2 M NH_4HCO_3 buffer (pH 8.5) using an AKTA Purifier (GE Healthcare, USA).

Assay of laccase activity. Laccase activity was assayed by measuring the oxidation of 2,7'-azinobis [3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt (ABTS). A modification of the method of Shin and Lee

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Abbreviations: DEAE, Diethylaminoethyl; CM, carboxymethyl Q-Sepharose, quaternary amine sepharose; FPLC, fast protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfatepolyacrylamide gel electrophoresis; HPLC, high performance liquid chromatography; ABTS, 2, 7'-azinobis [3-ethylbenzothiazolone-6-sulfonic acid] diammonium salt; HIV-1, human immunodeficiency virus type 1

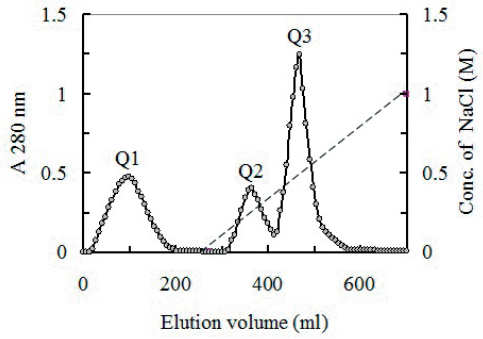

Figure 1. Ion exchange chromatography on Q-Sepharose. Sample: *P. nebrodensis* fruiting body extract adsorbed on DEAE-cellulose and then unadsorbed on CM-cellulose. Column dimensions: 2.5 × 20 cm. Starting buffer for eluting fraction Q1 10 mM Tris/HCl (pH 7.4). Buffer for eluting fractions Q2 and Q3: 0–1 M NaCl gradient in starting buffer.

(2000) was used. An aliquot of enzyme solution was incubated in 1.3 ml of 67 mM sodium acetate buffer (pH 4.5) containing 1.54 mM ABTS at 30°C. One unit of enzyme activity was defined as the amount of enzyme required to produce an absorbance increase at 405 nm of one per min per ml of reaction mixture under the aforementioned condition (Zhang *et al.*, 2011).

Molecular mass determination by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) and by FPLC gel filtration. SDS/PAGE was carried out in accordance with the procedure of Laemmli and Favre (1973), using a 12% resolving gel and a 5% stacking gel. At the end of electrophoresis, the gel was stained with Coomassie brilliant blue. FPLC gel filtration was carried out using a Superdex 75 HR 10/30 column which had been calibrated with molecular mass standards (GE Healthcare, USA).

Analysis of N-terminal amino acid sequence. N-terminal amino acid sequence analysis was carried out using an HP G1000A Edman degradation unit and an HP1000 HPLC system (Zhang *et al.*, 2011).

Substrate specificity of isolated laccase. To determine the substrate specificity of the purified laccase, several aromatic substrates were added to the assay media in place of ABTS. The substrates used (5.0 mM) were ABTS, *N,N*-dimethyl-1,4-phenylenediamine, catechol, hydroquinone, 2-methylcatechol, pyrogallol, and tyrosine. The enzyme assay was performed as described above and the test buffer was sodium acetate buffer (pH 4.5). The substrate oxidation rate was followed by measuring the absorbance change with the molar extinction coefficients (ε) obtained from the literature (Eggert *et al.*, 1996; Galhaup *et al.*, 2002).

Assay for temperature and pH optima. To determine the optimal temperature, the standard laccase activity assay was ran at 20, 25, 30, 40, 50, 60, 70, 80, and 100°C. The assay buffer was the same sodium acetate buffer used in the standard assay described above. In the assay for optimal pH value, enzyme activities were determined using the standard laccase assay above, but in a series of assay buffers in a pH range of 3.0–9.0 instead of the sodium acetate buffer (pH 4.5). The assay buffers were 50 mM NaOAc-HAc (pH 3.0–5.0), Mes (pH 5.0–7.0), and Hepes (pH 7.0–9.0).

Assay for bioactivities. HIV-1 reverse transcriptase inhibitory activity was carried out using an assay kit from Boehringer Mannhein (Germany) (Zhao *et al.*, 2009). Antiproliferative activity towards tumor cell lines including human breast cancer (MCF-7) and hepatoma (HepG2) was assayed using a standard method described by Lam and Ng (2001). Antifungal activity towards *Fusarium oxysporum*, *Rhizoctonia cerealis*, *Rhizoctonia solani*, and *Sclerotinia sclerotiorum* was carried out using Li's method (Li *et al.*, 2011).

RESULTS AND DISCUSSION

Purification of laccase. The fraction of *P. nebrodensis* fruiting body extract that was adsorbed on DEAE-cellulose (fraction D2) and subsequently unadsorbed on CM-cellulose (fraction C1) contained the bulk of laccase activity (Table 1). Ion exchange chromatography of fraction C1 on Q-Sepharose yielded an unadsorbed peak (fraction Q1), a small adsorbed peak (fraction Q2) and a large adsorbed peak (fraction Q3) (Fig. 1). Laccase activity resided in fraction Q2 (Table 1). Q2 was resolved on Superdex 75 into two peaks of similar size (Fig. 2). Laccase activity was enriched in the first peak SU1 (Table 1). In brief, the laccase resided in adsorbed fraction D2 of DEAE-cellulose, then unadsorbed fraction C1, then adsorbed fraction Q2 of Q-Sepharose, and finally fraction SU1 of FPLC.

Most of the laccases reported in the literature were isolated from mycelia and not from fruiting bodies of mushroom. It is known that straw mushroom myce-

Table 1. Yield and laccase activities of various chromatographic fractions (from 1.5 kg fresh fruiting bodies, 30°C)

Fraction	Protein (mg)	Laccase activity (u/mg)	Total activity (u)	Yield (%)	Purification fold
Extract	3810	3.0	11430	100	1
D1	1163	< 0.1			–
D2	1312	5.9	7741	68	2
CM1	547	9.2	5032	44	3
CM2	396	1.8			–
Q1	15	< 0.1			–
Q2	64	45.2	2893	25	15
Q3	165	2.0			–
SU1 (Purified laccase)	18.3	92.5	1693	15	31
SU2	21.4	2.1			–

Data corresponding to the most active fraction at each chromotographic step are shown in boldface.

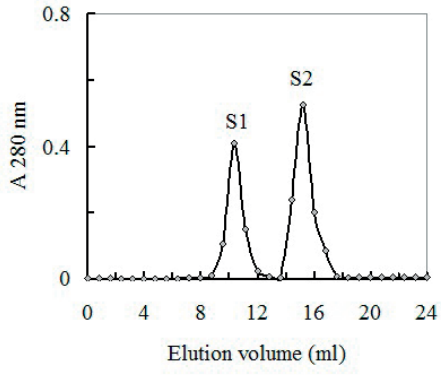

Figure 2. Gel filtration by fast protein liquid chromatography on a Superdex 75 HR 10/30 column using an AKTA Purifier System (GE Healthcare, USA).
Sample: fraction Q2. Eluent: 0.2 M NH_4HCO_3 (pH 8.5). Flow rate: 0.4 ml/min. Fraction size: 0.8 ml.

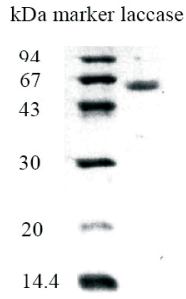

Figure 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Left lane: Molecular mass standards (GE Healthcare, USA). From top downward: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and α-lactalbumin (14.4 kDa). **Right lane**: *P. nebrodensis* laccase.

lia and fruiting bodies elaborate the same lectin (She *et al*., 1998). However, dissimilar lectins are extracted from *Tricholoma mongolicum* mycelia (Wang *et al*., 1996) and fruiting bodies (Wang *et al*., 1998). It is reported herein that a laccase can be purified from the fruiting bodies of the mushroom *P. nebrodensis*. The procedure employed in the present study was useful for isolating *Pleurotus nebrodensis* laccase. Proteins with little or no laccase activity were separated from the laccase-enriched fraction in each of the chromatographic steps on DEAE-cellulose, CM-cellulose, Q-Sepharose and Superdex 75. In contrast to some of the previously reported laccases, e.g., those from *Coriolus hirsutus* (Shin & Lee, 2000) and *Rigidoporus lignosus* (Cambria *et al*., 2000), which are adsorbed on cationic and anionic exchangers including DEAE-Sepharose, Q-Sepharose and Hitrap-SP, the *P. nebrodensis* laccase is adsorbed on DEAE-cellulose and Q-Sepharose but unadsorbed on CM-cellulose.

Molecular mass determination and N-terminal sequence analysis. SU1 appeared as a single band with a molecular mass of 64 kDa in SDS-PAGE (Fig. 3) and a single peak with the same molecular mass upon rechromatography on a Superdex 75 column (data not shown). It represented purified laccase. The N-terminal sequence of *P. nebrodensis* laccase showed only little similarity to other mushroom laccases (Table 2). Its molecular mass lies within the range of molecular masses found for mushroom laccases (Baldrian, 2006). Its N-terminal sequence does not, however, resemble previously isolated mushroom laccases to any great extent.

Table 2. N-terminal sequence comparison of laccases from *P. nebrodensis* and other mushrooms

Mushroom laccase	N-terminal sequence	Ref.
Pleurotus nebrodensis laccase	AIGPDDTINF	This study
Pleurotus eryngii laccase I (fruiting body)	AVGPVLGPDA	(Wang & Ng, 2006b)
Pleurotus eryngii laccase I (mycelial)	AXKKLDFHII	(Munoz *et al*., 1997)
Pleurotus eryngii laccase II (mycelial)	ATKKLDFHII	(Munoz *et al*., 1997)
Agaricus bisporus I	KTRTFDFDLV	(Perry *et al*., 1993)
Agaricus bisporus laccase II	DTKTFNFDLV	(Perry *et al*., 1993)
Albatrella dispansus laccase	AQPPNYHYN	(Wang & Ng, 2004b)
Basidiomycete PM1 laccase	SIGPVADLTI	(Coll *et al*., 1993)
Cantherellus cibarius laccase	GCCNCGHA	(Ng & Wang, 2004)
Ceriporiopsis subvermispora laccase	AIGPVTDLEI	(Fukushima & Kirk, 1995)
Coriolus hirsutus laccase	AIGPTADLTI	(Kojima *et al*., 1990)
Coriolus hirsutus laccase	GIGTKANLVI	(Shin & Lee, 2000)
Ganoderma lucidum laccase	GQNGDAVP	(Wang *et al*., 2006a)
Hericium erinaceum laccase	AVDDDAEQIP	(Wang & Ng, 2004a)
Phlebia radiata laccase	SIGPVTDFHI	(Saloheimo *et al*., 1991)
Pycnoporus cinnabarinus laccase	AIGPVADLTI	(Eggert *et al*., 1996)
Trametes versicolor laccase I	AIGPVASLVV	(Bourbonnais *et al*., 1995)
Trametes versicolor laccase II	GIGPVADLTI	(Bourbonnais *et al*., 1995)
Trametes versicolor laccase III	GIGPVADLTD	(Shin & Lee, 2000)
Tricholoma giganteum laccase	DDPQQAVIDD	(Wang & Ng, 2004c)

Amino acid residues identical to corresponding residues of *P. nebrodensis* laccase are underlined.

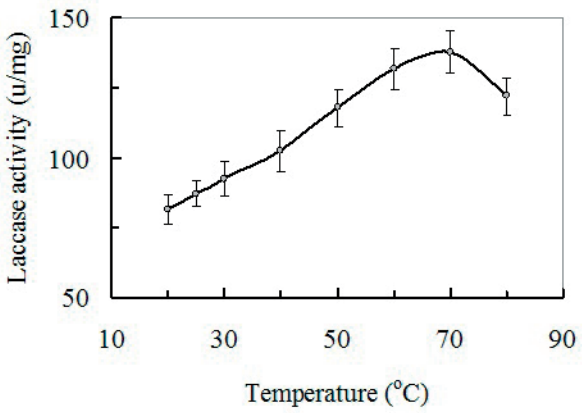

Figure 4. Dependence of *P. nebrodensis* laccase activity on temperature.

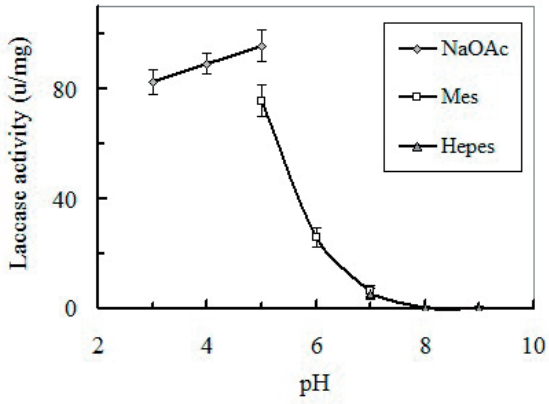

Figure 5. Dependence of *P. nebrodensis* laccase activity on pH.

Table 3. Activities of *Pleurotus nebrodensis* laccase assessed against various substrates

Substrate	Wavelength (nm)	Relative activity (%)
ABTS	420	100.0
N, N-Dimethyl-1,4-phenyl-enediamine	515	72.1
Catechol	450	26.7
Hydroquinone	248	18.2
2-Methylcatechol	436	13.5
Pyrogallol	450	7.3
Tyrosine	280	0.0

Substrate specificity of isolated laccase. The enzyme oxidized a range of substrates, including polyphenolic substrates (hydroquinone, pyrogallol, catechol), methoxy-substituted phenols (2-methylcatechol), aromatic diamines (*N,N*-dimethyl-1,4-phenylenediamine) and the non-phenolic heterocyclic compound ABTS (Baldrian, 2006). The highest activity was demonstrated toward ABTS, about 70% as much activity toward N, N-dimethyl-1,4-phenylenediamine, about 25% toward catechol, more than 10% toward hydroquinone and 2-methylcatechol, very little activity (~7%) toward pyrogallol, and no activity toward tyrosine (Table 3).

Temperature and pH dependence of laccase activity. The activity of the enzyme rose steadily when the temperature was raised from 20°C to 70°C. The activity dropped slightly when the temperature was further raised to 80°C (Fig. 4). Incubation of the enzyme at 100°C for 10 minutes brought about complete abolition of activity. The enzyme activity increased slowly when the pH was elevated from 3 to 5. A further rise in pH to 6 led to an abrupt drop in activity. Negligible laccase activity was detected at pH 7 while no activity was discerned at pH 8 and 9 (Fig. 5).

The *P. nebrodensis* laccase is unique in that its enzymatic activity undergoes a steady increase as the temperature is raised from 20°C to 70°C and that only a small decrement in activity ensues when the temperature is further elevated to 80°C. In contrast, *R. lignosus* laccase exhibits a temperature optimum at 45°C with only residual activity at 65°C (Cambria *et al.*, 2000). However, the enzyme is denatured after treatment at 100°C for 10 minutes and all activity is lost. *P. nebrodensis* laccase requires a pH of 5 for it to manifest maximal activity. This finding is at variance with the observation of an optimum pH of 2–3 from several other mushroom laccases (Shin & Lee, 2000). It is noteworthy that a drastic decline in laccase activity results when the pH is elevated to 6 and that total loss of activity occurs at pH 8 and 9. Since the present laccase manifests a considerably high temperature optimum, it has good potential for industrial applications.

Assay for other biological activities. The laccase did not display antifungal activity, anti-proliferative activity on tumor cells or inhibitory activity toward HIV-1 reverse

Table 4. Comprison of biochemical characteristics and activities of *P. nebrodensis* laccase and other *Pleurotus* laccases

	P. nebrodensis	*P. ostreatus*	*P. pulmonarius*	*P. eryngii*	*P. florida*
Molecular mass (kDa)	64	75	46	34	77
Chromatographic behavior on					
(i) DEAE-ion exchange	Adsorbed	Adsorbed	Adsorbed	Adsorbed	Adsorbed
(ii) Q- ion exchange	Adsorbed	Adsorbed	–	Adsorbed	–
(iii) CM- ion exchange	Unadsorbed	–	–	Unadsorbed	–
Optimum pH	5	Alkaline range	4.0–5.5	3–5	–
Optimum temperature	70°C	50°C	50°C	70°C	–
HIV-1 reverse transcriptase inhibitory avtivity (IC_{50})	No activity	–	–	2.2 μM	–

– not determined or not attempted. References: *P. florida* laccase (Das *et al.*, 2000); *P. ostreatus* laccase (Palmieri *et al.*, 2001); *P. pulmonarius* laccase (De Souza & Peralta, 2003); *P. eryngii* laccase (Wang & Ng, 2006b)

Table 5. Comprison of biochemical characteristics and activities of *P. nebrodensis* laccase and non-*Pleurotus* laccases

	P. nebrodensis	*G. lucidum*	*H. erinaceum*	*A. dispansus*	*T. giganteum*	*C. cibarius*
Molecular mass (kDa)	64	75	63	62	43	92
Chromatographic behavior on						
(i) DEAE-ion exchange	Adsorbed	Unadsorbed	Adsorbed	Unadsorbed	Unadsorbed	Unadsorbed
(ii) Q- ion exchange	Adsorbed	–	Adsorbed	–	–	–
(iii) CM- ion exchange	Unadsorbed	–	Unadsorbed	–	Adsorbed	–
4) Affi gel blue gel	–	Unadsorbed	-	Unadsorbed	Adsorbed	Unadsorbed
5) Con A-Sepharose	–	Adsorbed	-	Adsorbed	–	Adsorbed
Optimum pH	5	3-5	5	4	4	4
Optimum temperature	70°C	70°C	50°C	70 °C	70°C	50°C
HIV-1 reverse transcriptase inhibitory avtivity (IC50)	No activity	1.2 μM	9.5 μM	No activity	2.2 μM	–

– not determined or not attempted. References: *H. erinaceum* laccase (Wang & Ng, 2004a); *A. dispansus* laccase (Wang & Ng, 2004b); *T. giganteum* laccase (Wang & Ng, 2004c); *C. cibarius* laccase (Ng & Wang, 2004); *G. lucidum* laccase (Wang & Ng, 2006b).

transcriptase when tested at a concentration of 100 μM (not shown). Some mushroom products, e.g., lectins (Li *et al*., 2008), polysaccharopeptide (Wang & Ng, 2001), ribosome inactivating proteins (Lam & Ng, 2001) and ubiquitin-like proteins (Wang & Ng, 2000) manifest an inhibitory activity toward HIV-1 reverse transcriptase. Laccases from *G. lucidum*, *H. erinaceum* and *T. giganteum* also inhibit this retroviral enzyme. The *P. nebrodensis* laccase is devoid of this ability. The laccase isolated in this investigation lacks anti-proliferative and antifungal activities, in contrast to the observation that some mushroom lectins, antifungal proteins and ribonucleases show anti-proliferative activity (Wang & Ng, 2001), and mushroom antifungal proteins inhibit mycelial growth (Lam & Ng, 2001).

Comparisons with other *Pleurotus* and non-*Pleurotus* mushroom laccases. The comparisons are shown in Table 4 and Table 5. *P. nebrodensis* laccase resembled *P. eryngii* laccase (Wang & Ng, 2006b) to the greatest extent. However, the two laccases differed in molecular mass, N-terminal sequence, optimum pH and presence/absence of HIV-1 reverse transcriptase inhibitory activity. When compared with non-*Pleurotus* laccases, the *P. nebrodensis* laccase resembled only laccases from *Hericium erinaceum* (Wang & Ng, 2004a) and *Albatrella dispansus* (Wang & Ng, 2004b) in molecular mass. It was similar to only *H. erinaceum* laccase in optimum pH, and laccases from *Ganoderma lucidum* (Wang & Ng, 2006b), *A. dispansus* and *Tricholuma giganteum* (Wang & Ng, 2004c) in optimum temperature. Although the *P. nebrodensis* laccase resembled the *H. erinaceum* laccase in some aspects, they differed in optimum temperature and HIV-1 reverse transcriptase inhibitory activity.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interes.

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