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Characterization of a novel laccase purified from the fungus *Hohenbuehelia serotina* and its decolourisation of dyes

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A novel laccase was purified from the white rot fungus, *Hohenbuehelia serotina*, to investigate the applications of this laccase in the decoloration of various dyes. SDS-PAGE revealed a single band of this laccase corresponding to a molecular weight of approximately 57.8 kDa. The enzyme showed activity towards several substrates, the most sensitive of which was 2,2′-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS). The highest enzymatic activity using ABTS as a substrate was observed at pH 6.8 and 30°C. The enzyme activity was found to be significantly enhanced in the presence of Zn^{2+} ions and inhibited by Fe^{2+} ions. Moreover, SDS and β-mercaptoethanol were inhibitory, and inhibition by L-cysteine was observed while EDTA and DMSO had almost no inhibitory effect. The laccase could effectively decolorize seven different dyes within 30 minutes at 40°C.

Key words: *Hohenbuehelia serotina,* laccase. purification, decolouration

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INTRODUCTION

Laccase (EC 1.10.3.2), an oxidase belonging to the blue multi-copper oxidase family, is widely distributed in bacteria (Khandare *et al*., 2012), fungi (Wu *et al*., 2010; Freixo *et al*., 2012) and some plants (Gutierrez *et al*., 2012). Among these, white-rot fungi have received special attention due to their capability of lignin biodegradation through secretion of oxidative enzymes such as peroxidase (Saranyu, 2009) and laccases (Halaburgi *et al*., 2011). However, most of these identified laccases have low enzymatic activity and mesophilic stability (Baldrian, 2006; Hilden *et al*., 2009). Owing to their diversity, laccases are capable of oxidizing a broad range of substrates including phenols, diamines, and various non-phenolic compounds (Champagne & Ramsay, 2007; Asgher *et al*., 2008; Jia *et al*., 2012). Among these, complex azo and anthraquinone dyes are the most discussed (Enayatzamir *et al*., 2009; Meng *et al*., 2012).

The sewage produced by textile industries is often strongly colored due to the presence of dye waste. The dyes that are currently used cannot be degraded or removed with physical and chemical processes; besides, the degraded products are more toxic than the dyes (Mohorcic *et al*., 2006; Khlifi *et al*., 2010; Verma *et al*., 2010). These toxins cannot only be leached into the environment through contaminated water and adversely impact all organisms but they can also be inhaled and absorbed directly. Laccases are used in paper bleaching (Kahraman & Yesilada, 2001), bioremediation (Cea *et al*., 2010), and dye decoloration (Robinson & Nigam, 2008; Zhao *et al*., 2012). Since laccases eliminate a wide range of pollutants from waste water, the ability of laccase producing microorganisms and novel laccases is of immense interest to environmental biotechnologists. Consequently, discovery of novel laccases with higher enzymatic activity and enhanced stability is highly desirable. We describe the purification and characterization of a novel laccase from the white-rot fungus, *Hohenbuehelia serotina* in the present study. We also investigated the effects of pH, temperature, metal ions and putative inhibitors on the laccase. Furthermore, we investigated the applications of this laccase in the decoloration of various dyes and the optimun of decolouring conditions involved.

MATERIALS AND METHODS

Materials. ABTS, N,N-dimethyl-1,4-phenylenediamine, Pyrogallol, 2-methylcatechol, and tyrosine, DEAE-Cellulose and CM-Cellulose were bought from Sigma–Aldrich (St. Louis, MO, USA). Molecular mass standards were obtained from GE Healthcare, USA. All other chemicals were of analytical grade.

Dyes used in the experiment were bought from Zhenyang dye chemical industry Co. Ltd. (Yixing, Jiangsu, China).

Purification of *H. serotina* laccase. Purification of laccase from the fungus was conducted as described by Kim *et al.* (Kim & Shoda, 1999). Dried fruit bodies (50 g) were homogenized in saline. The homogenate was centrifuged at $18000 \times g$ for 15 min at 4°C. The supernatant was dialyzed with 10 mM Tris-HCl buffer (pH 7.5). It was then passed through a 2.5 cm × 30 cm DEAE-Cellulose column. After removal of the unabsorbed fraction D1, adsorbed proteins were desorbed stepwise with 150 mM NaCl, 300 mM NaCl and 1 M NaCl in Tris-HCl buffer to yield fractions D2, D3 and D4. Fraction D2 with laccase activity was then subjected to ion exchange chromatography on a 2.5 cm × 20 cm column of CM-cellulose in 10 mM CH_3COONa-CH_3COOH (NaAc–HAc) buffer (pH 5.4). The unadsorbed fraction

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Abbreviations: ABTS, 2,2′-Azinobis- (3-ethylbenzthiazoline-6-sulphonate); CBB, Coomassie Brilliant Blue; R-3G, Reactive scarlet; R-4GLN, reactive brilliant yellow; R-RV, reactive brilliant blue; RES, reactive emerald blue; RW, active super navy blue; RBBR, Remazol Brilliant Blue; M-GR, malachite green; A-OR, active orange ; X-3B, active red; K-BR, active black; CM, carboxymethyl; DEAE, diethylaminoethyl; FPLC, Fast Protein Liquid Chromatography; HPLC, High Performance Liquid Chromatography; Tris, N-tris (hydroxymethy) aminomethane; β-ME, β-mercaptoethanol

C1 was removed with the same buffer. Thereafter, the adsorbed proteins were eluted sequentially with 150 mM NaCl and 1 M NaCl in 10 mM NaAc–HAc buffer (pH 5.4) to yield fractions C2 and C3. Fraction C2 after ultrafiltration was further purified by gel filtration on a fast protein liquid chromatography (FPLC) Superdex 75 column (GE Healthcare) in 0.2 M NH_4HCO_3 buffer (pH 8.5). The first peak constituted purified laccase.

Molecular mass determination of the isolated laccase. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out following the procedure of Laemmli and Favre (1973), using a 12% resolving gel and a 5% stacking gel. The gel was stained with Coomassie Brilliant Blue (CBB). FPLC-gel filtration was conducted in a Superdex 75 column that had been calibrated with molecular mass standards.

Assay for laccase activity. Laccase activity was determined by the method involving oxidation of ABTS. The ABTS is oxidized by laccase to the more stable and preferred state of the cation radical. The concentration of the cation radical responsible for the intense blue-green color can be correlated to enzyme activity and is read at 420 nm (Kim & Shoda, 1999). Oxidation of ABTS was monitored by determining the increase in A_{420}. In brief, 10 μL laccase solution was incubated with 190 μL 1 mM ABTS (in 50 mM sodium acetate buffer, pH 5.2) at 30ºC for 5 min. Subsequently, the reaction was ended by adding 200 μL 10% (w/v) trichloroacetic acid. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank. One unit was defined as the amount of laccase that oxidized 1 μmol of ABTS substrate per min. All determinations were performed in triplicate.

Determination of physicochemical properties of the isolated laccase. The optimal pH value of the purified laccase was determined in buffers with different pH values (from pH 2.0 to pH 9.8) at 30ºC. The optimal temperature was tested by determining the activity of the enzyme at different temperatures ranging from 20 to 90ºC in sodium acetate buffer (pH 6.8). The effects of metal ions (K^+, Mg^{2+}, Ca^{2+}, Zn^{2+}, Mn^{2+}, Fe^{3+}, and Al^{3+}) and inhibitors (β-mercaptoethanol, EDTA, L-Cysteine, DMSO and SDS) on laccase activity were also investigated after incubation in sodium acetate buffer (pH 6.8) for 60 min at 30ºC.

Assay of substrate specificity of isolated laccase. Activity toward several aromatic substrates was tested to determine enzyme specificity. The following substrates were added to a final concentration of 10.0 mM: ABTS, N,N-dimethyl-1,4-phenylenediamine, 2-methylcatechol, Pyrogallol and tyrosine. The enzyme assay was performed as described above in 50 mM sodium acetate buffer, pH 6.8. The rate of substrate oxidation was determined by monitoring the change in absorbance of the substrate using the published molar extinction coefficient (ε).

Decolorization of dyes by the purified laccase. Reactive scarlet (R-3G, λ_{max} 510 nm), reactive brilliant yellow (R-4GLN, λ_{max} 430 nm), reactive brilliant blue (R-RV, λ_{max} 590 nm), reactive emerald blue (RES, λ_{max} 630 nm), active super navy blue (RW, λ_{max} 595 nm), Remazol Brilliant Blue (RBBR, λ_{max} 590 nm), malachite green (M-GR, λ_{max} 610 nm), active orange (A-OR, λ_{max} 475 nm), active red (X-3B, λ_{max} 517 nm), active black (K-BR, λ_{max} 597 nm) and coomassie brilliant blue (CBB, λ_{max} 580 nm) were used in this study. All maximum absorption wavelengths were detected with full wavelength scanning using a UV spectrophotometer.

The decolorization reaction was carried out at 30ºC for 5 h in a 6 ml reaction system containing 0.3 mg dye prepared in 25 mM sodium acetate buffer at pH 6.8 and 1.5 U purified laccase. Control containing heat-denatured enzyme was used to measure decolorization of dye. Choose dyes that have higher decolorizing efficiency to analyze optimal decoloring conditions. The optimal decoloring time was determined by spectrum detecting under 2 h, 4 h and 6 h, respectively. Also, the degradation rate was detected by changes of values at maximum absorption wavelengths.

Decolorization was determined as below:

$$\text{Decolorization (\%)} = 100\times \frac{\text{absorbance}_{t0} - \text{absorbance}_{tf}}{\text{absorbance}_{t0}}$$

Where absorbance_{t0} is the absorbance value at λ_{max} of each reaction mixture before incubation with enzyme and absorbance_{tf} is the absorbance value at λ_{max} after incubation with the enzyme.

It is not accurate enough to judge dyes decolorizing effect only depending on changes in absorbance value. In order to further confirm whether dyes have been degraded, we use the high performance liquid chromatography (HPLC) (Aglient 1100 series, Eclipse Hypersil ODS C^{18}, 5 μM, 4.6 × 150 mm) to analyze M- GR processed by laccase. The basic parameters were as follows:

Flowing phase: acetonitrile — 5mM pH4.5 Ammonium acetate buffer (volume 70:30); Flow rate: 0.5 mL/min; Column temperature: room temperature; detection wavelength: 600 nm; Sample volume: 20 μL.

RESULTS

Purification of *H. serotina* laccase

The *H. serotina* extract was resolved on DEAE-cellulose into four fractions D1, D2, D3 and D4, using NaCl at different concentrations (Fig. 1a). Only the adsorbed fraction D2 was found to possess laccase activity. Fraction D2 was further fractionated on CM-cellulose into three fractions C1, C2 and C3 (Fig. 1b). Fraction C2, which contained the bulk of laccase activity, was separated into two major fractions S1 and S2 on Superdex 75 column after ultrafiltration. Laccase activity was found in fraction S1 (Fig. 1c). Based on the elution volume, the molecular mass of S1 was deduced to be about 57.8 kDa. In addition, fraction S1 displayed a single band with a molecular mass of approximate 57 kDa in SDS–PAGE (Fig. 1d). The yields and specific laccase activities at various stages of purification are shown in Table 1.

Effects of pH and temperature on laccase activity

The temperature profile of the purified laccase with ABTS as substrate is shown in Fig. 2. The laccase activity demonstrated a smooth and stable increase as the temperature was increased from 4ºC to 30ºC, and the activity was found to be maximal at 30ºC. The laccase activity decreased drastically when the temperature was over 70ºC. The thermal stability of the purified laccase was determined upon pre-incubation within a temperature range of 30–60ºC for 3 h (Fig. 2). At temperatures below 40ºC, there was almost no change in enzymatic activity. It was only at temperatures over 50ºC that the remaining laccase activity dropped to below 70% of the initial activity. These results indicated that the laccase showed better thermo-stability properties than several other fungal laccases (Hilden *et al.*, 2009; Shraddha *et al.*, 2011).

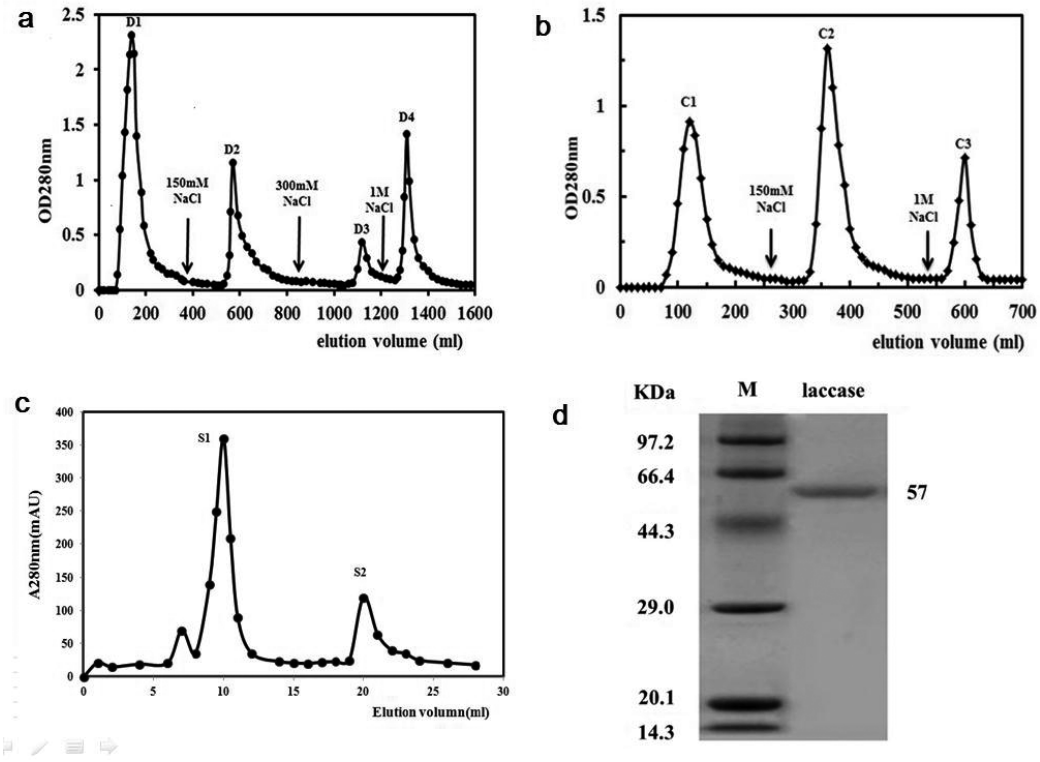

Figure 1. Elution profiles of laccase from *H. serotina*.
(**a**)Anion exchange chromatography of crude extract of *H. serotina* on a DEAE-cellulose column (2.5 cm × 30 cm, Sigma). Starting buffer: 10 mM Tris-HCl buffer (pH 7.5). Fractions D2, D3, and D4 were acquired by sequential elution with 150 mM NaCl, 300 mM NaCl, and 1 M NaCl in Tris-HCl buffer, respectively. Laccase activity resided in fraction D2. (**b**) Cation exchange chromatography of fraction D2 on a CM-cellulose column (2.5 cm× 20 cm). Starting buffer: 10mM NaAc-HAC buffer (pH 5.4). Arrows indicate successive elution with 150 mM NaCl in NaAc-HAC buffer and 1 M NaCl in NaAc-HAC buffer, respectively. Laccase activity was enriched in fraction C2. (**c**) Gel filtration by fast protein liquid chromatography on a Superdex 75 column. The molecular mass of S1 was estimated to be 60 kDa. Buffer: 0.2 M NH_4HCO_3 (pH 9.2). Flow rate: 0.5 ml/min. Fraction size: 0.8 ml. (**d**) SDS-PAGE of purified *H. serotina* laccase. M is the protein marker. Molecular mass of *H. serotina* laccase was estimated to be about 57 kDa. M: marker; Purified laccase is shown in the other lane.

The optimum pH value of the purified laccase for ABTS oxidation was found to be pH 6.8 (Fig. 3). The laccase activity declined continuously at every pH value and we got relative stability value at 6.8 (Fig. 3). These results revealed that *H. serotina* laccase had stability within a wide range of pH values, which is different from many of the previously described fungal laccases (Giardina *et al.*, 2010; Villalba *et al.*, 2010).

Effects of metal ions and inhibitors on isolated laccase

The laccase activity was not significantly affected in the presence of K^+. The enzyme was strongly inhibited by Fe^{2+} ions while Zn^{2+} ions increased the laccase activity at concentrations under 50 mM. Mg^{2+}, Mn^{2+}, Ca^{2+} and Al^{3+} ions brought about varying degrees of suppression (Table 2). We also tested the sensitivity of the laccase to several putative laccase inhibitors (Table 3). Laccase was inhibited by sodium dodecyl sulfate and β-mercaptoethanol, suggesting that disulfide bonds are essential for enzyme activity. EDTA and DMSO up to a concentration of 50 mM could not inhibit laccase activity, while a 10% inhibition was observed with L-cysteine at 25 mM.

Assay of substrate specificity of isolated laccase

We also analyzed the activity of *H. serotina* laccase toward various substrates, including polyphenolic substrates (pyrogallol), methoxy-substituted phenols (2-methylcatechol), aromatic diamines (N,N-dimethyl-1,4-phenylenediamine), and the non-phenolic heterocyclic compound ABTS to determine substrate specificity (Table 4). The highest activity was found with ABTS which was then

Table 1. Summary of purification of *H. serotina* laccase with ABTS as substrate (from 10 g dry fruiting bodies)

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification fold
Crude extract	2699.2	3696	1.37	100	1
precipitate	404.2	1792	4.44	48.48	3.24
D2	152.1	1177.2	7.74	31.85	5.65
C2	16.9	928	54.91	25.11	40.08
S2	3	247.9	82.7	6.7	60.36

Data are mean values from triplicate experiments.

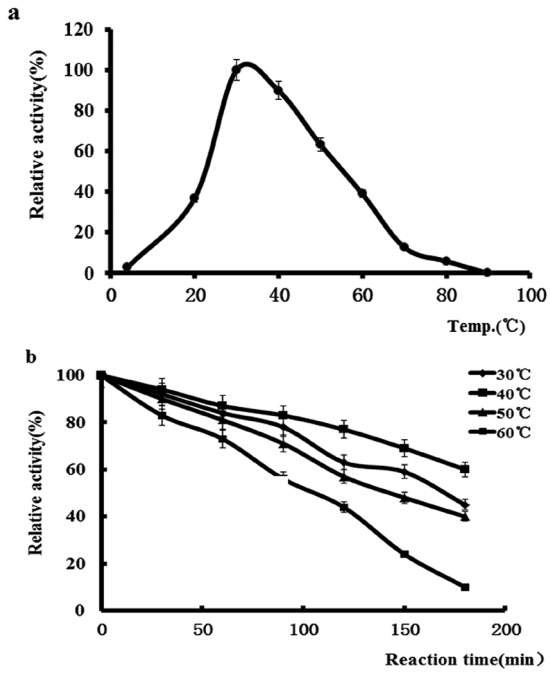

Figure 2. Optimum reaction temperature and thermostability of *H. serotina* laccase.
(**a**)The effect of different temperatures on the activity of *H. serotina* laccase in oxidation of ABTS substrate. (**b**) Relative activity was tested after pre-incubation of laccase at different temperatures (3 h at each temperature).

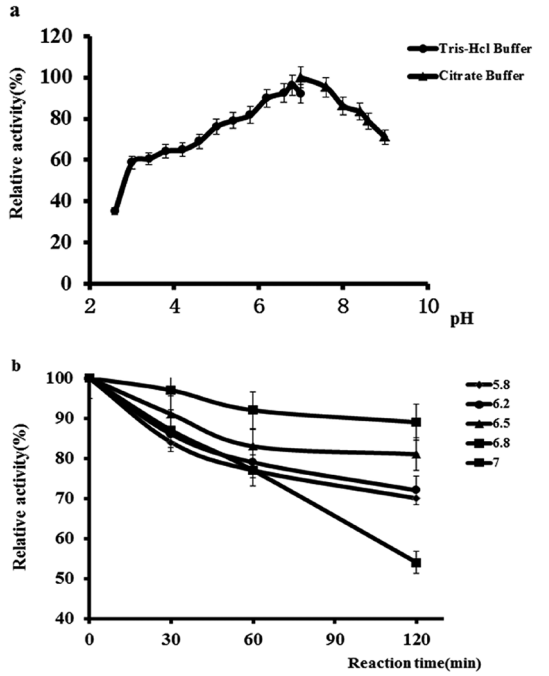

Figure 3. Optimum pH and pH stability of *H. serotina* laccase.
(**a**) The effect of different pH values on laccase activity in oxidation of ABTS substrate. (**b**) Relative activity was tested after pre-incubation of the laccase at different pH values. The incubation time was 2 h for each pH.

Table 2. Effects of metal ions on laccase activity. Relative laccase activities (%) of the purified enzyme (dissolved in 0.1 M Tris-HCl buffer, pH 6.8) were determined in the presence of designed metal concentrations using ABTS as a substrate.

Metal	Relative activity (% of control)					
ion	1.25 mM	2.5 mM	5 mM	10 mM	25 mM	50 mM
K+	95.13±2.3	94.77±1.9	93.26±1.4	92.54±0.7	91.61±0.2	89.49±1.1
Zn^{2+}	167.8±1.3	140.5±2.1	114.33±1.5	106.92±1.0	100.7±0.9	94.55±0.6
Mn^{2+}	96.47±1.8	92.64±1.7	86.51±2.2	62.28±2.3	51.18±1.4	51.08±0.5
Ca^{2+}	93.22±1.6	87.49±1.2	78.65±0.9	65.38±2.4	62.46±2.7	43.46±0.9
Mg^{2+}	83.59±2.1	71.82±1.9	67.92±1.2	61.81±0.3	26.91±2.2	28.41±1.3
Fe^{2+}	65.73±1.4	58.19±1.5	42.42±1.6	27.55±2.4	18.46±2.1	15.11±2.6
Al^{3+}	94.61±1.8	91.23±1.3	90.22±1.2	57.01±1	51.55±0.8	38.85±0.3

Table 3. Effects of inhibitors on laccase activity. Inhibition (%) was measured using ABTS as a substrate after adding each inhibitor to the assay mixture (purified laccase in 0.1 M Tris-HCl buffer, pH 6.8) to attain the final concentration of inhibitor.

Concentration	Inhibition (%)				
(mM)	DMSO	β-ME	EDTA	L-Cysteine	SDS
control	0	0	0	0	0
0.75	4.08±2.3	13.99±1.9	0	0	27.97±1.2
1.5	0	14.76±1.6	0	0	47.81±1.4
3	0	52.48±1.3	0	0	84.84±2.4
6	0	78.43±2.1	0	0	87.46±2.5
12.5	0	100	0	1.17±1.1	87.76±2.3
25	0	100	0	11.08±1.5	95.04±3.3
50	0	100	0	15.16±1.9	99.42±2.1

DMSO can interact with protein hydrophobic groups and break the hydrogen bonds; β-mercaptoethanol (β-ME) reduces disulfide bonds; EDTA is a metal ion chelating agent and can inhibit the metalloprotease activity; L-Cysteine can change the disulfide bond between protein molecules; sodium dodecyl sulfate (SDS) is an anionic detergent that can break intramolecular and intermolecular hydrogen bonds.

Table 4. Substrate specificity of the purified *H. serotina* laccase

Substrate	Wavelength (nm)	Relative activity (100)
ABTS	420	100
N,N-Dimethyl-1,4-phenylenediamine	515	42.6
2-Methylcatechol	436	17.2
Pyrogallol	450	7.7
Tyrosine (negative control)	280	0

The laccase activity towards ABTS was regarded as 100%.

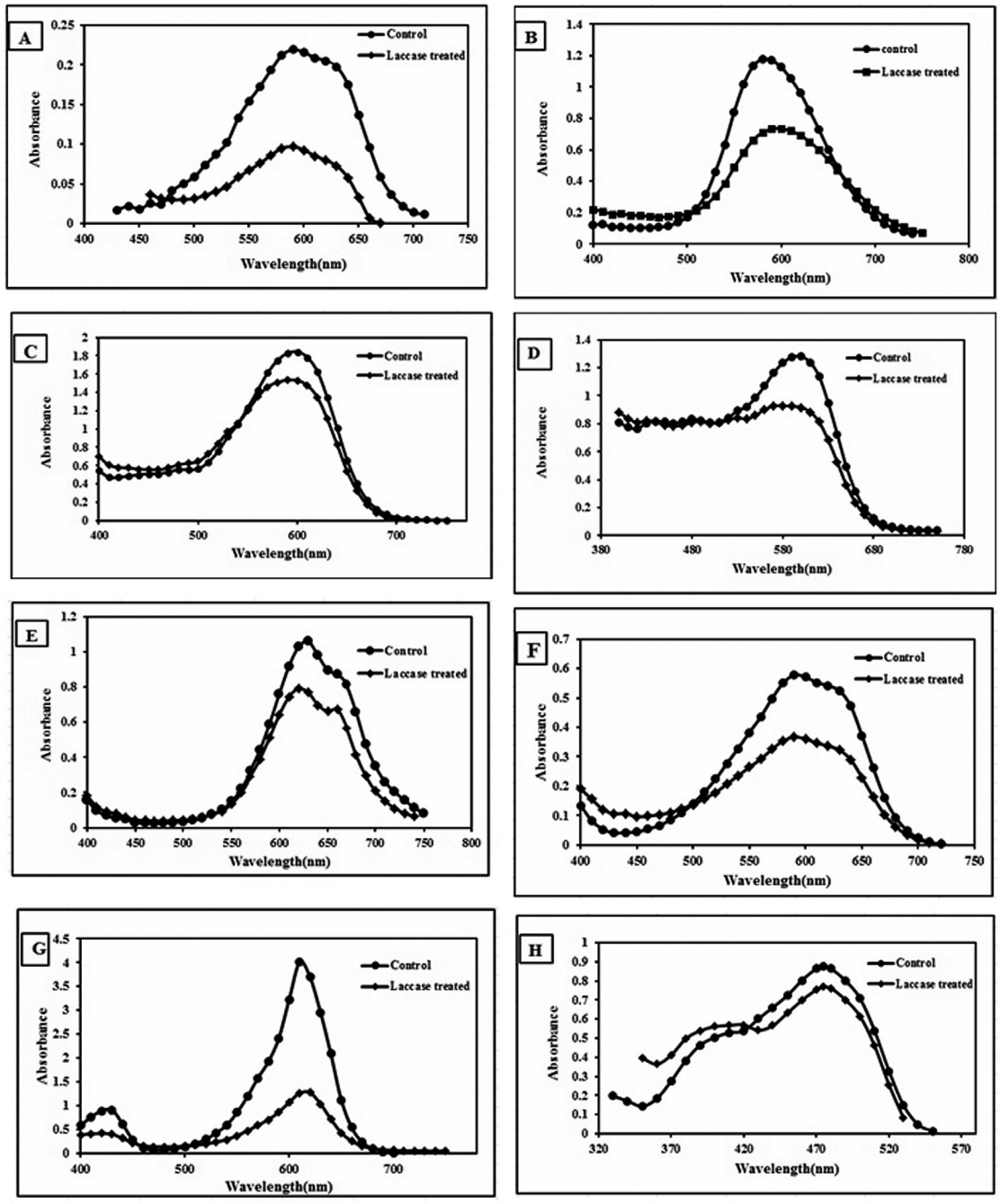

Figure 4. Full wavelength scanning of dyes before and after laccase treatment.
A: RBBR; B: CBB; C: RW; D: K-BR; E: RES; F: R-RV; G: M-GR; H: A-OR.

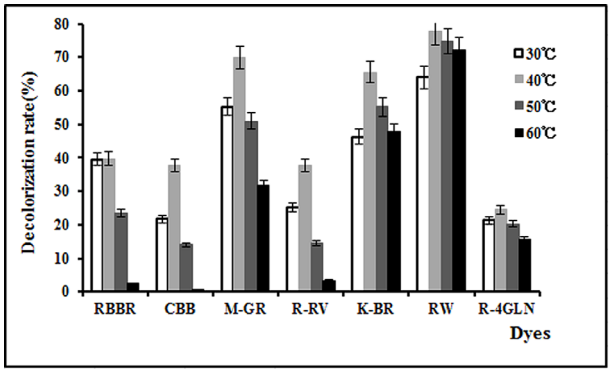

Figure 5. Optimum temperature of dye decolorization by purified laccase (0.25U ml^{-1}).
The data points represent the means of triplicate assay.

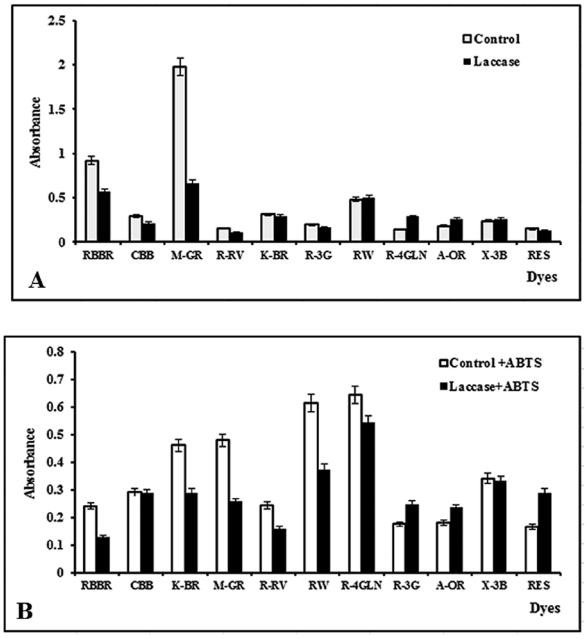

Figure 6. Media influence of dye decolorization by purified laccase (0.25U ml−1).
The data points represent the means of triplicate assay.

taken as the reference substrate. Approximately 42% relative activity toward N,N-dimethyl-1,4-phenylenediamine was observed whereas approximately 17% as much activity toward 2-methylcatechol was observed, and very little activity (~8%) pyrogallol was observed. No activity toward tyrosine was observed (Table 4).

Applications of purified laccase in decolorization

The purified laccase decolorized 11 structurally different dyes. Out of the 11 tested dyes, eight dyes, namely, R-RV, RBBR, K-BR, CBB, RW, RES, A-OR and M-GR showed decolorization efficient to some degree (Fig. 4) and the effect of R-RV, RBBR, K-BR, CBB and M-GR was obvious after treatment 2 h, 4 h and 6 h (Fig. 6A).

We select M–GR with best decolorizing efficiency to detect the decoloring results with HPLC. The maximum absorption wavelengths of the degradation products were different from the relevant substrate dye and the dyes were degraded into different materials.

DISCUSSION

In the present study, we isolated a novel laccase from the edible mushroom *H. serotina*. We used ion exchange chromatography on DEAE-cellulose and CM-cellulose, and gel filtration on Superdex 75 to remove inactive proteins from the laccase-enriched chromatographic fraction. *H. serotina* laccase resembles that of *Pleurotus sajor-caju* MTCC 141 (Sahay *et al.*, 2008) in that both are adsorbed on diethylaminoethyl (DEAE) cellulose, carboxymethyl (CM) cellulose, and Q-Sepharose and can be further purified by fast protein liquid chromatography-gel filtration on a Superdex 75 column. The purification protocol resulted in 60-fold purification of the laccase and 6.7% yield. In comparison, the laccase from the edible mushroom *Pleurotus sajor-caju* MTCC 141 was purified with a purification factor of 11-fold and a 3.4% yield (Sahay *et al.*, 2008). On the other hand, *H. serotina* laccase resembled laccases from other genera including *Pleurotus florida* (Palanivel & Thayumanavan, 2013) and *Pleurotus eous* (Rani *et al.*, 2008) laccase in optimum temperature and molecular mass, and *Pleurotus nebrodensis* (Tian *et al.*, 2012) laccase in optimum pH.

H. serotina laccase was a monomeric 57-kDa protein as evidenced by the results of SDS-PAGE and gel filtration on Superdex 75. The molecular mass of the laccase was within the range of molecular masses reported in laccases from other white-rot fungi (Forootanfar *et al.*, 2011). The laccase from *H. serotina* showed distinct characteristics such as intrinsically modest thermostability, and stability over a wide pH range, which render it extremely suitable for a wide scope of potential applications.

H. serotina laccase, like other fungal laccases (Call & Mucke, 1997), non-specifically oxidized a wide range of substrates, but not tyrosine. The nature and substitution of the phenolic ring affected the oxidation activity of the laccase. The degradation activity toward substrates was ABTS>N,N-dimethyl-1,4-phenylenediamine>2-methylcatechol>pyrogallol. In general, increasing the number of substituted methoxy groups increased the oxidation activity.

The enzymatic activity of the *H. serotina* laccase was found to be completely inhibited by SDS and β-ME, which can reduce hydrogen and disulphide bonds completely. L-cysteine, which is a strong reducing agent of disulphide bonds, partially inhibited the laccase. These results indicated the existence of a disulphide structure in the active domain, and that the hydrogen bond between laccase and water or in the interior of the laccase is essential for the stability of its active domain (Luzar & Chandler, 1993). Furthermore, EDTA had almost no effect on the laccase, thereby suggesting the absence of a metal-binding domain in the active domain. DMSO, which can interact with hydrophobic groups of proteins, also did not alter the activity of the enzyme in any way. The active domain consisted of disulphide bonds, which were important to control spatial conformation, while the hydrogen bond present in the laccase or between laccase and water was of no great importance (Hu *et al.*, 2009). A metal-binding domain was not observed in this laccase.

The laccase from *H. serotina* displayed the typical properties of an oxidoreductase as it had the capability to degrade structurally different dyes.

Most of the dyes used in our study had a complex structure and were aromatic compounds with conjugate structures (Casieri *et al*., 2010). The quantity of these dyes has been increasing gradually in the environment as these dyes are now used on a large scale. They are not easily oxidized and can cause pigment sedimentation in the digestive system and lead to liver cancer (Brown & Devito, 1993).

We chose the dyes with good decoloring effeciency to determine their optimal decoloring temperature,and the influence of ABTS on decoloring efficiency was analyzed. The efficiency of RW and R — 4GLN was improved obviously after addition ABTS, suggesting that the synthetic fixator ABTS mediated the degradation of RW and R — 4GLN. And results of HPLC have proved the validity of the conclusion.

This experiment analyzed chemical bonds maintained enzyme conformation from the angle of enzymology, and determined the characteristics of degradation about anthraquinone substrates by disposing different industrial dyes.

However, the laccase purified from *H. serotina* can specifically degrade anthraquinone dyes, and is highly advantageous in bioremediation and biotransformation of regions polluted by industrial waste containing toxic and recalcitrant dyes. These studies in conjunction with other mediators of interest can provide effective environmental engineering strategies for remediation.

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