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Contributed Paper

Purification and Characterization of Fungal Laccase from *Mycena purpureofusca*

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ABSTRACT

The extracellular laccase produced by *Mycena purpureofusca* was purified and characterized biochemically and biologically. The molecular mass of the laccase was determined from the analysis of SDS-PAGE following purification with anion exchange chromatography. The purified laccase showed a relative molecular mass of 61.7 kDa. Laccase activity was detected in culture filtrate of *M. purpureofusca* with ABTS as substrate, optimum pH being 2.2 and optimum temperature at 50°C. The enzymatic activity was stable at neutral pH and temperatures between 10 and 30°C. The laccase activity decreased rapidly when the temperature was above 30°C for 1h. The values of kinetic parameters *K*m and *V*max for purified laccase were 0.296 (mM) and 0.0645 (mM/min), respectively. Among the metal ions used, Fe^{3+}, Mn^{2+} , Cu^{2+}, Ag^{+}, Ca^{2+}, Ba^{2+} and Zn^{2+} were found to have a slightly stimulating effect on the enzyme activity at the concentrations of 0.05 mM. The enzyme activity can be enhanced by 18.7% and 130.5% when Ag^{+} was added to the medium at the concentration of 0.05 and 0.5 respectively. On the contrary, Fe^{2+} strongly inhibited enzyme activity up to 98% at a concentration of 0.05 and 0.5 mM.

Keywords: characterization, laccase, *Mycena purpureofusca*, purification

1. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases, EC 1.10.3.2) are blue multicopper oxidases that catalyze the oxidation of an array of aromatic substrates concomitantly with the reduction of molecular oxygen to water [5]. The majority of laccases are often found in white-rot fungi and higher plants such as *Rhus vernicifera* [11]. Among these resources, the glycosylation content of fungal laccase is generally lower than that of plant laccase [8]. In fungi, laccases play a variety of physiological roles in their life cycle, such as lignin degradation, pathogenesis, detoxification and morphogenesis [1, 7, 21]. Fungal laccases have many advantages, such as substrate non-specific, directly oxidizing various phenolic compounds, using molecular oxygen as the final electron acceptor instead of hydrogen peroxide, showing a considerable level of stability in the extracellular

environment. Therefore, fungal laccases have been widely applied in biotechnology and industry, such as delignification of lignocellulosics, paper pulping/bleaching, and degradation of different recalcitrant compounds, bioremediation, sewage treatment, dye decolorization and biosensors [14, 17, 20]. Laccases are of great potential in industrial applications, which point out the need of isolating high laccase-secreted microorganisms with different physicochemical and catalytic properties [6]. However, until now, there have been seldom reports on laccase from *Mycena* genus.

In this study, the purification and characterizations of laccase were studied with a fungal strain *M. purpureofusca*. Laccase is a singular lignin degradation enzyme produced by this strain. The aim of this work was to introduce a new source of extracellular laccase and demonstrate its physicochemical and catalytic properties, which are now indispensable in both fundamental and applied research. The data obtained in this work will contribute to a better knowledge of the enzyme secreted by this fungus and should be helpful for the highly efficient laccase production.

2. MATERIALS AND METHODS

2.1 Fungal Strain and Culture Conditions

The *M. purpureofusca* strain was obtained from the Fujian General Station of Technology Popularization for Edible Fungi (Fujian, China) and maintained on potato dextrose agar slant (PDA: potato 200 g/L, glucose 20 g/L, agar 20 g/L, pH is nature) at 25°C with a periodic transfer.

2.2 Enzyme Production

The liquid medium [21] contained (per litre) 4.26 g sucrose, 15 g yeast powder, 2.7 g KH_2PO_4, 4.83 g $MgSO_4 \cdot 7H_2O$, 5.625 mg $CuSO_4 \cdot 5H_2O$, and 0.1 g Vitamin B_1. The liquid seed was inoculated with mycelia mat (ca. 5mm^2) from a stock slant and grew in a 250 mL flask containing 100 mL of liquid medium at 24°C on a rotary shaker at 110rpm for 8 days. The broth was filtered through a nylon mesh, the filtrate further was clarified by centrifugation at 8,000rpm for 10 min at 4°C, and the supernatant was retained and stored at 4°C for further experiment.

2.3 Enzyme assays

Laccase activity assay was conducted in 3 mL reaction mixtures consisting of 2.7 mL of 0.1 M sodium acetate buffer (pH 4.5), 0.2 mL of 1 mM ABTS solution, and 0.1 mL culture supernatant. The reaction was monitored by measuring the change in A_{420} for 3 min at 40°C. One unit of enzyme activity was defined as the amount of enzyme that oxidized 1 μM ABTS per minute. The extinction coefficient of 3.6×10^4 mol^{-1} cm^{-1} was used for oxidized ABTS [13]. The reaction mixtures used to determine MnP activity contained 2.7 mL of 50 mM sodium lactate (pH 4.5), 0.1 mL of 1.6 mM manganese sulfate, 0.1 mL of 2.0 mM H_2O_2, and 0.1 mL culture supernatant. The reaction was monitored by measuring the change in A_{240} for 3 min at 40°C. The reaction mixtures used to determine LiP activity contained 2.7 mL of 0.5 M sodium tartrate (pH 4.0), 0.1 mL of 1.2 M methylene, 0.1 mL of 2.0 mM H_2O_2, and 0.1 mL culture supernatant. The reaction was monitored by measuring the change in A_{664} for 3 min at 40°C. One unit of enzyme activity was defined as the amount of enzyme that transformed 1 μM of substrate per min [19].

2.4 Purification of Laccase

The culture supernatant was precipitated with cold acetone at the ratio of 1:1 in order

to remove non-target proteins. Laccase preparation was precipitated with cold acetone at the ratio of 1:2, and then centrifuged at 10,000 rpm and 4°C for 20 min. The precipitate was redissolved in 5 mL of 10mM sodium phosphate buffer (pH 7.0) and then the concentrated sample was dialyzed against the same buffer overnight. Finally, the sample was loaded onto a DEAE-Sepharose anion-exchange column (cylindrical glass column with 30 cm height and 1 cm diameter) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). The column was washed with the same buffer, and the absorbed proteins were eluted by a linear concentration of gradient of NaCl from 0 to 0.5 mM at a flow rate of 0.5 mL/min. The sample was collected to one tube every 10 min. The fractions containing laccase activity were pooled and concentrated by acetone precipitation. The concentrated enzyme solution was applied to a Sephadex G-100 column (cylindrical glass column with 30 cm height and 1 cm diameter) equilibrated with 10 mM sodium phosphate buffer (pH 7.0). Proteins were eluted with the same buffer at a flow rate of 1.0 mL/min. The laccase-rich fractions were pooled, concentrated by acetone precipitation as described above, and stored at -20°C for further experiment.

2.5 Molecular Mass Determination

To determine the purity of the protein and its molecular weight, SDS-PAGE was performed according to the protocol of [24] with 5% stacking gel and 12% resolving gel using a vertical electrophoresis system containing 0.1% SDS. All samples (10 μg of protein) with 0.5% SDS and 5% β-mercaptoethanol were boiled at 100°C for 10 min before being loaded onto the gel. The protein was visualized by staining the gel with Coomassie brilliant blue R-250 (Solarbio, China) and used for determining relative molecular mass by calculating the relative mobility of standard protein markers run alongside the samples.

2.6 Laccase Characterization

The effect of pH on laccase activity was determined using ABTS as substrate in a pH range of 2.2 to 8.0 in 0.1 M citrate-phosphate buffer, which consisted of a 100 mM sodium citrate buffer (pH 2.5-6.0) and 100 mM sodium phosphate buffer (pH 6.5-8.0). The effect of pH on the enzyme stability was also investigated by measuring the activity remaining after 24 h incubation at various pH values at 4°C. Optimum temperature of the purified laccase was examined over the temperature range of 20 to 80°C with ABTS as substrate at its optimal pH value. The thermal stability of laccase was determined in 0.1 M citrate-phosphate buffer (optimum pH) for 1 h at 10-80°C, followed by determining the residual activity. The effects of metal ions (i.e. Cu^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Ca^{2+}, Ba^{2+}, Fe^{2+}, Zn^{2+} and Ag^{+}) with the concentrations of 0.05 and 0.5 mM on laccase activity were analyzed by adding them in to assay mixture prior to the determination of residual activity [4]. All experiments were performed in triplicate to ensure reproducibility and the data represent the mean of three independent experiments.

2.7 Kinetic Properties

The oxidation of substrates by the purified laccase was determined spectrophotometrically at the specific wavelength of each substrate [16]. The assay was performed by measuring the increase at the A_{420} for ABTS in a 100 mM sodium acetate buffer (pH 4.5). The reaction rate was determined at the substrate (ABTS)

in the concentration range of 0.01 to 0.1 mM. The kinetic constants, K_m and V_{max}, of the enzyme were determined using a Lineweaver-Burk plot with ABTS as the substrate. All experiments were performed in triplicate to ensure reproducibility and the data represent the mean of three independent experiments.

3. RESULTS AND DISCUSSION

3.1 Purification of Laccase

Crude enzyme was obtained after 8 days of fermentation by removing fungal mycelium through centrifugation at 8,000 rpm for 10min. Laccase was the main ligninolytic enzyme since there were no other ligninolytic enzymes detected in the culture filtrate.The initial laccase activity of the culture supernatant reached 443 U/L on the 8th day. The purification of laccase after precipitation with cold acetone resulted in a yield of 48% of the total units of enzyme activity initially present in the culture filtrate (crude enzyme fraction). The laccase in culture filtrate of *M. purpureofusca* was purified to homogeneity. Figure 1 showed the elution profile from the DEAE-Sepharose anion-exchange column of four fractions (F1-F4) with sodium phosphate buffer elution containing the concentration of sodium chloride (50, 100, 300, and 500 mM). The absorbance at 280 nm of the active fraction tubes was detected and the corresponding laccase activity was also assayed. The high absorbed fraction F1 exhibited no laccase activity. The tiny absorbed fraction F2 that eluted with 100 mM NaCl displayed the highest laccase activity. The fractions F3 and F4 have no laccase activity. DEAE-Sepharose anion exchange chromatography resulted in a yield of 21% of the initial enzyme activity. The fraction F2 (cube No. 26, 27, and 28) containing laccase activity was pooled, and then applied to Sephadex G-100. After this step, there was an approximate 26.8-fold increase in specific activity when the final purified enzyme was obtained with an overall yield of 9.0%. The purification steps and relevant details such

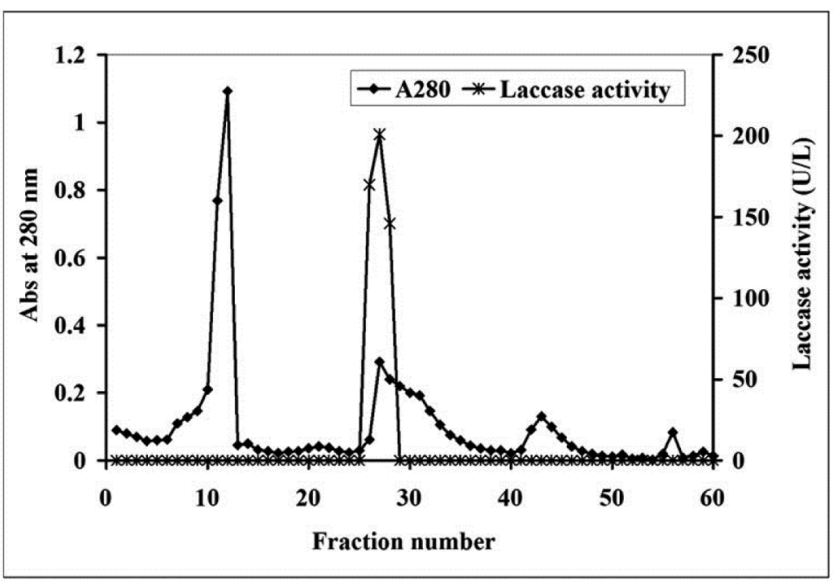

Figure 1. Elution profile for laccase from *M. purpureofusca* based on DEAE-Sepharose anion-exchange chromatography.

Table 1. Purification of laccase from *M. purpureofusca.*

Purification step Total protein	Total protein (mg)	Enzyme activity (U)	Specific activity (U/mg)	Yield (%)	Fold purification
Culture filtrate (crude enzyme)	1262	821	0.35	100	1.0
Precipitation with cold acetone	380	394	0.56	48	1.6
DEAE-Sepharose anion exchange chromatography	53	172	1.78	21	5.1
Sephadex G-100	7.8	74	9.38	9	26.8

as laccase activity, specific activity, and yield percentage and fold purification are outlined in Table 1.

To determine the molecular weight and verify the purity of laccase, the purified laccase was analyzed by SDS-PAGE. As shown in Figure 2, the purified enzyme and molecular weight marker was displayed in lane a and b, respectively. The appearance of a single protein band in SDS-PAGE indicates that the laccase purified by this scheme was a high purity enzyme. The relative molecular mass of the purified laccase was found to be 61.7 kDa.

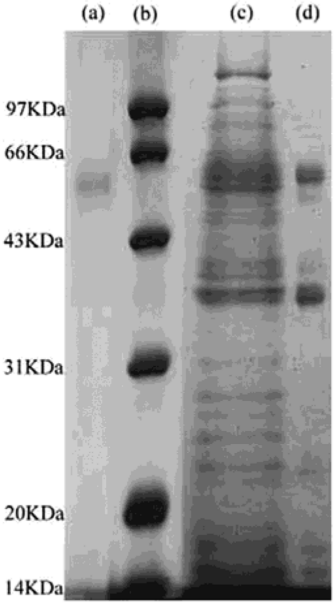

Figure 2. SDS-PAGE electrophoresis of the laccase from *M. purpureofusca.* Lane a: Laccase from Sephadex G-100 gel filtration; Lane b: Molecular mass marker standards; Lane c: Crude laccase; Lane d: Purified enzyme from DEAE-Sepharose anion-exchange chromatography.

3.2 Kinetic Study

The results showed that the K_m and V_{max} values of the purified laccase were 0.296 mM and 0.0645 mM/min, respectively, with ABTS as substrate (Figure 3). In general, the low K_m value may indicate a high affinity of the enzyme for its substrate, which also demonstrated that ABTS was a better substrate for the purified laccase.

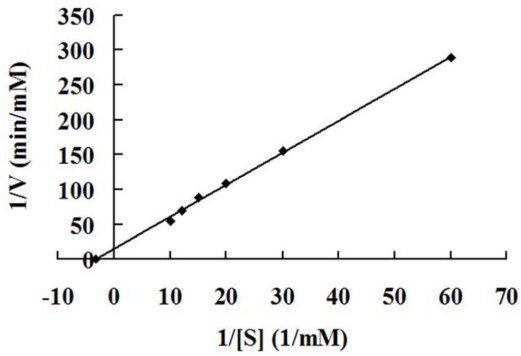

Figure 3. Lineweaver-Burk plot with ABTS as substrate

3.3 Laccase Characterization

The optimal pH value of the purified laccase was approximate 2.2. There was a sharp decline in the enzymatic activity when the pH value got close to the neutral range, reaching an almost undetectable level at above pH 7.0 (Figure 4A). As shown in Figure 4B, the purified laccase was stable after 24 hours of incubation at 4°C from pH 6 to 8. Thermal stability was measured after up to 1 h of incubation. The laccase had a half-life of 40 min at 40°C. It was stable for 1 h during incubation at 10°C to 30°C but for only 5 min during incubation at 50°C (Figure 5A). Incubation at 40 to 60°C increased enzyme activity compared to the control activity (the activity of the enzyme without incubation). Thus, we calibrated enzyme activity by using an enzyme preparation incubated for 2 min at 50°C (100% activity). The optimal temperature range for activity of the purified laccase was 40 to 50°C (Figure 5B). Enzyme activity was little at temperatures above 70°C, as determined during 3-min reactions. The effect of metal ions on the laccase activity was also studied using ABTS as the substrate. As described in Table 2, all metal ions at the concentration of 0.05 mM could affect enzyme activity, but Ag^{+} proved to be most effective to the increase of laccase activity in *Mycena purpureofusca* cultures. When the concentration of [Ag^{+}] was at 0.05 and 0.5 mM, laccase activity was enhanced by 18.7% and 130.5%, respectively. Compared with the control, laccase activity decreased when the concentration of other metal ions was 0.5 mM. On the other hand, Fe^{2+} ion inactivated the enzyme completely when its concentration was from 0.05 to 0.5 mM.

Laccases have recently drawn a lot of

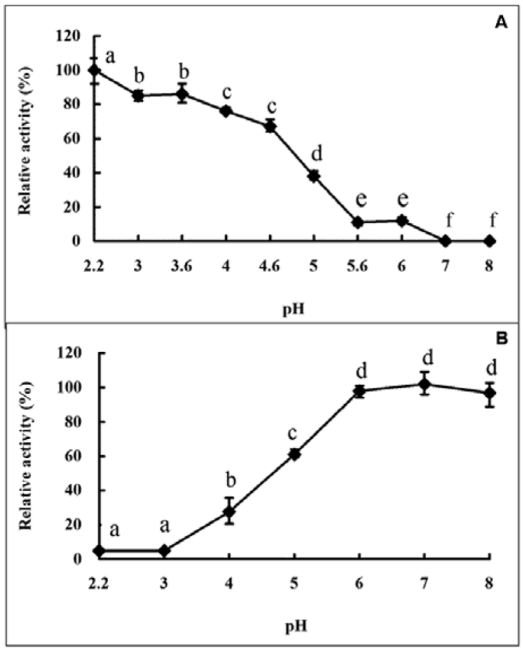

Figure 4. Effect of pH on activity and stability of purified laccase from *M. purpureofusca*. (A) Optimal pH, (B) pH stability.

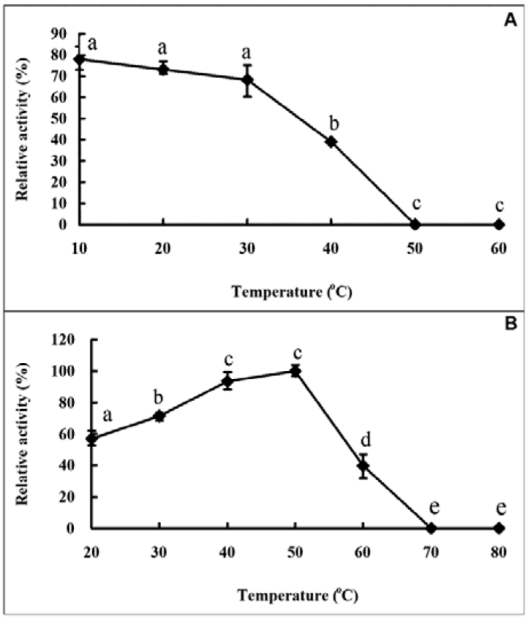

Figure 5. Effect of temperature on activity and stability of purified laccase from *M. purpureofusca*. (A) Thermal stability at 10, 20, 30, 40, 50, and 60°C after 1 h incubation, (B) Optimal temperature.

Table 2. Effect of metal ions on laccase activity.

Mental ions	Final Concentration (mM)	Relative activity (%)
Control	-	100
Mg^{2+}	0.05	97
	0.5	86
Fe^{3+}	0.05	108
	0.5	65
Mn^{2+}	0.05	110
	0.5	97
Cu^{2+}	0.05	101
	0.5	50
Ag^{+}	0.05	119
	0.5	231
Ca^{2+}	0.05	109
	0.5	84
Ba^{2+}	0.05	106
	0.5	84
Fe^{2+}	0.05	1
	0.5	2
Zn^{2+}	0.05	106
	0.5	81

attention in biocatalysis, biotransformations and cosmetics due to their stability and biological nature. Therefore, the availability of plentiful pure and bioactive enzyme samples is pivotal towards both basic research and technologically focused exploration. In this study, it is the first report that *M. purpureofusca* can produce laccase constitutively in submerged liquid culture. Based on this result, the purification and characterization of laccase produced by *M. purpureofusca* were investigated in the present work. The purified laccase from *M. purpureofusca* appeared as a single band with a molecular weight of 61.7 kDa in SDS-PAGE (Figure 3), which is in accordance with the other reports [2, 23, 25]. The parameters K_m for ABTS of the purified laccase from *M. purpureofusca* was 0.296 mM, The value was very similar to those of laccases from *Melanocarpus albomyces* (0.28mM) [11], *Pleurotus* sp. (0.25mM) [10], *P. ostreatus.* (0.28 mM) and *F. fraxinea* (0.27mM) [16]. The effect of pH on laccase activity as laccase enzymes tend to react differently was different with different substrates [9, 17]. The optimum pH of the purified laccase from *M. purpureofusca* was 2.2 for ABTS substrate, which was in accordance with the optimum pHs (2.0-5.5) of many other fungal laccases [1]. The purified laccase showed the maximum enzyme activity at 50°C, which was similar to the previously reported fungal laccase properties [12, 23, 28]. The purified laccase maintained its activity over a wide range of temperatures, and the typical optimum temperature range for this laccase is 50-60°C [9]. The interaction of metal ions and extracellular laccase is very important for the understanding of the

biotechnological processes of xenobiotic degradation [18]. ABTS was used as a substrate to test the effects of metal ions and inhibitors on laccase activity. The enzyme was not strongly inhibited by the tested metal ions except Fe^{2+}. Laccase activity was inhibited almost completely when the concentration of [Fe^{2+}] was 0.05 and 0.5 mM, which is similar to the results reported by other researchers [3]. On the contrary, there are some reports that Fe^{2+} can increase the laccase activity [22]. There is little publication to report the effect of Ag^{+} on laccase activity. Addition of Ag^{+} at 0.05 and 0.5 mM led to an increase of the laccase activity of 18.7% and 130.5%, respectively. However, for the *C. versicolor* laccase, its activity was strongly inhibited by the Ag^{+} [27]. Laccase as a member of multi-copper enzymes needs copper ions to perform catalysis, but Cu^{2+} did not enhance the laccase activity from *M. purpureofusca* at the concentration of 0.5 mM, which was similar to the research [15, 26]. However, Cu^{2+} can act as an inducing factor to improve laccase production by adding to the liquid medium.

4. CONCLUSIONS

The purified laccase from *Mycena purpureofusca* showed a relative molecular mass of 61.7 kDa. The laccase activity was stable at neutral pH and temperatures between 10 and 30°C. The laccase activity decreased rapidly when the temperature was above 30°C. The values of kinetic parameters *K*m and *V*max for purified laccase were 0.296 (mM) and 0.0645 (mM/min), respectively. The enzyme activity can be enhanced by 18.7% and 130.5% when Ag^{+} was added to the medium at the concentration of 0.05 and 0.5 respectively. This work will be helpful for the highly efficient laccase production by this fungus.

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