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Purification and characterization of two thermostable laccases with high cold adapted characteristics from *Pycnoporus* sp. SYBC-L1

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ABSTRACT

The white-rot fungus *Pycnoporus* sp. SYBC-L1 produced large amount of laccase in submerged fermentation. Two laccase isozymes (LacI and LacII) were purified using $(NH_4)_2SO_4$ fractionation, DEAE-cellulose and Sephadex G-100 column chromatography. The molecular masses of LacI and Lac II were 55.89 and 63.07 kDa, respectively by SDS-PAGE. Both the laccases showed acidic pH optima and high catalytic activities at low temperature for oxidations of 2,6-dimethoxyphenol (DMP), 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonate acid) (ABTS), syringaldazine and guaiacol. LacI and LacII were not only with high cold adaptation, but also fairly stable at high temperature. The half-lives of LacI at 50, 60 and 70 °C were 69.31, 2.58 and 0.13 h, respectively, whereas LacII was more stable with half-lives of 256.72, 21.00 and 2.06 h respectively. The best substrates for the enzymes were both found to be ABTS, in which the K_m values of LacI and LacII were 0.0166 and 0.0435 mM and the catalytic efficiencies were 19640.36 and 31172.64 S^{-1} mM^{-1}, respectively. EDTA and low concentration of Cu^{2+} and Mn^{2+} almost had non-inhibitions on their activities. LacII with syringaldehyde efficiently decolorized Remazol Brilliant Blue R. The high thermostabilities as well as cold adapted properties made *Pycnoporus* sp. SYBC-L1 laccases to be excellent candidates in harsh industry.

1. Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC1.10.3.2), belonged to a family of multicopper blue oxidases, can oxidize a broad range of organic and inorganic substrates, such as phenols, non-phenols, aromatic amines and their derivatives by transferring of electron with the concomitant reduction of oxygen to water [1]. Due to its low substrate specificity and strong oxidative ability, laccase can be investigated for a variety of applications including bleaching of paper pulp, decolorization of textile dyes, transformation of lignins or their derivatives, and detoxification of harmful substances [2,3].

Laccase was first found in *Rhus vernicifera* by Yoshida at the end of the 19th century. Thereafter, it has been found widely distribution in various fungi, some higher plants and bacteria [4–6]. The most efficient laccase producers are white-rot fungi, such as *Trametes versicolor, Coriolus hisutus, Pleurotus ostreatus*, and *Pycnoporus cinnabarinus* [7,8]. Among them, the genus of *Pycnoporus*, which has a simple ligninolytic system, has been described as model organisms for laccase production.

The purification and characterization of laccases from the leading producers of white-rot fungi had been greatly reported [9–12]. For commercial applications, it usually requires unique properties such as the thermal stability and resistant to acid or alkaline under some harsh conditions. Therefore, it is of great interest in obtaining enzymes with unique properties. *Thermus thermophilus* laccase was described as a thermophilic enzyme with a half-life of 14 h at 80 °C [13]. A halotolerant-alkaline laccase in *Streptomyces psammoticus* was reported, which was not only tolerant to NaCl with concentrations up to 1.2 M, but also stable in the pH range from 6.5 to 9.5 at 45 °C for 90 min [14]. Although the genus *Pycnoporus* seems to be the potential laccase producer, there are limited excellent laccases were reported, therefore, it has important significance to exploit some laccases with novel characteristics.

Synthetic dyes, classified as azo, anthraquinone, triphenylmethane and phthalocyanine, are being used increasingly in the textile. Among them, anthraquinone dyes occupy the most proportion of dyes [15,16]. It is well known that these dyes can cause serious environmental pollution, but unfortunately, they are

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unusually resistant to degradation. The important environmental benign and cost effective method is using white-rot fungi in the dye decolorization [17,18]. The degradation processes are involved in their secreted ligninolytic enzymes including lignin peroxidase, manganese peroxidase and laccase. Laccase is the most important enzyme which has been successfully applied in the decolorization of natural pigments or synthetic dyes [19,20].

In our previous study, a fungus of *Pycnoporus* sp. SYBC-L1 had been isolated and identified as a high laccase producer in submerged fermentation [21]. In this work, we aim to purify and characterize the laccase produced by this strain. Moreover, the purified enzyme was evaluated for its potential capability in the decolorization of anthraquinone dye.

2. Materials and methods

2.1. Organism and culture conditions

Pycnoporus sp. SYBC-L1 was a stock culture of the Laboratory of Biochemistry, School of Biotechnology, Jiangnan University, Wuxi, Jiangsu Province, China. The fungus was maintained on potato dextrose agar slants at 4 °C. The synthetic liquid medium (SLM) containing (per liter) 60 g barley bran, 60 g glucose, 10 g soybean meal powder, 0.5 g $NH_4H_2PO_4$, 2.0 g K_2HPO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, 0.075 g $CaCl_2$ and 7.0 ml trace element solution [22] was supplemented with $CuSO_4{\cdot}5H_2O$ (0–3.0 mM) as the laccase production inducer. *Pycnoporus* sp. SYBC-L1 was incubated in 250 ml flasks containing 50 ml of SLM on a rotary shaker at 200 rpm. After 12 days of cultivation at 30 °C, crude laccase was harvested and the mycelia were removed by centrifugation at 10,000 × *g* for 20 min (Himac CR22G, Hitachi, Japan). The supernatant was then used for laccase purification.

2.2. Laccase assay and protein determination

Laccase activity was determined spectrophotometrically by using 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonate acid) (ABTS, Sigma–Aldrich) as the substrate. A 3 ml of reaction mixture contained 0.5 ml of 1 mM ABTS, 2.4 ml of 100 mM citrate/phosphate buffer (pH 3.0) and 0.1 ml of appropriately diluted enzyme sample. Enzyme assay was carried out by monitoring the absorbance increase at 420 nm ($\varepsilon_{420\ nm}$ = 36,000 M^{-1} cm^{-1}). One unit of laccase activity was defined as the amount of enzyme that oxidized 1 μmol of substrate per min [23].

Protein contents of the broth and other samples were determined by the method of Bradford using bovine serum albumin as the standard [24].

2.3. Laccase purification

All the following purification procedures were carried out at room temperature and the protein content and laccase activity of each step were determined as described earlier. The culture supernatant was concentrated by adding ammonium sulfate to 40% saturation. After 5 h incubation, the solution was centrifuged at 10,000 × *g* for 20 min, meanwhile, the supernatant fluid was then increased to 80% saturation by the addition of ammonium sulfate. Storing the solution overnight, the precipitate obtained by centrifugation was dissolved in a small volume of citrate/phosphate buffer (20 mM, pH 6.0), and then dialyzed against the same buffer. The dialyzed protein was loaded onto a pre-equilibrated DEAE-cellulose anion exchange column (1.6 cm × 30 cm). The column washed with 20 mM citrate/phosphate buffer (pH 6.0) until the A_{280} reading was less than 0.02. Bound protein was then eluted with a linear salt gradient (0–1 M NaCl) at a flow rate of 1 ml per min. Laccase active fractions were pooled, dialyzed, concentrated and applied for further purification on Sephadex G-100 column (1.6 cm × 60 cm). The loaded proteins were eluted with 20 mM citrate/phosphate buffer (pH 6.0) at a flow rate of 0.5 ml per min and the laccase-rich fractions were then pooled, concentrated by vacuum freeze-drying and stored at −20 °C for further use.

2.4. Gel electrophoresis and absorption spectrum

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli [25] with 5% stacking gel and 12% resolving gel. The protein bands were visualized by staining the gel with Coomassie Brilliant Blue R-250 (Sigma–Aldrich) and the molecular mass was determined by calculating with standard protein markers (Code No.: D532A, TaKaRa).

Native-PAGE was prepared according to the same protocol of SDS-PAGE, but without SDS. Activity staining for the determination of laccase isozymes was carried out by incubating the gel in citrate/phosphate buffer (pH 4.5) with 0.5 mM α-naphthol for 30 min at room temperature. The proteins in the culture supernatant were analyzed on Native-PAGE, and after electrophoresis, the gel was then stained with Coomassie Brilliant Blue R-250.

The absorption spectrum of the purified laccase was recorded from 200 to 900 nm at room temperature in 20 mM citrate/phosphate buffer (pH 6.0) with a UV–vis spectrophotometer (U-3010, HITACHI, Japan).

2.5. Effect of pH and temperature on laccase activity and stability

Four substrates, such as ABTS (0.5 mM), 2,6-dimethoxyphenol (DMP, 1.0 mM), syringaldazine (1.0 mM) and guaiacol (1.0 mM), were used to determine the optimal pH and temperature of the purified laccase. The optimal pH was examined in 100 mM citrate/phosphate buffer by varying the pH value from 2.0 to 8.0. The effect of temperature on laccase activity was determined at a temperature range from 0 to 100 °C.

The stability of the purified laccase at different pH value was evaluated by incubating the enzyme in 100 mM citrate/phosphate buffer (pH 2.0–7.0) or Tris–HCl buffer (pH 7.0–10.0) at 30 °C. The thermostability of the purified laccase was analyzed in 20 mM citrate/phosphate buffer (pH 6.0) at 0–80 °C. The residual laccase activity was measured periodically with ABTS as the substrate. All data were collected from two separate experiments which were both with at least triplicates.

The enzymatic inactivation constant (k) of purified laccase at different temperature was determined according to Cui [26] by adjusting the data to a first-order decay model (dR/dt) = kR, where R is residual activity (%) at t (h). The adjusted data had a correlation >0.96 at all temperatures. The half-life ($t_{1/2}$) at different temperatures was calculated as $t_{1/2} = \ln 2/k$.

2.6. The kinetic parameters of purified laccase

The kinetic constant K_m, V_{max} and catalytic constant k_{cat} of the purified laccase for ABTS, DMP, syringaldazine and guaiacol were respectively determined at their optimal temperature and pH value. The reactions were all performed at least three times and the kinetic data were calculated from Lineweaver–Burk plots using the Michaelis–Menten equation.

2.7. Effect of metal ions and inhibitors on laccase activity

The purified laccase was incubated with 0, 5 or 10 mM (final concentration) of metal ion or inhibitor for 5 min at 30 °C and the laccase activity was then measured in triplicates as described above using ABTS as the substrate. Control tests were conducted in parallel in the absence of the metal ions or inhibitor.

2.8. Analysis of protein sequence by mass spectrometry

The purified laccase was loaded onto SDS-PAGE. After electrophoresis and protein visualization, the laccase band was cut up from the gel and then partially digested with trypsin (Sigma–Aldrich, Germany). The digest mixture was first separated by HPLC and then analyzed by electrospray ionization quadrupole-time of flight-mass spectrometry (ESI-Q-TOF-MS). The experimental mass values were compared with a database of nr-NCBI-fungi laccase. By using an appropriate scoring algorithm, the closest match or matches could be identified.

2.9. Dye decolorization by laccase

The decolorization of Remazol Brilliant Blue R (RBBR, Sigma–Aldrich) was examined by the purified laccase of LacII. Appropriate dye concentration (200 mg l^{-1}, final concentration) and laccase activity (2.0 U ml^{-1}, final concentration) were selected in order to obtain an appropriate absorbance at the maximal wavelength. The effects of pH and temperature on dye decolorization were determined as following: the reaction solutions (4 ml, final volume) containing RBBR and LacII were incubated in 100 mM citrate/phosphate buffer (pH 3.0–7.0) at 30–70 °C. Moreover, the effects of redox mediators on RBBR decolorization were also examined. Four redox mediators including syringaldehyde (1 mM, final concentration), *p*-hydroxybenzoic acid (5 mM, final concentration), vanillin (10 mM, final concentration) and 1-hydroxybenzotriazole (HBT, 10 mM, final concentration) were used in this study.

All the mixtures were incubated in static conditions and in complete darkness. Control samples were done in parallel with heat-denatured laccase and all assays were taken in triplicates. Dye decolorization was measured by monitoring the decrease of absorbance at the maximum wavelength of RBBR and expressed in terms of percentage.

3. Results

3.1. Laccase production

Laccase is a multicopper oxidase, therefore, copper is an essential metal for laccase synthesis [19]. The extracellular laccase produced by *Pycnoporus* sp. SYBC-L1 was testified to be stimulated significantly by adding copper ion to the culture medium (Fig. 1). Supplementation of $CuSO_4$ at 2.0 mM obtained a high laccase yield of 28.55 U ml^{-1}, which was 1.9-folds ($P < 0.05$) compared to the control. The onset of laccase activity occurred on day 6 and reached its maximum on day 12 (Fig. 1), therefore, the laccase obtained from 12-day culture was used for the next purification.