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Textile dye degrading laccase from *Pseudomonas desmolyticum* NCIM 2112

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ABSTRACT

A laccase requiring optimum temperature 60 °C, pH 4.0 for the activity and having apparent molecular weight 43,000 Da was purified from *Pseudomonas desmolyticum* NCIM 2112 by three steps, including heating, anion exchange, and molecular sieve chromatography. The purification fold and yield of laccase obtained through Biogel P100 were 45.75 and 19%, respectively. Staining of native gel with L-dopa showed dark brown color band indicating the presence of laccase. In relation to hydroquinone, the substrate specificity of laccase was in the following order: DAB > *o*-tolidine > ABTS > L-dopa. The absence of monophenolase activity in eluted fractions conformed that the purified protein is laccase. This laccase showed substrate dependent optimum pH character. Effect of inhibitor and metal ion on enzyme activity was analyzed. UV–vis analysis showed the decolorization of Direct Blue-6, Green HE4B and Red HE7B in the presence of laccase. The FTIR spectral comparison between the control dye sample and the metabolites extracted after decolorization by purified laccase have confirmed degradation of these dyes. This study contributes for the structural requirement of a dye to be degradable by *P. desmolyticum* laccase and is important in order to optimize potential bioremediation systems for industrial textile process water treatment.

1. Introduction

The biodegradation ability of the bacteria is assumed to be associated with the production of lignolytic enzymes such as lignin peroxidase [1] and laccase [2]. Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) is a multicopper oxidase, widely distributed among plants, fungi [3], and bacteria [4]. It catalyzes the oxidation of a broad range of organic and inorganic substrates, including diphenols, polyphenols, diamines, aromatic amines, and ascorbate by a one-electron transfer mechanism [5].

There are extensive studies focused on laccase from fungi like *Daedalea quercina* [6], *Sclerotium rolfsii* [7], *Ganoderma lucidum* [8], *Trametes trogii* [9], and *Pycnoporus sanguineus* [10]. Laccases can be applied extensively in many fields, including waste detoxification and textile dye transformation due to their low substrate specificity [2]. Although some bacterial laccases have been characterized [11,12], little information is available concerning their substrate specificities towards dye decolorization. Large amounts of chemically different dyes are used for textile dyeing and a significant proportion of these dyes enter the environment as waste water. Not all these dyes could be degraded and/or removed with physical and chemical processes, and sometimes the degradation products are more toxic [13]. Currently, one of the possible alternatives for treatment of textile effluents is the use of bacteria or their enzymes, which can oxidize a wide spectrum of synthetic dyes [13].

The demand for removal of synthetic dyes from the textile industrial waste using fugal/bacterial laccase is being increased tremendously. Laccase has been reported as an inducible enzyme during degradation of azo dyes by various bacteria [14,15]. *Pseudomonas desmolyticum* NCIM 2112 has been reported to decolorize and degrade reactive and benzidine based azo dyes at the static anoxic condition [16,17]. Even though the induction of dye degrading enzymes has been reported with proposed metabolic pathways, the individual role of enzymes is not discussed. There are several reports on effect of physicochemical parameters, cell aging, dye concentration, immobilization of whole cells and consortium on bacterial dye decolorization [15,18,19]. Studies on the action of purified bacterial laccase are scarce and its role in color removal remains poorly understood. To this end, we have purified and characterized the laccase from *P. desmolyticum* NCIM 2112. The degradation of three textile dyes by purified laccase has been studied.

Abbreviations: DAB, 3-3′-diaminobenzidine tetrahydrochloride; ABTS, 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonate; DB6, Direct Blue-6; GHE4B, Green HE4B; RHE7B, Red HE7B.

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2. Materials and methods

2.1. Chemicals

DEAE (diethyl aminoethyl) cellulose was purchased from Sisco Research Laboratories, India. Biogel P100 was purchased from Biorad, USA. 2,2′-azinobis (3-ethylbenzthiazoline-6-sulfonate (ABTS) was purchased from Sigma–Aldrich, USA. The textile dyes, Direct Blue-6 (DB6; C. I. Direct Blue 6), Green HE4B (GHE4B; C. I. Reactive Green 19A) and Red HE7B (RHE7B; C. I. Reactive Red 141) were the generous gift from local textile industry, Solapur, India. All chemicals used were of the highest purity available and of the analytical grade.

2.2. Microorganism and culture condition

P. desmolyticum NCIM 2112 was obtained from National Center for Industrial Microorganisms, NCL, India. As previous studies from our lab have reported induction of laccase activity in *P. desmolyticum* NCIM 2112 during degradation of reactive and benzidine based dyes [16,17], we selected this strain for the production of laccase. Pure culture was maintained on nutrient agar slants at 4 °C by transferring culture once in a month. Composition of nutrient medium used for decolorization studies was (g l^{-1}): NaCl 5, peptone 5, beef extract 3.

2.3. Laccase production

Time course of laccase production by *P. desmolyticum* was studied in 100 ml nutrient broth at 30 °C at static condition. Laccase activity (as mentioned in following section) was measured in crude cell extract of *P. desmolyticum* cells [20] grown at different time intervals. Dry weight of cells was measured as reported previously [20].

For higher laccase production, 10% inoculum of 12 h ($A_{660\,nm}$ 0.7) grown *P. desmolyticum* was inoculated in 3 l nutrient medium and incubated ~12 h at 30 °C. Cells were collected by centrifugation at 8000 × *g* for 15 min and suspended (150 mg ml^{-1}) in 50 mM sodium phosphate buffer (pH 7.0; buffer A) containing 5 mg ml^{-1} lysozyme. Cells were further incubated at 37 °C for 45 min in water bath and then disrupted by sonication as mentioned previously [20]. This cell free extract was solubilized in cholic acid (0.33 mg mg protein^{-1}) on magnetic stirrer at 4 °C for 30 min. The cell lysate obtained was centrifuged twice at 15,000 × *g* for 30 min at 4 °C and the clear supernatant used immediately or stored at −20 °C until its use to purify laccase.

2.4. Purification of laccase

All purification steps were carried out using Biorad protein purification system (EP 1-Econo pump) at 4 °C. The supernatant containing laccase activity 0.04 U (mg protein)$^{-1}$ min^{-1} was heated at 60 °C for 10 min and centrifuged at 8000 × *g* for 20 min. The clear supernatant obtained after centrifugation was loaded on a DEAE cellulose fast flow column (15 mm × 120 mm), equilibrated with buffer A. The column was washed with the same buffer by two times of the column volume and the enzyme was eluted with a linear gradient of 0–1.0 M NaCl. Fractions containing laccase activity were pooled and dialyzed against 1 mM sodium phosphate buffer (pH 6.0). The dialyzed sample was concentrated (1–2 ml) by ultrafiltration using YM10 cut-off membrane (Amicon, Boston, MA) and loaded on Biogel P100 column (10 mm × 500 mm) equilibrated with 50 mM sodium phosphate buffer (pH 6.0, buffer B). The protein elution was carried with the same buffer at 6 ml h^{-1} flow rate. Fractions containing laccase activity were pooled and stored at −20 °C until use.

2.5. Protein determination and enzyme activity

The protein concentration of each fraction was monitored by absorbance at 280 nm or Lowry methods with bovine serum albumin as a standard [21]. Laccase activity was determined at 30 °C by measuring increase in optical density at 420 nm in a reaction mixture of 2 ml containing 0.66 mM ABTS in 0.1 M acetate buffer (pH 4.9) and 100 μl enzyme [22]. One unit of enzyme activity was defined as a change in absorbance unit min^{-1} mg protein^{-1}.

The monophenolase activity of purified protein was determined using *p*-cresol and 4-hydroxyanisole (4HA) as substrate. Cresolase activity toward *p*-cresol was measured spectrophotometrically by the appearance of 4-methyl-*o*-benzoquinone at 400 nm (ε = 1350 M^{-1} cm^{-1}), as has been described by Mayer et al. [23]. The reaction mixture of 2 ml contained 100 μl enzyme and 1 mM *p*-cresol in 100 mM Na-PO_4 buffer (pH 7.0) at 30 °C. One unit of enzyme was defined as the amount of enzyme that produced 1 μmol of *tert*-butylquinone min^{-1}. 4HA was used as substrate to determine the monophenolase activity of purified protein [24]. The 100 μl enzyme was incubated with 50 mM sodium acetate, pH 5.5, 2% (v/v) DMF and 50 mM 3-methyl-2-benzothiazolinone hydrazone hydrochloride (MBTH) at 25 °C for 5 min. The reaction was started by addition of 100 mM 4HA and monitored spectrophotometrically at 500 nm. One unit of enzyme activity (U) is defined as the amount of enzyme producing 1 μmol of MBTH-quinone adducts per minute during the linear phase of the reaction.

2.6. Characterization of purified laccase

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out following the protocol of Laemmli [25] with 4% (w/v) stacking and 10% (w/v) resolving gel, run on a slab gel unit (Genetech Laboratories, India). Protein bands were stained with Coomassie brilliant blue R-250 at 0.1% (w/v) in methanol/acetic acid/water (v/v/v) (4:1:5) for 1 h at room temperature followed by destaining using same solution. The molecular mass of the purified laccase was determined by calculating the relative mobility of standard protein markers (Genie, India) run alongside (205,000 Da myosin rabbit muscle; 0.5 mg ml^{-1}, 97,400 Da phosphorylase b; 0.5 mg ml^{-1}, 66,000 Da bovine serum albumin; 0.5 mg ml^{-1}, 43,000 Da ovalbumin; 0.75 mg ml^{-1}, 29,000 Da carbonic anhydrase; 0.5 mg ml^{-1}, 20,100 Da soyabean trypsin inhibitor; 2.0 mg ml^{-1}, 14,300 Da lysozyme; 0.75 mg ml^{-1}). Zymogram analysis for laccase activity was performed on native-PAGE using 1 mM L-Dopa in 0.1 M acetate buffer (pH 4.9), after washing the gels for 1 h with the same buffer.

Substrate specificity of the purified laccase was determined spectrophotometrically at 30 °C in a reaction mixture of 2 ml containing either substrate (5 mM hydroquinone, 5 mM DAB, 2 mM *o*-tolidine, 0.66 mM ABTS, 5 mM L-dopa) in 0.1 M acetate buffer (pH 4.9) and 100 μl enzyme. The rate of oxidation of various substrates catalyzed by purified laccase was determined by measuring the absorbance change with the molar extinction coefficient (ε) obtained from literature [26,27].

The optimum pH for the purified laccase was examined in the pH range 2.0–7.0 (0.1 M of KCl–HCl buffer; pH 2.0, glycine–HCl buffer; pH 3.0, sodium-acetate buffer; pH 4.0–5.0 and sodium-phosphate buffer; pH 5.0–7.0) with 0.66 mM ABTS, and 5 mM 3-3′-diaminobenzidine tetrahydrochloride (DAB) and hydroquinone as a substrate separately. The optimum temperature and the effect of putative laccase inhibitors and metal ions on laccase activity were determined using 5 mM hydroquinone as substrate in 0.1 M sodium-acetate buffer (pH 4.0). For temperature study (30–80 °C), the reaction mixture was incubated at each temperature for 10 min before enzyme addition. The individual inhibitors tested were 1 mM Ethylene diamine tetraacetic acid (EDTA), Dithiothreitol (DTT), and metal ions used at 1 mM concentration were $MgCl_2$, $CaCl_2$, $HgCl_2$, and $CuSO_4$. The purified laccase was incubated with metal ions and inhibitors for 10 min and the enzyme activity was assayed in triplicate as described above. Control samples were maintained without the metal ions and laccase inhibitors.

2.7. Decolorization and degradation of dyes by purified laccase

One benzedine based dye, DB6 (MW 932.76; λ_{max} 540 nm) and two reactive azo dyes, GHE4B (MW 871.07; λ_{max} 630 nm) and RHE7B (MW 1070; λ_{max} 552 nm) were used in this study. These dyes are previously reported to be decolorized by *P. desmolyticum* NCIM 2112 [16,28,17]. The decolorization reaction was carried out at 30 °C for 24 h in 2 ml reactions mixture containing 100 mg l^{-1} dye prepared in 50 mM acetate buffer (pH 4.8) and 0.5 U ml^{-1} purified laccase. Control containing heat-denatured enzyme was used to measure decolorization of dye at different time interval. The decolorization was monitored by scanning the UV–vis spectrum between 200–800 nm using Hitachi (U-2800) double beam spectrophotometer.

The above decolorized samples were centrifuged at 1000 × *g* for 1 min and laccase degraded metabolites of DB6, GHE4B and RHE7B were extracted by adding twice equal volume of ethyl acetate. After drying over anhydrous Na_2SO_4, the biodegraded metabolites were characterized by Fourier Transform Infrared Spectroscopy (PerkinElmer, Spectrum one) and compared with control dye. The FTIR analysis was done in the mid IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out.

3. Results

3.1. Purification of laccase

P. desmolyticum achieved maximum intracellular laccase activity of 0.012 ± 0.0003 U mg^{-1} protein in the crude cell free extract of 24 h growth (Fig. 1). Laccase production was more between 18 and 24 h of incubation period. Although dry cell weight increased after 24–36 h incubation period, significant loss in the laccase activity was observed. The purification of laccase from *P. desmolyticum* consisted of three steps including heating, anion exchange and molecular sieve chromatography. Heating of cell free extract at 60 °C for 10 min proved to be effective for removing large amounts of proteins as laccase was stable for this period and there was no significant loss of the enzyme activity. The specific activity of laccase was increased from 0.04 to 0.081 U mg^{-1} proteins on heating of the cell free extract. Further it was increased to 0.424 U mg^{-1}