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Original Research Paper

A novel laccase from newly isolated *Cotylidia pannosa* and its application in decolorization of synthetic dyes

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ABSTRACT

The present study reports an extracellular laccase from *Cotylidia pannosa* sequestered from North Western Himalayas, grown in submerged culture and used to investigate its ability to decolorize synthetic dyes. The laccase production was optimized in yeast extract peptone (YEP) broth supplemented with 2% wheat bran at an inoculum level of $2.9x10^8$ fungal spores/mL for different parameters like temperature, pH, incubation time and agitation rate. The maximum activity of laccase (10 U/mL) was obtained with wheat bran as the substrate at pH of 5.0 when incubated at 30 °C for 72 h at 120 rpm. The partial characterization revealed a laccase with a molecular mass of ~43 kDa and a K_m and V_{max} of 3.5 mg/mL and 88.6 U/mL, respectively. The crude enzyme was found to perform optimally at a temperature of 50 °C and a pH of 5.0. The fungus as well as the crude laccase preparation decolourized the synthetic dyes such as congo red, bromophenol blue and coomassie brilliant blue R-250 to different extent. A decolorization efficiency of 94% by fungal biomass and 40% by crude laccase was observed for congo red. The decolorization of synthetic dyes by the novel laccase from *C. pannosa* makes it an ideal candidate for the treatment of wastewater from industrial effluents.

1. Introduction

Laccases (EC 1.10.3.2, benzenediol:oxygen oxidoreductase) are copper-containing polyphenol oxidases which mediate the oxidation of phenolic/non-phenolic lignin-related compounds and recalcitrant environmental pollutants. The laccases are widely distributed in plants, fungi, bacteria and insects (Madhavi and Lele, 2009). Among all these sources, laccases from white-rot fungi are of special interest because of their aptitude to degrade lignocellulosic biomass by elaborating extracellular laccase enzyme (Ramírez-Cavazos et al., 2014). As the laccases have low substrate specificity, these can oxidize a wide variety of substrates like phenols, diphenols, polyphenols, substituted phenols, diamines, aromatic amines, and various non-phenolic compounds (Claus, 2004; Kurniawati and Nicell, 2008). The laccases provide green route in various biochemical processing used during paper pulping and bleaching, textile refining, dye decolorization, bioremediation, organic synthesis, juice and wine clarification (Gonzalez et al., 2013), as a tool for medical diagnostics and as a bioremediation agent to clean up of herbicides, pesticides and certain explosives in soil (Couto and Herrera, 2006).

Intense research has been carried out on laccases in the recent years to explore sources of laccases with easy availability, high catalytic efficiency, broad substrate specificity, tolerance to various activity parameters depending on their wider utility. White-rot fungi such as *Pleurotus ostreatus* and *Trametes versicolor* are being used as model organisms for laccase activity. The laccases have also been reported in other fungi such as *Phanerochaete chrysosporium*, *Ganoderma lucidum*, *Coriolus versicolor* (Manavalan et al., 2015) and *Polyporus brumalis* (Kim et al., 2012). However, laccase from the newly isolated white-rot fungus, *Cotylidia pannosa* (Gene Accession no. KT008117) has not been investigated yet. The newly identified fungus isolated from North-Western Himalayan ranges is reported to produce efficient cellulolytic enzyme system, which can be used for degrading the cellulose content in lignocellulosic waste materials resulting in free glucose residues which can be readily fermented into ethanol (Sharma et al., 2015). The ethanol yield obtained by the isolated fungus was found to be far much better than other white rot fungi such as *P. chrysosporium* and *Trametes hirsuta* using wheat bran under submerged fermentation suggesting that *C. pannosa* could be used as a suitable candidate for use in the production of ethanol from lignocellulosic biomass.

Apart from the role of white rot fungi in bioconversion of lignocellulosic material to bioenergy, this fungal group is an effective bioremediator for toxic textile industrial pollutants because of their dye adsorption and pollutant degradation capabilities due to their efficient enzymatic machinery. Therefore, the biodegradation

Abbreviations: YEP, Yeast extract peptone; ABTS, 2,2-azinobis (3-ethylbenzathiazoline-6-sulfonic acid); PAGE, Polyacrylamide gel electrophoresis

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abilities of fungi are given special emphasis in management of environment through green route. The present study reports the optimization and characterization of laccase from a newly identified strain of *C. pannosa* followed by its application in removing synthetic dyes by enzymatic biodegradation or mineralization.

2. Materials and methods

2.1. Microbial culture and its maintenance

The culture of newly identified *C. pannosa* (Sharma et al., in press) used in this study was obtained from Chaudhary Sarwan Kumar Himachal Pradesh Krishi Vishvavidyalaya (CSKHPKV), Palampur, India. The stock culture was maintained on yeast extract peptone (YEP) containing 1% yeast extract, 2% peptone with 2% agar at 4 °C and routinely sub-cultured. For production of inoculums, the fungus was grown on YEP agar plates for 4 days at 30 °C.

2.2. Laccase activity in C. pannosa

From YEP agar plates, five agar plugs (equivalent to 2.9×10^8 spores/mL) were transferred aseptically into 250 mL Erlenmeyer flasks containing 100 mL YEP broth and incubated at 30 °C for 72 h at 120 rpm. After incubation, the cell free supernatant was used for determination of laccase activity qualitatively by agar well plate assay (Fu et al., 2013) and quantitatively by spectrophotometric analysis (Bourbonnais and Paice, 1990). Briefly, the plate assay was performed on potato dextrose agar (PDA) medium containing 14 μmol of ABTS [2,2-azinobis (3-ethylbenzathiazoline-6-sulfonic acid); Sigma Chemical Co., St. Louis, Mo.] per ml in 50 mM glycine–HCl buffer (pH 3.0). The agar wells made were filled with 25 μL of cell free supernatant. The plates were incubated overnight and observed for bluish-green colouration. For the quantitative analysis, the assay mixture contained 2 mL ABTS dissolved in 0.5 mM sodium acetate buffer (pH 5.0) and 2 mL aliquots of appropriately diluted culture fluid. The oxidation of ABTS was monitored by following the increase in absorbance at 420 nm ($\varepsilon = 3.6 \times 104\ M^{-1}\ cm^{-1}$). One unit of laccase activity was defined as the amount of enzyme required to oxidize one μmol of ABTS per minute.

2.3. Lignocellulosic substrate selection

The biomass sources *viz.* lolium grass and bromus grass were obtained from Institute of Himalayan Bioresource Technology (IHBT), Palampur (H.P.) while dried pine needles, filter paper, wheat grains and wheat bran were obtained from Jaypee University of Information Technology, Waknaghat, Solan (H.P.).

The cellulose and hemicelluloses content in the selected substrate (wheat bran) was determined by the method of Crampton and Maynard (Crampton and Maynard, 1938); while lignin content was estimated by the method of Goering and van Soest (1970).

For laccase production, all the above substrates were grounded to a fine powder and filtered through a sieve of 0.5 mm. A 2.0 g of each substrate was added to their respective flasks which contained 100 mL of growth medium (YEP). A spore inoculum of *C. pannosa* (2.9×10^8 fungal spores/mL) was inoculated in 100 mL of sterilized growth medium followed by incubation for 96 h at 30 °C. A 5 mL aliquot from fermentation broth was withdrawn from the flasks at different time intervals till 96 h and centrifuged at 4 °C at 6300*g* for 15 min. The supernatant was collected and analyzed for laccase activity as discussed in Section 2.2.

2.4. Optimization of fermentation parameters

For laccase production, submerged fermentation was optimized in 250-mL flasks containing 100 mL of YEP containing 2% wheat bran inoculated with an inoculum size of 2.9×10^8 fungal spores/mL. The enzyme production was optimized at different fermentation parameters such as temperature (25 °C, 30 °C, 37 °C and 42 °C), incubation time (24, 48, 56, 72 and 96 h) and pH (3.0, 4.0, 5.0 and 6.0) at an agitation rate of 120 rpm.

2.5. Preparation and characterization of crude laccase

The cell free supernatant obtained from fermentation of YEP containing 2% wheat bran after 96 h of incubation at 30 °C, 120 rpm and pH 5.0 was precipitated with 100% ice cold acetone (1:2). The mixture was kept at −20 °C overnight for complete precipitation followed by centrifugation at 15,000*g* for 15 min at 4 °C. The pellet obtained was air dried to remove any residual acetone and stored at −80 °C until further use. The protein content of the precipitated sample was estimated by Lowry's method (Lowry et al., 1951).

A 0.5 mM ABTS substrate was used to determine the optimal pH for crude laccase. The pH optimum was examined using 0.5 mM sodium acetate buffer with a pH range from pH 3.0 to 7.0 at 30 °C. The effect of temperature on laccase activity was determined using ABTS as substrate, in a temperature range from 25 °C to 60 °C, in 0.5 mM sodium acetate buffer (pH 5.0). Three replicates of each experiment were performed.

The thermostability of crude laccase enzyme was analyzed in 0.5 mM sodium acetate buffer (pH 5.0) at 30–60 °C for upto 192 h. The stability of crude laccase at different pH values was evaluated by incubating the sample in 0.5 mM sodium acetate buffer (pH 3.0–6.0) at 30 °C temperature for upto 192 h. The residual laccase activity was measured periodically using ABTS as substrate. All stability experiments were performed in triplicates.

Michaelis constant (K_m) and maximal velocity (V_{max}) of the crude enzyme was determined from Michaelis–Menten equation derived by measuring laccase activity of crude enzyme at different substrate concentrations (0, 5.0, 10.0, 15.0, 20.0 and 30.0 mg/mL).

The molecular weight and zymogram analysis of crude laccase was estimated under both non-denaturing Native-PAGE and denaturing SDS-PAGE gel systems consisting of 12% resolving gel and 5% stacking gel using broad range molecular weight standards (Bio-Rad Laboratories, Hercules, CA) according to a modified method given by Laemmli (1970). After both Native and SDS-PAGE, each gel was divided into two pieces; one was stained with 0.05% coomassie brilliant blue R-250 for molecular weight determination and the second was incubated with 20 mM ABTS dissolved in sodium acetate buffer of pH 5.0 for laccase activity determination.

2.6. Decolorization of synthetic dyes

2.6.1. Dye decolorization by fungal mycelia

For decolorization experiments, five agar plugs (6 mm diameter) of active mycelium from PDA plate were transferred aseptically into 500 mL Erlenmeyer flasks containing 100 mL of potato dextrose broth with 60 mg/L of Congo red dye (CR), bromophenol blue dye (BB), coomassie Brilliant Blue R-250 (CBB R-250) and orange G (OG) dye and incubated at 30 °C for 96 h at 120 rpm. A biotic (sterilized medium, without dye addition) and abiotic (sterilized medium containing the dye but not inoculated with the fungus) control experimental were also prepared and maintained in parallel with the decolorization experiments.

Culture samples were withdrawn at defined interval of 24 h upto 96 h, centrifuged at 10,000*g* for 20 min at 4 °C and the supernatants obtained were used for decolorization assay. The