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Purification and characterization of yellow laccase from *Trametes hirsuta* MTCC-1171 and its application in synthesis of aromatic aldehydes

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ABSTRACT

A yellow laccase from the culture filtrate of *Trametes hirsuta* MTCC-1171 has been purified. The purification methods involved concentration of the culture filtrate by ammonium sulphate precipitation and an anion exchange chromatography on diethylaminoethyl cellulose. The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and native polyacrylamide gel electrophoresis gave single protein band indicating that the enzyme preparation was pure. The molecular mass of the enzyme determined from SDS-PAGE analysis was 55.0 kDa. Using 2,6-dimethoxyphenol, 2,2′[azino-bis-(3-ethylbonzthiazoline-6-sulphonic acid) diammonium salt] and 3,5-dimethoxy-4-hydroxybenzaldehyde azine as the substrates, the K_m, k_{cat} and k_{cat}/K_m values of the laccase were found to be 420 μM, 13.04 s^{-1}, 3.11×10^4 M^{-1} s^{-1}, 225 μM, 13.03 s^{-1}, 1.3×10^5 M^{-1} s^{-1} and 100 μM, 13.04 s^{-1}, 5.8×10^4 M^{-1} s^{-1}, respectively. The pH and temperature optima were 4.5 and 60 °C, respectively while pH and temperature stabilities were pH 4.5 and 50 °C. The activation energy for thermal denaturation of the enzyme was 18.6 kJ/mol/K. The purified laccase has yellow colour and does not show absorption band around 610 nm like blue laccases. The purified laccase transforms toluene, 3-nitrotoluene, 4-nitrotoluene, 3-chlorotoluene, 4-chlorotoluene and 3,4-dimethoxytoluene to benzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde and 3,4-dimethoxybenzaldehyde in the absence of mediator molecules in high yields.

1. Introduction

Laccase [benzenediol: oxygen oxidoreductase; E.C. 1.10.3.2] is a polyphenol oxidase, which belongs to the superfamily of multicopper oxidases and catalyzes the four electron reduction of molecular oxygen to water [1–5]. Laccases are dimeric or tetrameric glycoproteins. To perform their catalytic functions, laccases depend on Cu atoms that are distributed at the three different copper centres viz. type-1 or blue copper centre, type-2 or normal copper centre and type-3 or coupled binuclear copper centres, differing in their characteristics electronic paramagnetic resonance (EPR) signals [6]. The organic substrate is oxidized by one electron at the active site of the laccase generating a reaction radical which further reacts non-enzymatically. The electron is received at type 1 Cu and is shuttled to the trinuclear cluster where oxygen is reduced to water.

More than 100 fungal laccases have been purified and characterized. Their different physiochemical properties like temperature optima, pH optima, K_m and molecular masses for different substrates have also been studied [4]. Molecular masses of fungal lccases have been found in the range of 43–383 kDa [4]. Most of the studies reported so far are on blue laccases. Yellow or white laccases have rarely been studied [7,8]. Yellow or white laccases differ from blue laccases in two aspects. Blue laccases have the absorption band around 610 nm [7] while yellow or white laccases lack it. Yellow or white laccases oxidize non-phenolic substrates in the absence of mediator molecules [8], which are required in the case of blue laccases. Thus, yellow or white laccases are better biocatalysts than blue laccases. Ortho and para diphenols, aminophenols, polyphenols, polyamines, lignins, and arylamines and some of the

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inorganic ions are the substrates for laccases. The ability of laccases to catalyze the oxidation of various phenolic as well as non-phenolic compounds, coupled to the reduction of molecular oxygen to water makes it valuable from the point of view of their different applications [4,9–12]. The biotechnological importance of laccases have increased after the discovery that oxidizable reaction substrate range could be further extended in the presence of small readily oxidizable molecules called mediators [13,14]. During the last two decades, laccases have turned out to be the most promising enzymes for industrial uses [11,12] having applications in food, pulps, paper, textile and cosmetic industries and in synthetic organic chemistry like selective oxidation of aromatic methyl group, novel penicillins synthesis, coupling of amines, oxidation of amides etc. [9,15–18].

Laccases purified from different sources exhibit different properties and are suitable for different applications. Keeping these points in view, authors have initiated studies on purification, characterization and biotechnological applications of laccases from different sources. It is already reported in the literature that some fungal strains which secrete blue laccases in submerged liquid cultures, secrete yellow laccases in the presence of culture media containing natural lignin substrates [7,8,11,19–21]. Moreover, blue laccases transform toluene to benzaldehyde in the presence of mediator molecules [22,23] whereas some of the yellow laccases perform same reactions in the absence of mediator molecules [24]. Only conversion of toluene to benzaldehyde was done previously [24]. Thus, the objective of this communication was to purify and characterize a novel yellow laccase from *T. hirsuta* MTCC-1171 and to demonstrate the conversion of toluene, 3-nitrotoluene, 4-nitrotoluene, 3-chlorotoluene, 4-chlorotoluene and 3,4-dimethoxytoluene to benzaldehyde, 3-nitrobenzaldehyde, 4-nitrobenzaldehyde, 3-chlorobenzaldehyde, 4-chlorobenzaldehyde and 3,4-dimethoxybenzaldehyde in the absence of mediator molecules.

2. Materials and methods

2.1. Materials

3,5-Dimethoxy-4-hydroxybenzaldehyde azine (syringaldazine), 3-chlorotoluene, 4-chlorotoluene and diethyl amino ethyl (DEAE) cellulose were from Sigma Chemical Company, St. Louis (USA). 2,2′-Azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) diammonium salt (ABTS) and 2,6-dimethoxy phenol (DMP) were from Fluka, Chemi new Ulm (Switzerland). All other chemicals used in these investigations were either from Himedia laboratory Ltd., Mumbai (India) or from E. Merck Ltd., Mumbai (India) and were used without further purifications. The chemicals used in the gel electrophoresis of the protein samples were from Bangalore Geni Pvt. Ltd., Bangalore, India. DEAE Cellulose column used in purification of enzyme was from Pharmacia, Upssala, Sweden.

2.2. The fungal strain and its growth

The fungal strain was procured from the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh, India and was maintained on agar slant as reported in MTCC Catalogue of strains-2000 [25]. The growth medium for the fungal strain *T. hirsuta* MTCC-1171 consisted of malt extract 20.0 g, and agar 20.0 g in 1.0 L Milli-Q water. The pH of the growth medium was adjusted to 6.5.

In order to detect the extracellular secretion of laccase by *T. hirsuta*, the liquid culture growth medium reported by Coll et al. [26] was used. This medium consisted of glucose 10.0 g, asparagine 1.0 g, yeast extract 0.5 g, $MgSO_4{\cdot}7H_2O$ and $FeSO_4{\cdot}7H_2O$, 0.01 g in 1.0 L of Milli-Q water. This liquid culture growth medium containing natural lignin substrates like coir dust, corn cob, wheat straw, saw dust and bagasse particles were separately prepared by adding 0.5 g of one of the natural lignin substrates to 25 mL of growth medium in 100 mL culture flasks which were sterilized. The sterilized growth media were inoculated with small pieces of mycelia (0.5 cm × 0.5 cm) under aseptic condition and the fungal cultures were grown under stationary culture conditions at 25 °C in a biological oxygen demand (BOD) incubator. In order to monitor the production of laccase in the liquid culture medium, 0.5 mL aliquots of the growth medium were withdrawn at the regular intervals of 24 h and filtered through sterilized Millipore filter 0.22 μm. The filtered extract was analyzed for the activity of laccase using DMP as substrate by the method [26] given in assay section. Extracellular secretion of laccase in the liquid culture medium by *T. hirsuta* MTCC-1171 was determined by plotting enzyme unit/mL of the growth medium against the number of days after inoculation of the fungal mycelia. Each point on the curve is an average of three measurements. The growth medium for control experiment has the same composition except that no natural lignolytic substrate has been added. In order to optimize the conditions for maximum production of laccase by *T. hirsuta* MTCC-1171 in the liquid culture medium, the amount of the best inducer wheat-straw particles were varied from 100 mg to 1000 mg in 25 mL of the growth medium. The amount of inducer in the growth medium which gave the maximum height of the enzyme activity peak was taken as the optimal amount of the inducer.

2.3. Enzyme assay

The assay solution using DMP as the substrate [26] contained 1.0 mM DMP in 50 mM sodium malonate buffer (pH 4.5) at 37 °C, using ABTS as the substrate [27] contained 0.5 mM ABTS in 0.1 M sodium acetate buffer (pH 5.0) at 25 °C and using syringaldazine as the substrate [28] contained 0.1 mM syringaldazine in 50 mM sodium phosphate buffer (pH 6.0) at 50 °C. In case of DMP, the reaction was monitored by measuring the absorbance change at $\lambda = 468$ nm ($\varepsilon = 49.6 \times 10^3\ M^{-1}\ cm^{-1}$) while in case of ABTS and syringaldazine, the reactions were monitored by measuring the absorbance change at $\lambda = 420$ nm ($\varepsilon = 36.0 \times 10^3\ M^{-1}\ cm^{-1}$) [27] and $\lambda = 530$ nm ($\varepsilon = 65.0\ mM^{-1}\ cm^{-1}$) [28], respectively. The UV/Vis spectrophotometer Hitachi (Japan) model U-2900 fitted with electronic temperature control unit was used for absorbance measurement. The least count of absorbance measurement was 0.001 absorbance unit. One enzyme unit produced 1 μmol of the product per minute under the specified assay conditions.

2.4. Purification of laccase

For the purification of laccase, *T. hirsuta* MTCC-1171 was grown in ten 100 mL culture flasks each containing 25 mL sterilized growth medium with optimal amount 600 mg of the inducer, wheat-straw particles, under stationary culture condition in a BOD incubator at 30 °C. The maximum activity of laccase appeared on 7th day of the inoculation of fungal mycelia. On 7th day, all the cultures in 10 flasks were pooled, mycelia were removed by filtration through four layers of cheese cloth. The culture filtrate was saturated up to 30% with ammonium sulphate and centrifuged using refrigerated centrifuge Sigma (Germany) model 3K-30 at 5480 × *g* for 20 min at 4 °C. The precipitate was discarded and the supernatant was saturated up to 90% by further addition of ammonium sulphate. The resulting suspension was centrifuged by repeating the same process of centrifugation and the supernatant was discarded. The precipitate was dissolved in 100 mM sodium acetate buffer pH 4.5