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RESEARCH PAPER

Purification and Characterization of Extracellular Laccase Secreted by *Pleurotus sajor-caju* MTCC 141

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Abstract: The effect of lignin containing natural substrates corn-cob, coir-dust, saw-dust, wheat straw, and bagasse particles on the extracellular secretion of laccase in the liquid culture growth medium of *Pleurotus sajor-caju* MTCC 141 has been studied. The culture conditions for maximum secretion of laccase by *Pleurotus sajor-caju* MTCC 141 have been optimized. Homogeneous preparation of laccase from the culture filtrate of the fungus has been achieved using ammonium sulphate precipitation, anion exchange chromatography on DEAE, and gel filtration chromatography on Sephadex G-100. The purified enzyme preparation gave a single protein band in SDS-PAGE analysis indicating a molecular weight of 90 kD. The enzymatic characteristics K_m, K_{cat}, pH, and temperature optima of the purified laccase have been determined using 2, 6-dimethoxyphenol as the substrate and have been found to be 35 μM, 0.30 min^{-1}, 4.5 and 37°C, respectively. The K_m values for the other substrate like catechol, m-cresol, pyrogallol, and syringaldazine have also been determined, which were found to be 216 μM, 380 μM, 370 μM, and 260 μM, respectively.

Keywords: laccase; *Pleurotus sajor-caju*; lignolytic enzymes; metalloenzymes; Cu-enzymes; lignolytic fungi

Introduction

Laccases [E.C.1.10.3.2] are multicopper enzymes belonging to the group of blue oxidases[1−3]. They catalyse the oxidation of a variety of phenolic compounds with concomitant reduction of molecular oxygen to water. A general reaction scheme has been proposed as:

$$4RH+O_2 \longrightarrow 4R+2H_2O$$

Since laccase recycles on molecular oxygen as an electron acceptor and does not require any other co-substrate, it is the most promising enzyme of oxidoreductases group for industrial applications[3−5]. 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[6,7]. Lu and Xia[8] have reviewed the applications of laccase mediated systems, which comprise of pulp bleaching, textile biofinishing, and environmental protection processes. During the past two decades, laccases have turned out to be most promising enzymes for industrial uses[4,5] having applications in food, pulp and paper, textile, cosmetics industries, and in synthetic organic chemistry[9]. Different applications will require different laccases with properties more suited to those applications. Keeping these points in view, the authors have initiated studies on purification and characterization of laccases from different fungal sources[10]. In this communication, we reported the purification and characterization of a laccase from a white-rot indigenous fungus *Pleurotus sajor-caju* MTCC 141.

1 Materials and methods

Syringaldazine (4-hydroxy-3, 5-dimethoxybenaldehydeazine), DEAE Cellulose, and Sephadex G-100 were from Sigma Chemical Company, St. Louis USA. DMP (2, 6-dimethoxy phenol) was from Fluka, Chemie, New Ulm, Switzerland.

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The chemicals used in the gel electrophoresis of protein samples were from Geni Pvt. Ltd. Bangalore. All other chemicals used in these investigations were either from Himedia laboratory Ltd, Mumbai or from E. Merck (India) Ltd. Werli Road Mumbai and were used without further purifications.

The fungal strain was procured from the Microbial Type Culture Collection Center and Gene Bank, Institute of Microbial Technology, Chandigarh and was maintained on agar slant as reported in MTCC Catalogue of strains-2000[11]. The growth medium for the preparation of slant of fungal strain *Pleurotus sajor-caju* MTCC 141 consisted of malt extract 20.0 g and agar 20.0 g in 1.0 L Milli-Q water, and pH of the medium was maintained at 6.5.

The liquid culture growth medium reported by Coll *et al*[12] was used for screening the fungal strain for the production of extracellular laccase. 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. The liquid culture medium containing natural lignin substrates coir dust, corn cob, wheat straw and saw dust, and bagasse particle were separately prepared by adding 0.5 g of one of the natural lignin substrates to 25 mL of the above mentioned growth medium in 100 mL culture flasks, which were sterilized. The sterilized growth medium was inoculated with small piece of mycelium (0.5 cm × 0.5 cm) under aseptic condition, and the fungal culture was grown under stationary culture condition at 30°C in a 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 a regular intervals of 24 h and filtered through sterilized millipore filter 0.22 μm and were assayed for the activity of laccase using the following method.

1.1 Assay method

The filtered extract was analyzed for the activity of laccase using DMP and syringaldazine as the substrates. The assay solution 1.0 mL for DMP as the substrate contained 1 mM 2,6-Dimethoxy phenol (DMP) in 50 mM sodium malonate buffer pH 4.5 and 37°C and for syringaldazine as the substrate 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 λ= 468 nm and using the molar extinction coefficient value of 49.6 mM/cm. In case of syringaldazine, the reaction was monitored by measuring the absorbance change at λ= 530 nm and using molar extinction coefficient value of 64.0 mM/cm. The UV/Vis spectrophotometer Hitachi (Japan) model U-2000 was used for absorbance measurement, which was fitted with electronic temperature control unit. The least count of absorbance measurement was 0.001 unit. One enzyme unit produced 1 μmole of the product per minute under the specified assay condition.

1.2 Secretion of laccase and optimization of the condition

Extracellular secretion of laccase in the liquid culture medium by *Pleurotus sajor-caju* MTCC 141 was determined by plotting the enzyme unit/mL of the growth medium against the number of days after inoculation of the fungal mycelium. Each point on the curve is an average of three measurements. The growth medium for the control experiment has the same composition as other except that no natural lignolytic substrate has been added. The best inducer of the laccase was the natural lignin containing substrate addition, which gave the maximum enzyme unit/mL. In order to optimize the conditions for maximum secretion of laccase by *Pleurotus sajor-caju* MTCC 141 in the liquid culture medium, the amount of the best inducer (bagasse in this case) was varied from 100 mg to 1000 mg in 25 mL of the growth medium. In this case, the enzyme unit/mL of the growth medium was also plotted against the number of days after the inoculation of the fungal strain. The amount of the inducer in the growth medium, which gave the maximum height of the enzyme activity peak, was taken as the optimal amount of the inducer.

1.3 Purification of laccase

For the purification of laccase, *Pleurotus sajor-caju* MTCC 141 was grown in 8 × 25 mL sterilized growth medium in eight 100 mL culture flasks containing optimal amount of the inducer (bagasse in this case) under stationary culture condition in a BOD incubator at 30°C. The maximum activity of laccase appeared on 9th day of the inoculation of the fungal mycelia. On the 9th day, all the cultures in the 8 flasks were pooled, mycelia was 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 12 500 rpm for 25 min, at 4°C. The precipitate was discarded and the supernatant was saturated up to 80% 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 1.0 M Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0) and dialyzed against 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0) in volume ratio 1:1000 with three changes at the intervals of 8 h. The dialyzed enzyme sample 15 mL containing 0.515 mg/mL protein was loaded on to a DEAE column (size 1.0 cm × 37 cm), which was equilibrated with 10 mM Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0). The enzyme was eluted by application of linear gradient of 1.0 M NaCl in the same buffer (50 mL buffer+50 mL buffer with 1.0 M NaCl). Fractions of 6.3 mL size were collected and analyzed for laccase activity and protein concentration. The active laccase fractions were pooled and concentrated using Amicon concentration cell with YM-10 ultrafiltration