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研究报告

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 μmol/L, 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 μmol/L, 380 μmol/L, 370 μmol/L and 260 μmol/L 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 last 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. The chemicals used in the gel electrophoresis of protein samples were from Geni Pvt. Ltd. Banglore. 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

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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 agar 20.0 g in 1.0 L Milli-Q water and pH of the medium was maintaining 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 mmol/L 2, 6-Dimethoxy phenol (DMP) in 50 mmol/L sodium malonate buffer pH 4.5 at 37°C and for syringaldazine as the substrate contained 0.1 mmol/L syringaldazine in 50 mmol/L 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 mmol/L/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 mmol/L/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 of 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 also the enzyme unit/ml of the growth medium was 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 r/min 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 mol/L Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0) and dialyzed against 10 mmol/L Na_2HPO_4 / NaH_2PO_4 buffer (pH 7.0) in volume ratio 1:1000 with three changes at the intervals of 8 hrs. 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 mmol/L Na_2HPO_4/NaH_2PO_4 buffer (pH 7.0). The enzyme was eluted by application of linear gradient of 1.0 mol/L NaCl in the same buffer (50 mL buffer + 50 mL buffer with 1.0 mol/L 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 membrane having molecular weight cut off value 30.0 kD.

The concentrated enzyme (6.0 mL) containing 0.309 mg/mL protein was loaded on to a Sephadex G-100 column (size 1.5 cm × 67 cm) which was equilibrated with 10 mmol/L Na_2HPO_4 /NaH_2PO_4 buffer (pH 7.0). The enzyme was eluted using the same buffer at a flow rate 9.0 mL/h. Fractions of 4.5 mL size were collected and analyzed for laccase activity and protein concentration. The active laccase fraction were pooled and concentrated using Amicon concentration cell with YM-10 ultrafiltration membrane as described above. Protein estimation was done by Lowry method[13].

1.4 SDS-polyacramide gel electrophoresis

The homogeneity of the enzyme preparation was checked by SDS-PAGE using the method of Weber and Osborn[14]. The separating gel was 12% acrylamide in 0.375 mol/L Tris·HCl buffer pH 8.8 and stacking gel was 5% acrylamide in 0.063 mol/L Tris·HCl buffer 6.8. The molecular weight marker were phosphorylase (97.4 kD), Bovine serum albumin (68 kD), Ovalbumin (43 kD), Carbonic anhydrase (29 kD), and Lysozyme (14.3 kD), and were procured from Bangalore Genei Pvt. Ltd. (Bangalore India) Gel was run at a constant current 20 mA[15].

2 Results and discussion

Fig. 1 shows the secretion of laccase in the liquid culture growth medium amended with various lignin containing natural substrates corn-cob, coir-dust, saw-dust, wheat straw and bagasse particles and inoculated with *Pleurotus sajor-caju* MTCC 141. It is obvious from the figure that lignin containing natural substrates when present in the liquid culture growth medium enhanced the secretion of the laccase. This enhancement of the extracellular laccase is maximum in the case of the growth medium containing bagasse particles.

Fig. 2 shows the results of optimization of culture conditions for maximum secretion of laccase by *Pleurotus sajor-caju* MTCC 141. It is obvious from the figure that maximum laccase production occurs in the liquid culture growth medium containing 800 mg of bagasse particles in a culture volume of 25 mL at 30°C under stationary culture condition. The maximum level of the laccase produced in the culture medium was 0.32 IU/mL.

The purification chart for the purification of the laccase from the liquid culture filtrate of *Pleurotus*

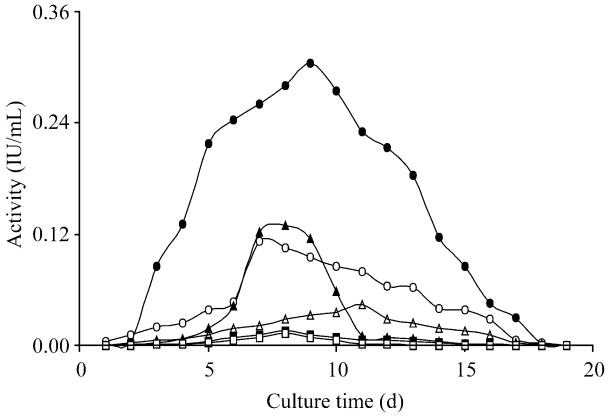

Fig. 1 Secretion of laccase by *Pleurotus sajor-caju* MTCC 141 in the liquid culture medium supplemented with different natural lignin containing substrate

(●) bagasse; (○) coir dust; (▲) corn cob; (△) wheat straw; (■) saw dust; (□) control

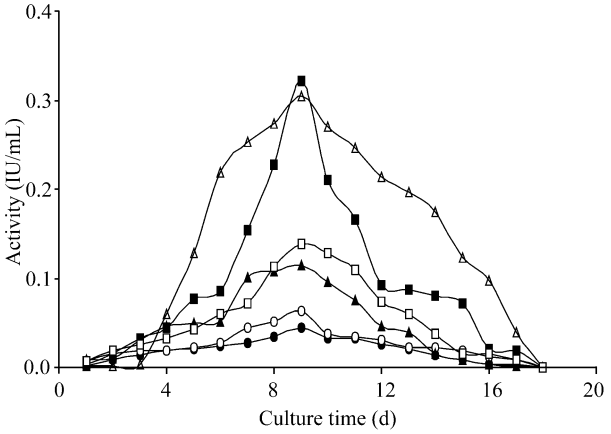

Fig. 2 Optimization of laccase *Pleurotus sajor-caju* MTCC 141 in the liquid culture medium supplemented with different concentration of bagasse particles

(●) 100 mg; (○) 200 mg; (▲) 400 mg; (△) 500 mg; (■) 800 mg; (□) 1000 mg

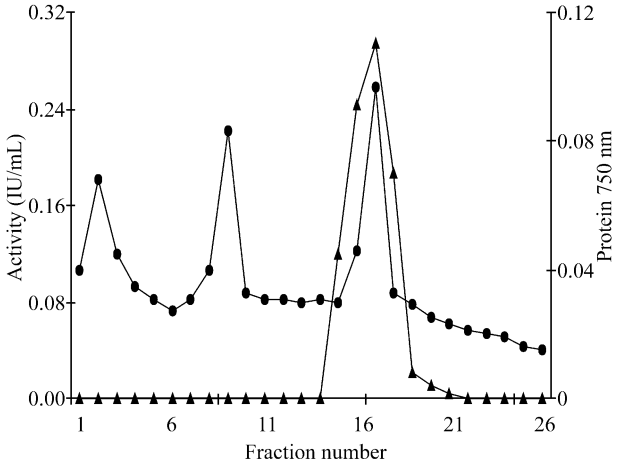

Fig. 3 Elution profile from Sephadex G-100 column

(▲) activity; (●) protein

sajor-caju MTCC 141 is shown in Table 1. The method gave 10.71-fold purification with 3.46% recovery of the enzyme activity. The elution profile of the laccase activity from Sephadex G-100 column is shown in Fig. 3. A single activity peak on gel filtration column chromatography coinciding with a protein peak indicates that the enzyme is relatively pure.

The results of SDS-PAGE are shown in Fig. 4. The presence of single band in lane 2 clearly shows that the enzyme preparation is pure and the determined relative molecular weight is 90.0 kD. The relative molecular masses of the laccases purified from the culture filtrates of *Ganoderma lucidium, Chaetomium thermophilium, Neurospora crassa*, and *Gaeumannomyces graminis* var. *tritici* are 68 kD, 77 kD, 64.8 kD and 60 kD, respectively[16–20]. It is worth mentioning that Soden and Doson[21] have also reported laccase from a different strain of *Pleurotus sajor-caju* but the molecular weight of the laccase reported by them is 59 kD. The difference in the molecular weight of the two laccases from the same species may be due to different strains.

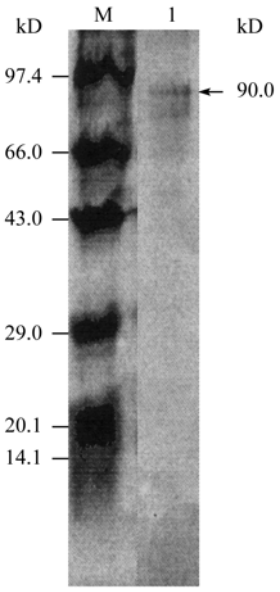

Fig. 4 SDS-polyacrelamide Gel-electrophoresis
M: protein molecular weight marker; 1: purified laccase

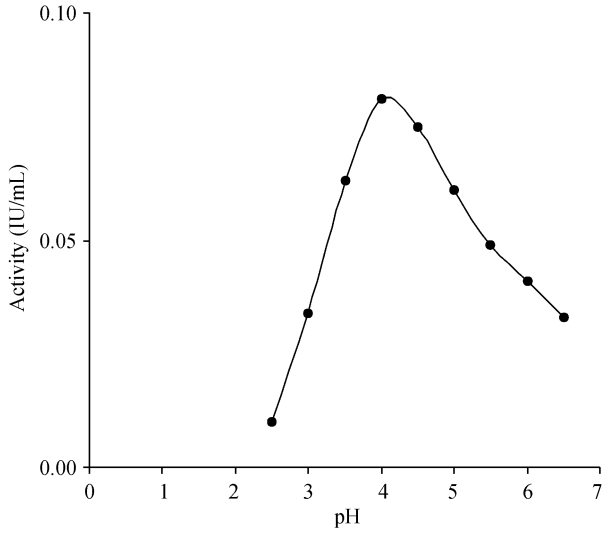

Fig. 5 Variation of activity of laccase of *Pleurotus sajor-caju* MTCC 141 with variation of reaction pH

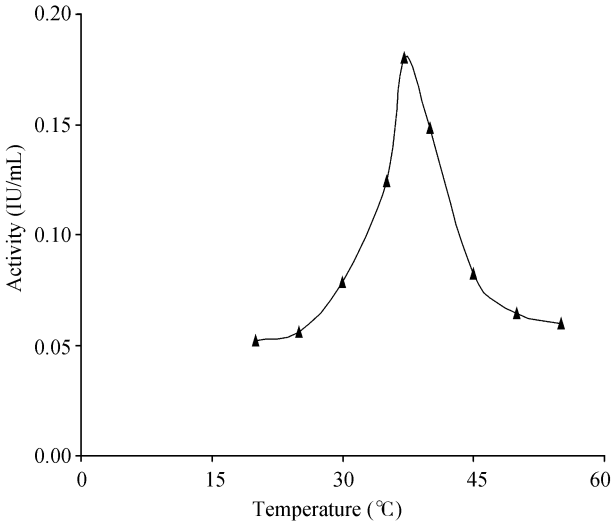

Fig. 6 Variation of activity of laccase of *Pleurotus sajor-caju* MTCC 141 with variation of reaction temperature

Table 1 Purification chart

Steps	Volume (mL)	Activity (IU/mL)	Protein (mg/mL)	Total activity	Total protein	Specific activity	Purification fold	Yield (%)
Crude	800	0.083	0.255	66.400	204.00	0.325	1.000	100.000
Ammonium sulphate	10	0.713	1.245	7.300	12.45	0.572	1.760	10.730
DEAE	6	6.540	0.309	3.920	2.33	1.682	5.176	5.903
Gel filtration	3	0.766	0.220	2.298	0.66	3.481	10.710	3.460

The double reciprocal plot using 2,6-dimethoxyphenol, catechol, m-cresol, pyrogallol and syringaldazine as the variable substrates were done. The calculated K_m values for the above substrates were 35 μmol/L, 216 μmol/L, 380 μmol/L, 370 μmol/L, and 260 μmol/L respectively. The K_m values for the laccase purified from *G. graminis var. tritici* using 2, 6-dimethoxyphenol, catechol and pyrogallol are 26 μmol/L, 250 μmol/L and 310 μmol/L respectively[20]. Thus the purified laccase from *Pleurotus sajor-caju* MTCC 141 has very low affinity for 2,6-dimethoxyphenol as compared to the laccase purified from *G. graminis var.* tritici where as the affinities of the both laccases for catechol and pyrogallol are of the same order of magnitude. Soden and Dobson[21] have reported a K_m value 0.12 μmol/L using 2,6-dimethoxyphenol as the substrate for the laccase of an other strain of *Pleurotus sajor-caju*. Thus the laccase purified from *Pleurotus sajor-caju* MTCC 141 has better affinity for 2,6-dimethoxyphenol as compared to the laccase reported by Soden and Dobson[21]. The k_{cat} value using 2,6-dimethoxyphenol as the substrate for the laccase of *Pleurotus sajor-caju* MTCC 141 is 0.30 min^{-1} which is higher than the value of k_{cat} 0.00058 min^{-1} reported by Soden and Dobson[21] using the same substrate for the laccase of another strain of *Pleurotus sajor-caju*.

The variation of the activity of the laccase with the variation of pH of the reaction medium is shown in Fig. 5, using 2,6-dimethoxyphenol as the substrate. It is obvious from the fig that the pH optimum of the purified laccase is 4.5 but the enzyme retains more than 50% activity in the pH range 4–6. The pH optima of the laccase purified from *G. lucidium*, *C. thermophilium* and *G. graminis* var. *tritici* reported in the literature are 3.5, 6.0 and 4.5 respectively[16, 20, 22]. Soden and Dobson[21] have reported that the pH optimum of the laccase depends on the substrate used. Though we have not performed such studies but the pH optimum reported by Soden and Dobson[21] for the laccase using 2,6 dimethoxyphenol of their *Pleurotus sajor-caju* strain is 6.0 as compared to 4.5 found in case of the laccase of *Pleurotus sajor-caju* MTCC 141.

The variation of activity of the laccase purified from the culture filtrate of *Pleurotus sajor-caju* MTCC 141 with temperature is shown in Fig.6. The activity is maximum at 37°C and the enzyme has more than 50% of its maximum activity at temperature above 40°C. The temperature optimum of the laccases purified from the culture filtrates of *Ganoderma lucidium*, *Pleurotus eryngii*, *Daedalea quercina* and *Streptomyces cyaneus* CECT-3335 are 40°C, 65°C, 60°C, and 70°C, respectively[22–25]. Thus the temperature optimum of the laccase purified from *Pleurotus sajor-caju* MTCC 141 is lower than the temperature optima reported for the laccases of the other fungal strains.

This communication reports a laccase for which purification procedure is relatively simpler and the enzymatic characteristics of the laccase are different from the laccases reported from other fungal strains.

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《生物工程学报》对摘要的写作要求

1. 研究报告摘要：基本要素包括研究目的、方法、结果和结论（不用单列标题书写）。目的(Purpose)：主要说明作者写此文章的目的，或说明本文主要要解决的问题；方法(Methods)：重点说明作者的主要工作过程及使用的方法。应用性文章如需要，可注明条件、使用的主要设备和仪器。结果(Results)：本文最后得出的结果（实验数据部分）。结论(Conclusions)：如系基础研究，应写明本文的创新之处，及文章在讨论部分表述的观点；如系应用性研究，应尽可能提及本文结果和结论的应用范围和应用情况或应用前景。

2. 综述摘要：包括论述内容的发展水平、自己的评论及展望，尤其要注意结合自己的研究工作。

3. 英文摘要的撰写要点：英文摘要的内容应与中文摘要一致，但比中文摘要更详尽。英文摘要完成后，务必请英文较好、且专业知识强的专家审阅定稿后再返回编辑部。

凡不符合要求的，即使学术上可以达到刊出的水平，本刊也将推迟发表。

(1) 建议使用第一人称，尽量不使用第三人称和被动语态。

(2) 建议用主动语态，被动语态表达拖拉模糊尽量不用，这样可以免好多长句，以求简单清晰。

(3) 尽量使用清晰简练的短句，避免很长的句子。注意正确使用英文写作习惯和语法。

(4) 摘要应当使用过去时态，语法正确，句子通顺。

(5) 摘要中避免使用缩写语，除非是那些人人皆知的（如DNA、ATP等），或者确实是非常长，而且出现多次的短语才允许用缩写语，并且在第一次出现时要写出全称。

(6) 在英语摘要中，不要使用任何汉字字符，包括标点、括号、温度、希腊字母等。

(7) 句子的开头处最好不要使用数字。