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Partial Purification of Laccase Enzyme from a newly Isolated *Lenzites sp.* Fungus and its use in Decolorization of Textile Dye

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ABSTRACT

A basidiomycetous fungus *Lenzites sp.* isolated from a live jujube (*Zizyphus mauritiana*) tree from the suburbs of Mumbai, with significant laccase activity could rapidly decolorize reactive blue 160 textile dye. The laccase produced in the culture medium was16.88 U/ml. Partial purification of laccase was performed by precipitating the enzyme with 70% ammonium sulphate saturation, followed by dialysis and gel filtration chromatography using Sephadex G-75. The presence of two laccase isoenzymes was detected with molecular weights of 97.4 kD and 70.794 kD respectively.

KEYWORDS : *Lenzites sp.*, Laccases, isoenzymes, Sephadex-G75.

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INTRODUCTION

Laccase (EC 1.10.3.2) is a multicopper blue oxidase that couples the four e^- reduction of oxygen with the oxidation of a broad range of organic substrates, including phenols, polyphenols, anilines, and even certain inorganic compounds by a one-e^- transfer mechanism[1]. Laccases can be roughly divided into two major groups with clear differences, i.e. those from higher plants and those from fungi[2]. The presence of laccase have been reported in bacteria[3] as well as in insects[4]. Laccases occur widely in fungi as constitutive and inducible enzymes[5]. White-rot basidiomycetes are microorganisms able to degrade lignin efficiently. However, the degree of lignin degradation with respect to other wood components largely depends on the environmental conditions and the fungal species involved. The ability of laccase producing microorganisms or purified laccases to eliminate a wide range of pollutants is currently one of the most interesting subjects for researchers in environmental biotechnology[6]. It is known that 90% of reactive textile dyes entering activated sludge sewage treatment plants will pass through unchanged and will be discharged to rivers [7]. Not all dyes currently used are degraded or removed with physical and chemical processes, and sometimes the degradation products are more toxic [8].

Therefore, the objective of the present work was to isolate laccase producing fungi from nature and identify new laccase producers. Here fungal laccase produced in the culture medium was partially purified and its ability to decolorize reactive blue 160 dye was investigated.

MATERIALS AND METHODS

Sample collection

Naturally grown and sun dried fruiting body of wood decay fungi from live jujube(*Zizyphus mauritiana*) tree in suburbs of Mumbai was collected aseptically in polythene bag and transported to the laboratory for further study.

Dye

Textile dye reactive blue 160 was procured from local textile company.

Isolation of white rot fungi and its molecular identification

To obtain pure culture of fungal fruiting body, it was cut open with sterile scalpel and knife into peices. These pieces were surface sterilized with tween 80 for 10 mins, 0.01% mercuric chloride for 30s and 70% ethanol for 1 min. This tissue was then inoculated on malt extract agar plates and incubated at 30^0C for a week. The fungal isolate was molecularly identified as *Lenzites sp.* at Agarkar Research Institute, Pune, India by ITS sequencing.

Production of Laccase

The medium for laccase production had the following composition : 4.5% (wt/vol) wheat bran, 1.5% yeast extract, 1% maltose, 0.75% meat extract, 0.124% ammonium sulphate $(NH_4)_2SO_4$, 0.1% potassium phosphate (KH_2PO_4), 0.05% thiamine dichloride, 0.05% $MgSO_4.7H_2O$, 0.01% $CaCl_2$, 0.05% KCl, 1mM 2,6-xylidine and 1mM $CuSO_4$. The pH was adjusted to 5.0. Incubation was carried out at 30°C (± 4 °C) on a rotary shaker (150 rpm) in 250 ml Erlenmeyer flask containing 100 ml of media, inoculated with one agar disc taken from active borders of MEA fungal cultures. Cultures were harvested on 7th day, filtered through whattman filter paper to remove the mycelia and centrifuged at 8,000 x g for 20 min and the enzyme activity determined.

Assay of extracellular laccase activity

Laccase activity was determined using 2, 6 - dimethoxyphenol (DMOP) as a substrate. The reaction mixture contained 1 mM DMOP in 50 mM sodium malonate (pH 4.5). The formation of 2, 2',6, 6' dimethoxyphenoquinone (orange /brown color) at 30°C was followed spectrophotometrically at 468 nm wavelength. Laccase activity was calculated from the molar extinction co-efficient (ε) of 49.6 mM cm^{-1} [9]. One unit of laccase activity was defined as activity of an enzyme that catalyzes the conversion of 1 μmole of DMOP per minute [10]. All values are mean of triplicates.

Partial Purification of laccase

The culture filtrate was fractionated in a sequence saturation of ammonium sulphate from 0-20%, 20-40%, 40-60%, 60-80% and 80-100% saturations. The ammonium sulphate precipitate was dissolved in 1M Na_2HPO_4/NaH_2PO_4 buffer (pH 5.0) and dialyzed against 10 mM Na_2HPO_4 / NaH_2PO_4 buffer (pH 5.0) in volume ratio 1:1000 with three changes at the intervals of 8 hrs. The dialyzed

enzyme sample 6 mL containing 0.311 mg/mL protein was loaded on to a Sephadex G-75 column (size 1 cm x 20 cm) which was equilibrated with 10 mM Na_2HPO_4 /NaH_2PO_4 buffer (pH 5.0). The enzyme was eluted using the same buffer at a flow rate 9.0 mL/h [11]. Fractions of 2 mL were collected and analyzed for laccase activity and protein concentration. Protein estimation was done by Lowry method [12] with crystalline bovine serum albumin as the standard.

SDS Poly Acrylamide Gel Electrophoresis of laccase enzyme

Molecular weight of partially purified laccase was determined by SDS-PAGE (12% polyacrylamide gels) using the method of Weber and Osborn [13]. The molecular weight markers were procured from Bangalore Genei Pvt. Ltd. (Bangalore India) Gel was run at a constant current 20 mA [14].

Activity staining

The partially purified laccase was then analysed by Native PAGE using 12% gels. After electrophoresis, the slab gels were subjected to the activity staining for laccase. The fixed slab gels were then stained by 300 mM ABTS solution for 5 min for color development [15].

Enzymatic treatment of reactive blue Textile dye

4 ml test-tubes containing 100mg/L reactive blue 160 dye and 4 U of laccase ml^{-1} (10 mM Na_2HPO_4 /NaH_2PO_4 buffer (pH 5.0)) were incubated on a rotary shaker at 30°C for 1 hr. Heat-inactivated enzymes were used as control, and decolorization was measured spectrophotometrically at 616 nm. The decolorization percentage was calculated using following equation :

$$\text{Decolorization (\%)} = \frac{A_0 - A_t}{A_0} \times 100$$

Where A_0 is an absorbance at λ_{max} of the control and A_t is the absorbance of the dye degraded by the active enzyme.

RESULTS AND DISCUSSION

In the present study, newly isolated strain *Lenzites sp.* had been found to produce 16.88 U/ml of laccase in the wheat bran medium in the presence of combination of inducers 2,6-xylidine and $CuSO_4$ on the 7^{th} day of incubation. Mycelia was removed from the culture filtrate by centrifugation at

8000xg for 20 mins and then subjected to ammonium sulphate precipitation. Most protein containing laccase activity was salted out at around 60-80% ammonium sulphate saturation (Table 1).

Table 1. Ammonium sulphate precipitation for laccase purification

S. NO	Ammonium Sulphate Precipitated Samples (%)	Laccase Activity (U/ml)
1	20-40%	2.029
2	40-60%	3.894
3	60-80%	23.190
4	80-100%	2.91

Table 2. Purification of laccase enzyme

S. NO	Purification Step	Total Volume (ml)	Protein (mg/ml)	Enzyme Activity (U/ml)	Total Activity	Specific Activity (U/mg)	Yield (%)	Purification factor (fold)
1	Culture Supernatant	140	0.344	16.88	2363.2	49.06	100	1.000
2	Ammonium Sulphate precipitate (70%)	11	0.508	25.22	277.42	49.64	11.73	1.011
3	Dialysis Bag	15	0.311	20.809	312.13	66.90	13.20	1.363
4	Sephadex G-75	2	0.171	23.190	46.38	135.61	1.96	2.764

Table 2 depicts the purification of laccase from the *Lenzites sp*. The enzyme activity, protein content, specific activity, percentage yield and purification fold have been presented here. The first ammonium sulphate precipitation helped to remove the pigments. Ammonium sulphate was removed after three

buffer changes during dialysis. The dialysate was further purified on Sephadex G-75 column and the elution profile is shown in Figure 1.

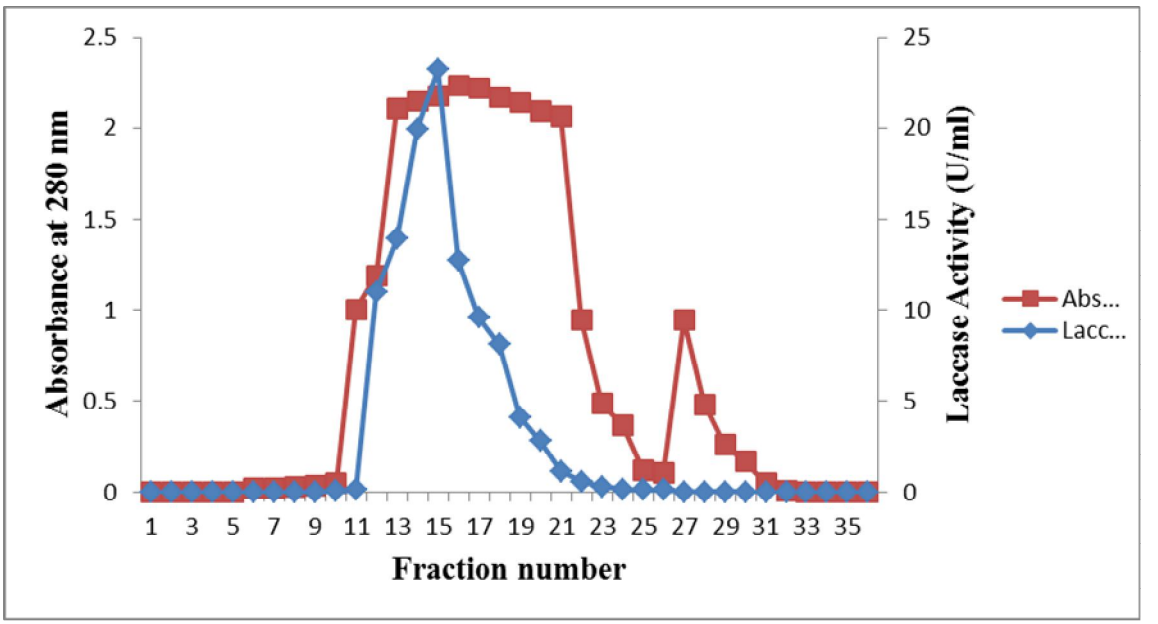

Figure 1. Elution profile of laccase from *Lenzites sp*. Based on Sephadex G-75 column.

It was observed that the enzyme started eluting from the 12th fraction onwards. Maximum laccase activity was noted in 14th and 15th fraction which gave 19 U/ml and 23 U/ml. The method gave 2.764 fold purification with 1.96% recovery of the enzyme activity. Laccase from *Cerrena sp.* HYB07 has been shown to be purified by ammonium sulphate (40-60%) followed by DEAE FF column and Phenyl FF chromatography with 1.8, 2.8 and 3.1-fold purification respectively [16]. Khammuang and Sarnthima purified laccase from fruiting bodies of *Ganoderma sp. MK05* with (40-70%) ammonium sulphate precipitation followed by DEAE cellulose chromatography and obtained 1.34 and 3.07 purification fold respectively[17]. Laccase from *Aspergillus sp.* was purified with ammonium sulphate precipitation (40-60%), followed by dialysis and sephadex G-100 gel filtration and obtained purification fold of 1.09, 1.56 and 2.04 respectively[18] (Table 3). In our study laccase from *Lenzites sp*. shows specific activity of 135.61 U/mg with 2,6-DMP as substrate. The specific activity however can vary with different substrates and assay conditions. The units of enzyme activity can also be defined differently. In spite of these differences, laccase from *Lenzites sp*. in our study shows a much higher specific activity as compared to laccase from other species reported. For example laccase from *Coriolopsis rigida* showed specific activity of 110 U/mg [19], and that from *Lachnocladium* sp showed

17.1 U/mg [20]. Laccase from Pycnoporus sanguineus CS-2 showed 69.8 U/mg [21], whereas that from *Coriolopsis floccosa* MTCC-1177 was reported to show 8.33 U/mg specific activity [22].

Table 3. Comparison of purification folds of each purification step and specific activities obtained for laccase

S.NO	Laccase	Purification steps	Purification fold	Specific activity
1	*Ganoderma sp. MK05* lacc [17]	1) Ammonium sulphate precipitation 40-70%) 2) DEAE cellulose chromatography	1.34 3.07	1.028 U/mg 2.349 U/mg
2	*Aspergillus sp.* lacc [18]	1) Ammonium sulphate precipitation (40-60 %) 2) Dialysis 3) Sephadex G-100 gel filtration	1.09 1.56 2.04	1.12 U/mg 1.62 U/mg 1.97 U/mg
3	*Coriolopsis rigida* lacc [19]	1) Ultrafiltration 2) Q-Cartridge 3) Mono-Q (LacI) 4) Mono-Q (LacII) 5) Mono-Q (Total)	1.1 1.8 2.5 2.5 2.5	49 U/mg 79 U/mg 108 U/mg 112 U/mg 110 U/mg

Activity staining of the enzyme on native-PAGE using ABTS showed presence of two laccase isoenzymes in Figure 2. The bands of protein exhibiting laccase activity stained green in a white background. Raghukumar et al.,[23] have shown presence of 2 isoenzymes with molecular masses 43 and 99kDa from *Flavodon Flavus*. It has been reported that several fungi have more than one laccase-encoding gene. Five laccase gene sequences from *Trametes sanguine* [24], three genes from *Pleurotus ostreatus* [25], six laccase genes from *Fusarium oxysporum* [26] and three laccase isoenzyme genes were identified from the basidiomycete *Trametes* sp. AH28-2 [27]. Thus, the biochemical diversity of laccase isoenzymes appears to be due to the multiplicity of laccase genes. Moreover, regulation of the expression of these genes is substantially different in different species [28].

The results of SDS-PAGE are shown in Figure 3, in which three bands of protein were observed. Then two laccase isoforms (Lcc1 and Lcc2) bands of molecular weight approximately 97.4 kDa and 70.794 kDa were observed in *Lenzites sp*. Molecular weights of most laccase isoforms have been reported to be in the molecular weight range of 60kD-100kD [29]. Almost all fungi that have been examined produce more than one isoform of laccase [30]. Laccase enzyme purified from *Trametes versicolor* by Han *et. al* showed molecular mass of approximately 97kDa [31].

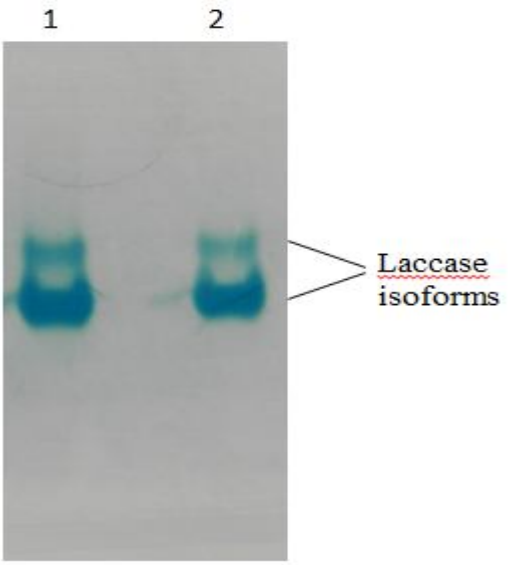

Figure 2. Native-PAGE (12% polyacrylamide gels) of *Lenzites sp*. Laccases after different purification steps.

Laccase activity was detected by staining the gel with ABTS solution (300mM). Lanes : 1, culture supernatant (344 µg of protein); 2, Sephadex G-75 gel filtration eluate (171 µg).

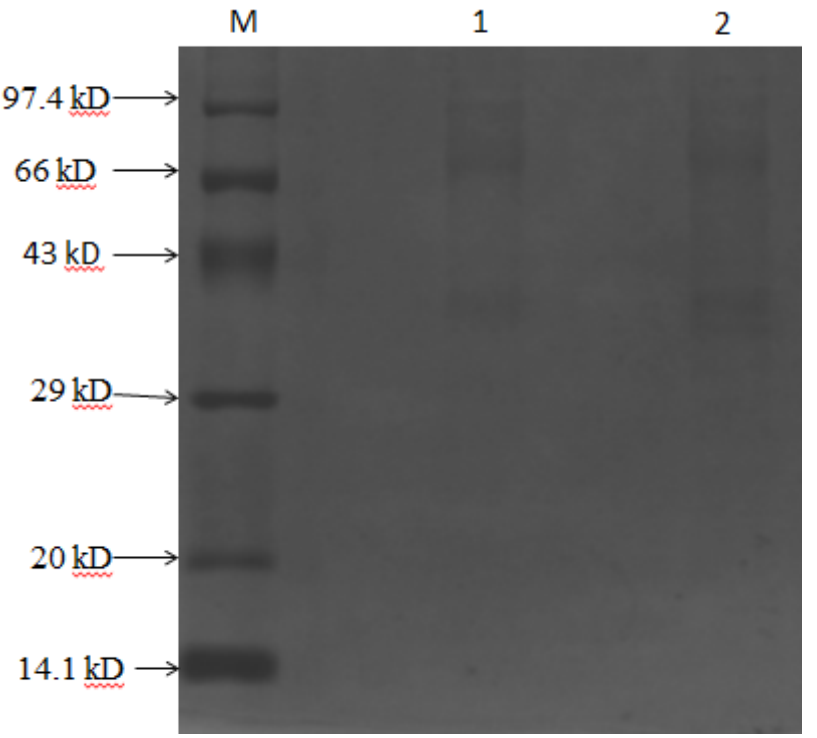

Figure 3. SDS-PAGE (12% polyacrylamide gels) of *Lenzites sp*. Laccases after different purification steps.

Protein was stained with Coomassie brilliant blue R-250. Lanes : M, protein molecular weight marker; 1 and 2, Sephadex G-75 gel filtration eluate 14 and 15 (tracks loaded with approximately 171 µg of protein).

The partially purified extracellular laccase was also investigated for its decolorization potential of a Reactive blue 160 dye, procured from the textile industry. This enzyme (4U/ml) could completely decolorize reactive blue 160 dye (100mg/L) in 30 mins of incubation. Eight bacterial strains along with co-metabolism of yeast extract (0.5%) in minimal medium have been reported to decolorize reactive blue 160 (100mg/L) within 4 hrs [32]. The decolorization potential of laccase enzymes depends on the species and also the strain from where the enzyme has been isolated. The nature of the dyes and the reaction conditions used for the decolorization process also plays a significant role in the rate of decolorization [33, 34, 35, 36, 37, 38] . Therefore the laccase isolated here has proved to be an eco-friendly solution for pollution.

CONCLUSION

Thus the present study, has shown that the partially purified laccase from *Lenzites sp.* is highly efficient in decolorizing industrially important dye i.e reactive blue 160, and is being further explored for decolorizing more such dyes. Currently industrial dye effluents from textile industry are being treated with this enzyme to check its potential in decolorization and detoxification.

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