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Production of Laccase Enzyme By *Trichoderma erinaceum*

KEYWORDS: laccase, molecular mass, SDS-PAGE, Trichoderma erinaceum

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ABSTRACT *Trichoderma erinaceum was isolated from decomposing coconut coir and was screened for its ability to produce laccase enzyme. Production of extracellular laccase by T. erinaceum was carried out under submerged fermentation. The extracellular laccase produced by T. erinaceum was purified and the molecular mass was determined from the analysis of SDS-PAGE. The purified laccase enzyme showed a molecular mass of 38 kDa as estimated by SDS-PAGE. ABTS has been used as substrate for determination of laccase activity for crude enzyme as well as the pure precipitated enzyme. The purified enzyme showed almost double enzyme activity (2.286 U/L) compared to the crude form (1.429 U/L). The present work represents the first report on the ability of Trichoderma erinaceum to produce laccase enzyme.*

INTRODUCTION:

Lignin depolymerization is necessary to get access to cellulose and hemicellulose fibers. The degradation of lignin is brought about by the lignin degrading enzymes. The ligninolytic enzymes include lignin peroxidase, manganese peroxidase and laccase [17].

Laccase (E.C.1.10.3.2, p-benzenediol:oxygen oxidoreductase) is a multi-copper enzyme belonging to the group of blue oxidase that catalyzes the one electron oxidation of a broad range of organic substrates including phenols, polyphenols, anilines, benzene thiols and even certain inorganic compounds with a concomitant four electron reduction of oxygen to water [21].

Laccases find enormous application in food, animal feed, textile, fuel, chemical industries, pulp and paper industries, bioremediation etc [14].

Among microorganisms, fungi are the efficient producers of lignocellulolytic enzymes. It is well known that over 60 fungal strains belonging to Ascomycetes, Basidiomycetes and Deuteromycetes show laccase activity [6]. Among basidiomycetes, white rot fungi produce laccase enzyme more efficiently such as *Trametes versicolor* and *Pleurotus eryngii*. *Chaetomium thermophilum,* an ascomycetous fungus produces laccase efficiently [19]. It has been reported that *Trichoderma* species (Deuteromycetous fungus) also possesses ability to produce polyphenol oxidase [10]. *Trichoderma atroviride*, *T. harzianum* and *T. longibrachiatum* have also been reported to produce laccase [1, 8, 18]. However, there are no previous studies demonstrating production of laccase by *Trichoderma erinaceum.*

The present study reports *T. erinaceum* as an efficient producer of laccase enzyme.

MATERIALS AND METHODS:

I. Isolation of fungi:

Trichoderma erinaceum was isolated from decomposing coconut coir using a tenfold serial dilution-plating technique on potato dextrose agar (PDA) plates. The plates were incubated at 28°C. The pure culture was then transferred to PDA slants and maintained at 4°C and sub-cultured every month.

II. Morphological and Molecular Identification:

For the study of microscopic characteristics slide culture technique was used. Morphological identification of the fungal form was carried out based on morphological features such as colour, appearance, diameter of colonies etc. by referring Barnett and Hunter monograph [2] and the results were authenticated from Agharkar Research Institute, Pune, Maharashtra, India.

Molecular identification of *Trichoderma erinaceum* was also carried out at Agharkar Research Institute, Pune, Maharashtra, India. The obtained sequence data was aligned with online publicly available NCBI-BLAST tool and compared with NCBI accession number KJ439115 [9].

III. Screening for Laccase production by qualitative methods:

1. ABTS – Plate screen test:

Plates containing Lignin-agar basal medium supplemented with 0.1% ABTS and 0.01% of 20% w/v aqueous glucose solution [16] were inoculated with test fungus and incubated at 28° C. The formation of green halo around the fungal colony indicates the production of laccase enzyme.

2. Guaiacol assay: Guaiacol assay was performed by the method given by Kiiskinen *et al.*, [10] with slight modifications. The test fungus was inoculated on to the plates containing PDA medium supplemented with 0.01% guaiacol and then incubated at 28°C for about 6 days. Appearance of reddish brown halos around the colony suggested laccase positive strain.

IV. Inoculums Preparation for submerged fermentation:

Four mycelial plugs (8mm diameter) from a 7 day old culture PDA plate were cut with the help of a potato borer. The mycelial mats on the plugs were scraped and aseptically added to 250ml Erlenmeyer flasks containing 10ml of Sabouraud's broth. The inoculated flasks were incubated at 28° $\pm$ 2°C on an orbital shaker at 150 rpm for 48 hrs. to obtain large quantity of active mycelia.

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V. Cultivation Media for Laccase enzyme Production:
Low Nitrogen (LN) medium [22] with slight modification was prepared and the pH was adjusted to 4.5 using Citrate phosphate buffer. The volume was brought upto 1 litre using distilled water.

VI. Enzyme Production by Submerged Fermentation:
100 ml of the media was dispensed into 250 ml of Erlenmeyer flask and autoclaved. The flasks were inoculated with 5ml of spore suspension and then incubated for 6 days at 28°C on rotary shaker at 150 rpm.

VII. Enzyme extraction:
After six days of cultivation the contents of the flasks were filtered through Whatman No. 1 filter paper. The filtrate was centrifuged at 5,000 rpm for 15 mins. The supernatant was used as the crude enzyme extract for further analysis.

VIII. Partial purification and SDS-PAGE:
Partial purification of laccase was carried out with slight modifications [20]. The culture supernatant was precipitated with cold acetone at the ratio of 1:4. It was then centrifuged at 7,000 rpm for 20 mins, to pellet down the precipitate. The precipitate was then resuspended in 20mM Tris pH 8.0. To determine the purity of the protein and its molecular weight, SDS-PAGE was performed [12] with 10% polyacrylamide gel and the protein was visualized by staining the gel with silver staining [3] using standard molecular weight markers.

IX. Laccase assay:
Laccase activity was determined by monitoring the oxidation of ABTS (ε = 29,300 M^{-1} cm^{-1}) (2, 2'- azinobis-3-ethyl-benzothiozoline-6-sulfonic acid) [15]. The reaction mixture contained 0.5 ml of 0.2 mM ABTS in 50 mM sodium acetate buffer pH 4.5 and 0.5 ml enzyme extract. The oxidation of ABTS was measured spectrophotometrically at 405 nm as an increase in absorbance at 1 min interval. One unit of enzyme activity (U) is defined as the amount of enzyme that released 1µmole of oxidized product per minute, expressed as µmole/ min /L.

X. Protein determination
Protein concentration was determined by Bradford method using BSA as standard [4].

XI. Statistical analysis:
All experiments were performed in replicates of five and the average values were given with standard deviation.

RESULTS AND DISCUSSION:
I. Isolation and identification of fungal isolate:

1. Morphological characteristics:
Trichoderma erinaceum forms flat filamentous colonies, initially white, turning green; conidiophore branches at right angles or less with respect to the main branch; phialides in whorls of 2 or 3, almost cylindrical to swollen in the middle (6.0 to 8.0µm long); conidia 1.3- 1.5 (L/W) ellipsoidal to broadly ellipsoidal , smooth (Fig.1a and b).

2. Molecular identification :
The homology search of the isolate against the GenBank data base revealed a 99% similarity to the ITS region of *Trichoderma erinaceum*. The NCBI accession number for the analysed sequence of the isolate is KJ439115, The isolate was thus authenticated as *Trichoderma erinaceum* [9].

II. Qualitative screening for laccase production:
ABTS Plate screen test and Guaiacol assay for laccases:
The formation of a green halo in the ABTS supplemented plates and reddish brown halo in guaiacol supplemented plates indicated laccase production by *Trichoderma erinaceum* (Fig.2: a and b). *Trichoderma* strains have been reported to produce polyphenol oxidases [1]; recently *Trichoderma atroviride* and *T. harzianum* have showed positive results for laccase [8, 10].

Guaiacol and ABTS were considered as best substrates for laccase activity [21]. The chromogen ABTS is a very sensitive substrate that allows rapid screening of laccase producing fungal strains by means of a color reaction [15].

III. Molecular mass
SDS-PAGE analysis was carried out for both the crude and purified enzyme extracts. The purified laccase showed a single band on SDS-PAGE with a mobility corresponding to the relative molecular mass of 38 kDa as visualized by Silver nitrate staining (Fig.3); this is very close to the molecular weight of laccase from *Pleurotus* sp. having molecular weight of 40 kDa as previously reported [13]. Assavanig *et al.*, (1992) [1] has reported molecular weight of 71 kDa for *Trichoderma* sp.

It has been reported that fungal laccases usually have molecular weights ranging from 50 to 100 kDa [11] and are covalently linked to carbohydrate-moiety, which may contribute to high stability of the enzyme[5].

IV. Quantitative estimation of enzyme activities:
The laccase enzyme activities for the crude extract and the purified precipitate are given in the Table 1. The purified enzyme showed almost double enzyme activity (2.286 U/L) compared to the crude form (1.429 U/L). Gochev and Krastanov (2007) [7] have demonstrated laccase activity in four *Trichoderma* strains viz. *T. atroviride* (1.5 U/mL), *T. longibrachiatum* (1.7 U/mL), *T. viride* (2 U/mL) and *T. reesei* (0.2 U/mL).

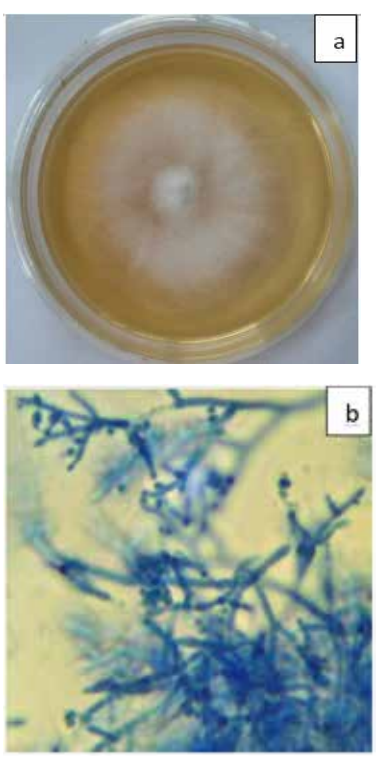

Figure 1: T. erinaceum (a) Colony morphology (b) Mycelia bearing conidiophores and conidiospores

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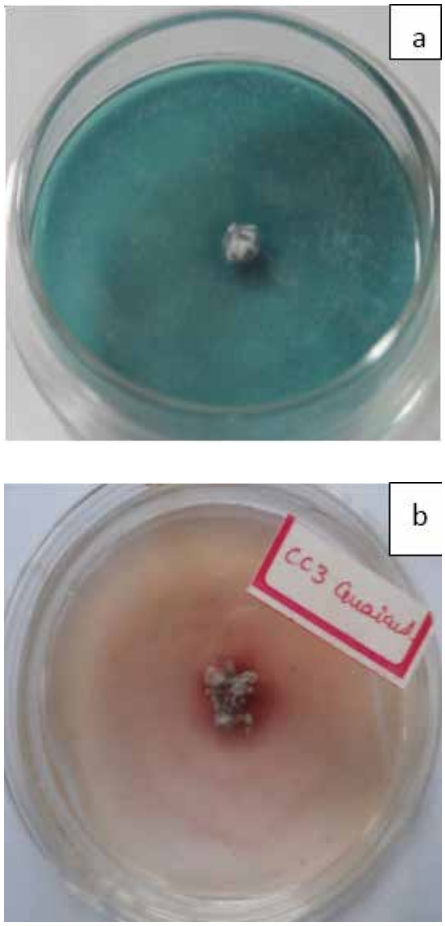

Figure 2: Qualitative screening (a) ABTS plate assay; (b) Guaiacol plate assay.

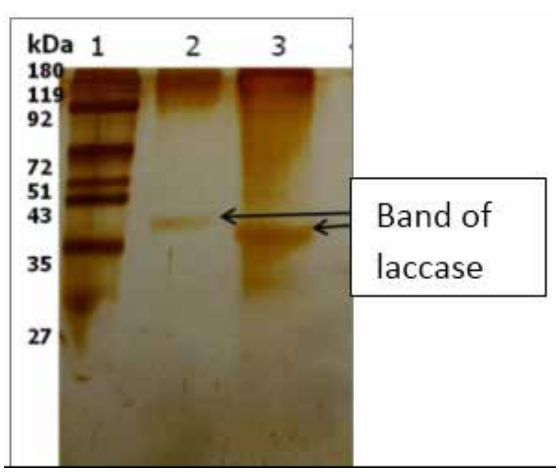

Fig. 3: 10 % PAGE, silver stained, under reducing condition showing Acetone precipitation of Laccase. Lane 1: Protein Ladder, Lane 2: Crude protein, Lane 3: Precipitated protein

Table 1: Laccase activity profile

Sample	Specific Activity U/mg	Enzyme Activity U/L
Crude enzyme extract	48.344	1.429
Precipitated enzyme	77.350	2.286

CONCLUSION:

The results of the study clearly indicate that the isolate *Trichoderma erinaceum* produces extracellular laccase that has a molecular weight of 38kDa. To the best of our knowledge this is the first report on laccase production by *T. erinaceum*. Optimization of culture conditions may enhance laccase production by the fungus. Therefore, more studies need to be carried out on optimization of various parameters.

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