RESEARCH ARTICLE

Studies on copper induction for laccase enzyme production by *Trichoderma erinaceum*

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ABSTRACT

Trichoderma erinaceum was isolated from decomposing coconut coir and was screened for its ability to produce laccase enzymes. Production of extracellular laccase enzyme from *Trichoderma erinaceum* was carried out by submerged fermentation. The laccase enzyme was partially purified by acetone precipitation from the culture filtrates of *Trichoderma erinaceum*. SDS-PAGE analysis showed the purified laccase to be a monomeric protein of 38 kDa. Laccase assay was carried out using ABTS as substrate. *Trichoderma erinaceum* exhibited laccase activity both under constitutive and copper induced conditions. Copper sulphate with 300 µm concentration added to the media highly induced production of laccase.

Keywords: *induced, laccase, SDS-PAGE, submerged fermentation, Trichoderma erinaceum*

INTRODUCTION

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 (Sandhu and Kalra, 1982). Laccases catalyze the oxidation of a variety of phenolic compounds, diamines and aromatic amines (Solomon *et al*., 1996).They are widely distributed in nature in higher plants, bacteria and fungi (Mayer and Staples;2002).Laccases find enormous industrial, biotechnological and environmental applications. They are used in food industry, pulp and paper industries, textile industry, cosmetics, bioremediation and biodegradation of environmental pollutants (Rao et *al*., 1993).Due to its versatile importance, there is a crucial need to induce both its expression and production through up-regulation of the enzyme coding genes. Although genetic manipulation is an effective tool, it is highly complex and expensive technique. Therefore increasing the enzyme yield by adding inducer is perceived as simple and

cost- effective (Skorobogat'ko *et al*., 1996; Levin *et al*., 2010). There are many different inducers for laccase production such as aniline (Bollag and Leonowicz, 1984), methoxy phenolic acids (Rogalski and Leonowicz, 1992,), lignin preparations (Rogalski *et al*., 1991) but the most common is copper. Copper is considered to be an efficient putative inducer for laccase production (Palmieri *et al*., 2000).

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 (Gianfreda *et al*., 1999). Among Basidiomycetes, white rot fungi produce laccase enzyme more efficiently (Ruiz-Dueñas, and Martínez, 2009). *Trametes versicolor*, *Chaetomium thermophilum* and *Pleurotus eryngii* produce laccase and it has been reported that *Trichoderma* species also has the ability to produce polyphenol oxidase (Gochev and Krastanov, 2007). *Trichoderma* species actively participates in delignification and biodegradation of lignocellulosic compounds in nature. Only a few publications are concerned with laccase producing *Trichoderma* species (Holker *et al*., 2002).*Trichoderma atroviride*, *T*. *harzianumT*. *longibrachiatum* and *T*. *erinaceum* have been reported to produce laccase (Blum *et al*., 1987; Gianfreda *et al*.,1999; Niku-Paavola *et al*.,1988; Ingle and Mishra, 2016).

The present study focuses on isolation and screening of laccase producing *Trichoderma erinaceum* and also the effect of copper on the production of laccase by submerged fermentation.

MATERIALS AND METHODS

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.

Primary screening for Laccase production by qualitative methods:
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 were inoculated with test fungus and incubated at 28º C (Pointing,1999). The formation of green halo around the fungal colony indicates the production of laccase enzyme.

Guaiacol assay:
Guaiacol assay was performed by the method given by Kiiskinen *et al*., (2004) with slight modifications. The test fungus was inoculated on to the plates containing PDA medium supplemented with 0.01% guaiacol and then incubated at 28ºC for about 6 days. Appearance of reddish brown halos around the colony suggested laccase positive strain.

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 carefully scraped so as to remove agar and aseptically added to the sterilized 250ml Erlenmeyer flasks containing 10ml of Sabouraud's broth. The inoculated flasks were incubated at 28º $\pm$ 2ºC on an orbital shaker at 150 rpm for 48 hrs to obtain large quantity of active mycelia.

Cultivation Media for Laccase enzyme Production:
Laccase enzyme production was carried out by using two media:

Media A: The culture broth as given by Tien and Kirk (1988) was prepared with slight modifications. The media was supplemented with 300 µm $CuSO_4$.

Media B: Tien and Kirk's medium was prepared.
The pH of the media was adjusted to 4.5 .The volume was brought up to 1000ml using distilled water.

Enzyme Production by Submerged Fermentation:
25 ml of the medium was dispensed into 250 ml of Erlenmeyer flask and autoclaved at 121°C for 15 minutes. The flasks were inoculated with 5ml of spore suspension (inoculum) and then incubated for 6 days at 28°C on rotary shaker at 150 rpm.

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

Partial purification and SDS-PAGE:

Partial purification of laccase was carried out with slight modifications (Sun *et al*., 2013) for both the samples i.e. culture filtrate from media A and media B. Cold acetone precipitation was carried out and the precipitate was then re-suspended in 20mM Tris pH 8.0. To determine the purity of the protein and its molecular weight, SDS-PAGE was performed (Laemmli, 1970) with 10% polyacrylamide gel and the protein was visualized by staining the gel with silver staining (Blum *et al*., 1987) using standard molecular weight markers.

Protein determination

Protein concentration was determined by Bradford method using BSA as standard (Bradford, 1976).

Laccase assay:

Laccase assay was carried out for the crude enzyme extract and precipitated enzyme from both the media. 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) (Niku-Paavola *et al*., 1988). 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 spectrophotome-trically 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.

Statistical analysis:

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

RESULTS AND DISCUSSION

Primary 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.1: a and b). *Trichoderma* strains have been reported to produce polyphenol oxidases (Blum *et al.,* 1987). Recently *Trichoderma atroviride* and *T. harzianum* have showed positive results for laccase (Gianfreda *et al*., 1999; Gochev and Krastanov 2007).

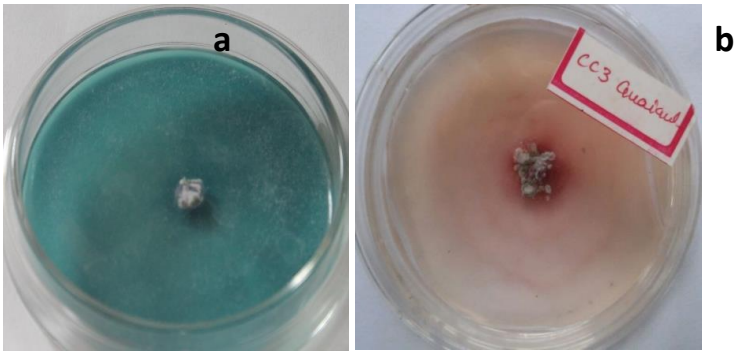
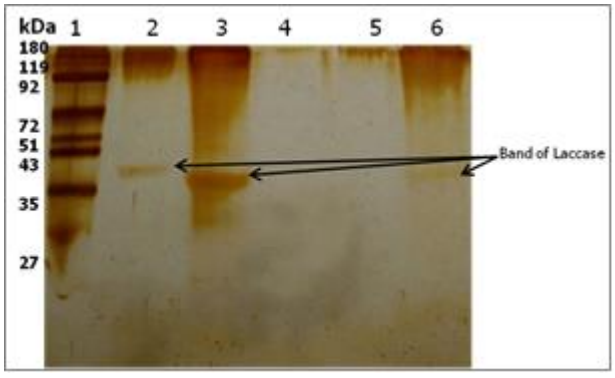


Figure 1: Qualitative screening (a) ABTS plate assay; (b) Guaiacol plate assay.



Lane No.	Sample ID
Lane 1	Protein Ladder
Lane 2	Crude A
Lane 3	Precipitated A
Lane 4	Blank
Lane 5	Crude B
Lane 6	Precipitated B

Fig 2: 10 % PAGE, silver stained, under reducing condition showing Acetone precipitation of Laccase.

Table 1 : Laccase activity profile before precipitation and after precipitation from Media A and Media B

Sample		Specific Activity (U/mg)	Laccase activity (U/L)
Media A: (with inducer)	Crude	48.344	1.429
	Precipitated	77.350	2.286
Media B: (without inducer)	Crude	6.397	0.429
	Precipitated	27.594	1.857

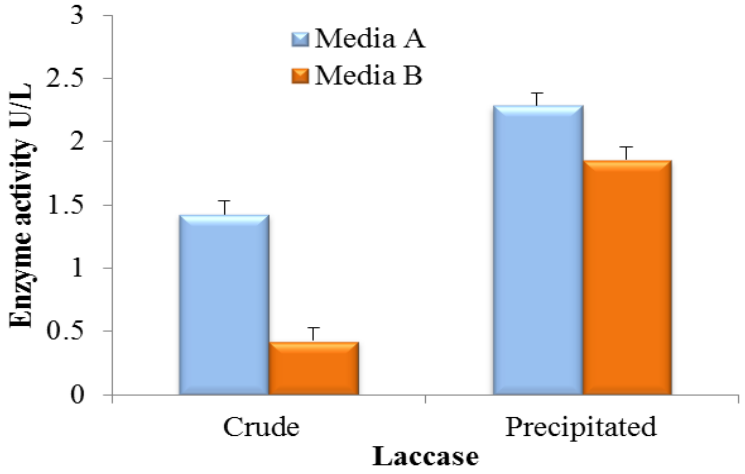


Fig. 1: Laccase production media

ABTS and Guaiacol are considered as best laccase substrates in the absence of hydrogen peroxidase, therefore confirming that the enzyme is true laccase (Sivakumar *et al*., 2010; Sandhu and Kalra, 1982). The chromogen ABTS is a very sensitive substrate that allows rapid screening of laccase producing fungal strains by means of a color reaction (Laemmli, 1970]. Guaiacol is used as a marker for extracellular oxidative enzymes, supports the above result (De Jong *et al*., 1994).

Molecular mass

SDS-PAGE analysis was carried out for both the crude and purified enzyme extracts obtained from both the media. 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 (More *et al*., 2011]. Assavanig *et al*., (1992) has reported molecular weight of 71 kDa for *Trichoderma* sp. According to Kunamneni *et al*. (2007) fungal laccases usually have molecular weights ranging from 50 to 100 kDa and are covalently linked to carbohydrate-moiety, which may contribute to high stability of the enzyme (Duŕan *et al*., 2002). From Fig.3; it is observed that laccase produced in the media A showed a darker band than media B. This indicates that the media supplemented with 300 µm CuSO4 has an inducing effect on laccase production. Lane 5 did not show any band of laccase, which might be due to negligible production of laccase, however Lane 6 (precipitated protein) showed a light band of laccase.

Laccase enzyme activities:

The laccase enzyme activities of *Trichoderma erinaceum* for the crude extract and the purified precipitate obtained from media A and media B are given in the Table 1. The laccase enzyme production in the Media A and Media B showed marked variations (Graph 1). Media A supplemented with 300 µm CuSO4 had an inducing effect on laccase production. It is worth noting that the addition of Cu+2 in the cultivation media stimulated laccase production increasing it almost three times higher than the control .This may be due to the filling of Type-2 copper binding sites with Copper ions (Nagai, *et al*., 2002).

Copper has been reported to be a strong inducer in several species such as *T. versicolor* (Collins and Dobson, 1997) and *P. chrysosporium* (Dittmer *et al*., 1997).

Similar observations have been recorded by Sivakumar *et al*. (2010) while working with *Ganoderma* sp. Niladevi and Prema (2007) have reported maximum laccase activity when Copper sulphate was used at a concentration of 1mM. However, Sadhasivam *et al*., (2008) reported that Copper at concentration of 10mM had a great inducing effect on laccase production by *Trichoderma harzianum* WL1 strain.

Also it was observed that the purified enzyme (media A) showed almost double enzyme activity (2.286 U/L) compared to the crude form (1.429 U/L).

CONCLUSION

The purified laccase from *Trichoderma erinaceum* showed molecular mass of 38kDa.The production of laccase by *Trichoderma erinaceum* occurs constitutively as well as in presence of inducer. The metal ion copper effectively increases the production of laccase by submerged fermentation. Further studies on standardization of concentration of copper for laccase production are needed

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