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Comparative studies on laccase enzyme production by two different species of *Trichoderma*

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ABSTRACT

Two different species of *Trichoderma viz., T. atroviride and T. erinaceum* were isolated from decomposing coconut coir and were screened for their laccase producing abilities. Production of extracellular laccases by *Trichoderma atroviride and Trichoderma erinaceum* was carried out under submerged fermentation at varying pH of the culture medium and different lignocellulosic agro industrial residues. The optimum pH of the culture medium for laccase production was reported to be 5. Sawdust was found to be the most efficient substrate for laccase enzyme production, followed by sugar cane bagasse. Maximum production of laccase enzyme has been noticed with regard to natural carbon sources than synthetic one.

Keywords: *agro industrial, laccase, lignocellulolytic, Trichoderma atroviride, Trichoderma erinaceum*

INTRODUCTION

The lignocellulosic biomass consists of cellulose, hemicellulose and lignin. Enzymatic hydrolysis of cellulose and other related oligo-saccharides is catalyzed by cellulase enzyme system that includes exoglucanases, endoglucanases and β-glucosidases acting in a synergistic manner (Acharya *et al.*, 2008). Hemicellulose being heterogeneous in nature is hydrolyzed by hemicellulases. Lignin depolymerization is necessary to get access to cellulose and hemicellulose fibers. However, the recalcitrance nature of lignin makes its degradation difficult. The degradation of lignin is brought about by the lignin degrading enzymes.

The ligninolytic enzymes include lignin peroxidase, manganese peroxidase and laccase (Ruiz-Duenas and Martinez, 2009).

Laccase (E.C.1.10.3.2, p-benzenediol: oxygen oxido-reductase) 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 (Thurston, 1994).

Fungi have been recognized as efficient producers of lignocellulolytic enzymes among microorganisms. 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 (Shraddha *et al*., 2011). *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 (Kiiskinen *et al*., 2004). *Trichoderma atroviride*, *T. harzianum, T. longibrachiatum* and *T. erinaceum* have also been reported to produce laccase (Assavanig *et al*., 1992; Hölker *et al*., 2002, Sandhu and Kalra, 1982).

The present study deals with the effect of pH of cultivation medium and different lignocellulosic agro wastes on the production of laccase by *Trichoderma atroviride* and *T. erinaceum*.

MATERIALS AND METHODS

I. **Isolation of fungi:**

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

II. Morphological Identification:

The macroscopic characters such as colour, appearance, and diameter of colonies and microscopic (microstructures) characteristics were studied. The fungal isolates were identified according to Barnett and Hunter (1972) and the results were confirmed from Agharkar Research Institute, Pune, Maharashtra, India.

III. Primary Screening for Laccase production:

ABTS – Plate screen test:

Plates containing Lignin-agar basal medium (Pointing, 1999) supplemented with 0.1% ABTS and 0.01% of 20% w/v aqueous glucose solution were inoculated with test fungi and incubated at 28° C. The formation of green halo around the fungal colonies indicates the production of laccase enzyme (Niku- Paavola *et al*., 1988).

IV. Preparation of Inoculum 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 28° $\pm$ 2°C on an orbital shaker at 150 rpm for 48 hrs. to obtain large quantity of active mycelia.

V. Cultivation Media for Laccase enzyme Production:

Tien and Kirk medium (1988) with slight modifications was prepared. The pH of the culture media was adjusted to 4.5 using Citrate phosphate buffer.

VI. Enzyme Production by Submerged Fermentation:

25 ml of the media was dispensed into 250 ml of Erlenmeyer flask and autoclaved at 121°C for 15 mins. The flasks were inoculated with 5 ml of spore suspension and then incubated for 6 days at 28°C on rotary shaker at 150 rpm.

VII. Enzyme extraction:

The contents of the flasks were filtered through Whatman No. 1 filter paper after six days of cultivation. The filtrates were then centrifuged at 5,000 rpm for 15 mins. The supernatants were used as the crude enzyme extracts for further analysis.

VIII. 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) (Sandhu and Kalra, 1982). 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 oxidi-zed product per minute, expressed as µmole/ min /L.

IX. Effect of pH variation:

To study the effect of pH of the culture medium on enzyme production, the culture media was adjusted to different pH using 0.1N HCl and 0.1N NaOH. For laccase enzyme production the pH range of the culture medium used was 3, 4, 5, 6 and 7.

X. Effect of varying carbon sources:

Various agro industrial residues like sugarcane bagasse, fibrous mesocarp of coconut and saw dust were used as carbon sources.

XI. **Statistical analysis:**

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

RESULTS AND DISCUSSION:

I. Morphological characteristics:

***Trichoderma atroviride*:**

The white mycelial colony appears uniformly dispersed, granular with 1-2 concentric rings showing green conidial production. White pustules produced on the green mat of conidia. Conidiophores branching typically unilateral; Phialides 6.0 -10 x 1.0 - 3.0 µm, straight or sinuous , sometimes hooked, whorls of 2-4 often cylindrical; Conidia 1.0- 1.3 µm long sub globose to ovoidal; Chlamydospores produced within 7 days, globose to sub globose, terminal or intercalary. (Fig.1.a, b)

Trichoderma erinaceum:

The colony is flat filamentous, 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 8.0 µm long), conidia 1.3- 1.5 (L/W) ellipsoidal to broadly ellipsoidal , smooth (Fig.2.a, b).

II. Qualitative screening for laccase production:

ABTS Plate screen test

ABTS has been considered as best substrates for laccase activity (Thurston, 1994). The formation of a green halo in the ABTS supplemented plates indicated laccase production by *Trichoderma atroviride* and *T. erinaceum* (Fig.3). *Trichoderma* strains have been reported to produce polyphenol oxidases (Assavanig *et al*., 1992). Studies have demonstrated laccase activities by *Trichoderma atroviride* and *T. harzianum* (Hölker *et al*., 2002; Kiiskinen *et al*., 2004).

III. Quantitative estimation of enzyme activities:

a. Effect of pH:

The pH of the culture medium influences laccase production by *Trichoderma atroviride* and *Trichoderma erinaceum* (Fig. 4: a). Both *Trichoderma atroviride* and *Trichoderma erinaceum* showed maximum laccase production at pH 5.0 indicating the acidic condition necessary for enzyme secretion. Decrease in laccase activity was noticed below pH 4. A sharp decline in the enzymatic activity with increasing pH was also noticed in the present study. No laccase activity was detectable at pH 7 under the given assay conditions. Studies have shown initial pH for laccase production by fungi to be between 4.5 and 6.0 (Shraddha *et al*., 2011).

Most reports indicated initial pH levels set between pH 4.5 and pH 6.0 prior to inoculation, but the levels are not controlled during most cultivations (Mtui,2012). Nyanhongo *et al*. (2002) reported that an initial pH of 7.0 was the best for optimal growth and laccase production by a newly isolated strain of *T. modesta*.

b. Effect of lignocellulosic substrates on laccase enzyme production enzyme:

It was observed that amongst sugarcane bagasse, saw dust and coconut coir used as a carbon sources in the culture medium, saw dust appeared to be a better lignocellulosic substrate for the production of laccase enzyme. *Trichoderma atroviride* and *Trichoderma erinaceum* showed 1.79428 (U/L) and 1.6228 (U/L) laccase enzyme activity when saw dust was used in the medium. (Fig.4 b)

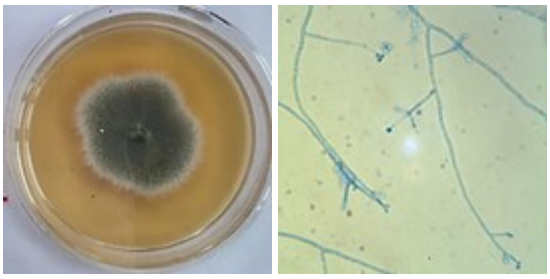

Figure 1: *T. atroviridae* **(a)** Colony morphology (b) mycelia bearing conidiophores and conidiospores after staining and mounting with Lactophenol Cotton Blue

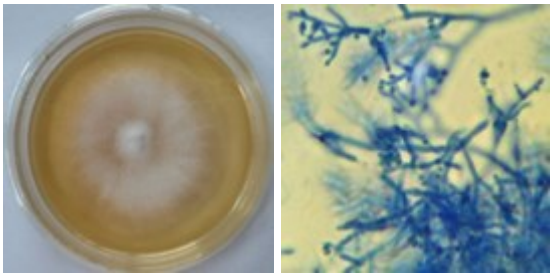

Figure 2: *T. erinaceum* **(a)** Colony morphology (b) mycelia bearing conidiophores and conidiospores after staining and mounting with Lactophenol Cotton Blue

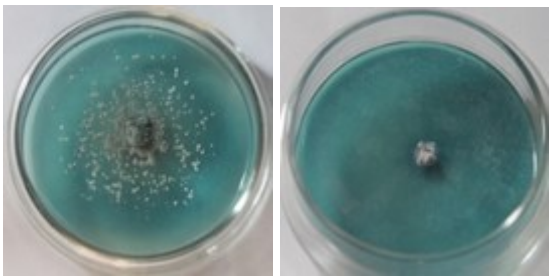

Figure 3: ABTS plate assay : **a.** *T. atroviridae* **b.** *T. erinaceum*

Table 1: Effect of lignocellulosic substrates in the production medium (Laccase)

Sr. No.	Fungal strain	Enzyme activities (U/L)		
		Sugarcane bagasse	Saw dust	Coconut coir
1	*Trichoderma atroviride*	1.44	1.79428	1.31428
2	*Trichoderma erinaceum*	1.16571	1.6228	0.9714

Table 2 : Laccase enzyme activities (U/L) at varying pH of the production medium

Sr.No.	Fungal strains	pH			
		3	4	5	6
1	*Trichoderma atroviride*	0.4457	0.8685	0.6057	0.2514
2	*Trichoderma erinaceum*	0.418	0.7234	0.5257	0.1828

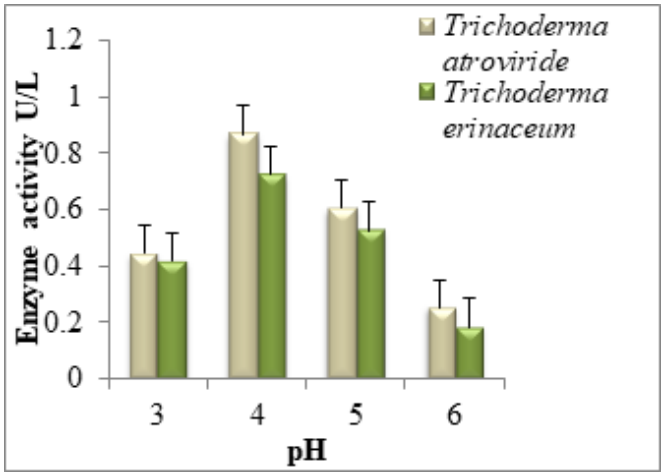

Figure 4. a: Effect of pH on laccase enzyme production

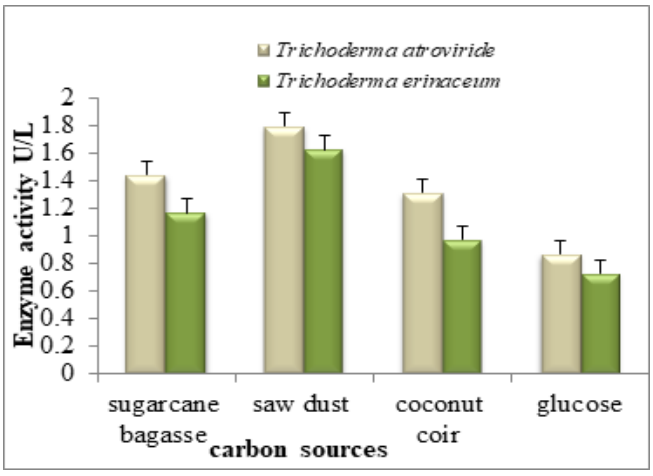

Figure 4 b: Effect of lignocellulosics (carbon) sources on laccase enzyme production

CONCLUSION

The results of the study clearly indicate that the isolates *Trichoderma atroviridae and T. erinaceum* have the ability to produce laccase enzymes, pH optima at 4.0. Sawdust served to be the best substrate for enzyme production.

Conflicts of interest: The authors stated that no conflicts of interest.

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