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# LIGNOCELLULOLYTIC ENZYME PRODUCTION BY SOFT –ROT FUNGI ISOLATED FROM DECOMPOSING COCONUT COIR PITH

**Botany**

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## ABSTRACT

Fungi were isolated from decomposing coconut coir and screened for their abilities to produce lignocellulolytic enzymes. The fungal strains that showed positive results were selected and utilized for production of extracellular lignocellulolytic enzymes viz., cellulase and laccase by submerged fermentation using defined media and culture conditions. The crude enzyme extracts were used for determining FPASe, endoglucanase, β-glucosidase and laccase enzyme activities. *Trichoderma atroviride* exhibited highest FPAse (54.343 U/mL) and endoglucanase (58.973U/mL) enzyme activities whereas β-glucosidase activity was very low (32.323 U/mL). However, *Aspergillus flavus* showed maximum β-glucosidase activity (75.863U/mL). Of all the nine fungal isolates, only *Trichoderma atroviride* and *T. erinaceum* showed laccase enzyme activity.

## KEYWORDS

agro industrial, cellulase, laccase, lignocellulolytic, *Trichoderma*

## INTRODUCTION

The coconut coir is the unique natural fibre obtained from the mesocarp of coconut fruit *(Cocos nucifera)*. The estimated annual production of coir pith in coir industry of India is about 7.5 million tons [16].The coconut coir pith is composed of 40-50% cellulose, 15-35% hemicellulose and 20-40% lignin. The coir pith and the coconut husk are difficult to degrade due to its high lignin content under natural conditions, taking over eight years to decompose thus causing environmental problems [4].

Enzymatic hydrolysis of cellulose and other related oligo-saccharides is catalyzed by cellulase. The cellulase enzyme system is a mixture of hydrolytic enzymes including exoglucanases, endoglucanases and β-glucosidases acting in a synergistic manner [1]. Hemicellulose being heterogenous in nature is hydrolyzed by hemicellulases. Lignin depolymerization is necessary to get access to cellulose and hemicellulose fibers. The degradation of lignin is brought about by the ligninolytic enzymes which includes lignin peroxidase, manganese peroxidase and laccase [18].

Lignocellulolytic enzymes are used in food, animal feed, beverages, pharmaceuticals, textiles and bioremediation of highly toxic aromatic and aliphatic xenobiotic compounds such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), pesticides and herbicides, [11].

Among microorganisms, fungi are the efficient producers of lignocellulolytic enzymes. Production of cellulolytic enzymes by various fungal genera such as *Aspergillus, Penicillium, Trichoderma* has been reported [2]. The white rot fungi like *Pleurotus, Agaricus* have been extensively studied for their ligninolytic activities compared to soft rot fungi . However, still many fungi have remained unexplored.

## MATERIALS AND METHODS:

### I. Isolation and identification of fungi:

Isolation of fungal species was carried out from decomposing coconut coir using a tenfold serial dilution-plating technique on potato dextrose agar plates. The pure culture obtained were then transferred to PDA slants and maintained at 4°C and sub-cultured every month.

The macroscopic characters colour, appearance, and diameter of colonies and microscopic characteristics were studied for identification of fungi [3] and the results were confirmed from Agharkar Research Institute, Pune, Maharashtra, India.

### II. Qualitative screening for lignocellulolytic activity:

Screening of lignocellulolytic fungi were carried out following the methods of Pointing, (1999) with slight modifications [17].

1. Congo red dye staining method for cellulase
2. Azure-B agar clearance for peroxidase and
3. ABTS plate assay for laccase enzyme activity.

### III. Inoculums Preparation:

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

### IV. Cultivation Media for Enzyme Production:

Mandel and Weber's medium (modified) was used for the production of cellulase enzymes [12]. Microcrystalline cellulose (1%) was used as carbon source. For ligninolytic enzyme production Low Nitrogen (LN) modified medium was used [19].

### V. Enzyme Production by Submerged Fermentation:

250 ml of Erlenmeyer flasks containing 25 ml of sterilized media were inoculated with 4ml of fungal inoculum and incubated at 28°C on rotary shaker at 150 rpm for 6 days. 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 mins. The supernatant was used as the crude enzyme extract for further analysis. The experiments were performed in triplicates.

### VI. Analytical methods:

The total cellulase (FPase) and Endoglucanase activities were determined according to the methods of the International Union of Pure and Applied Chemistry (IUPAC) Commission of Biotechnology [7]. For FPase 50 mg Whatman No.1 filter paper used as a substrate whereas for endoglucanase activity, 0.5 ml 1% CMC served as substrate.

The β – glucosidase assay was performed using 1% Salicin as substrate [20]. After incubation the amount of reducing sugars released from the substrates was determined by Dinitrosalicylic acid (DNS) method [13]. The absorbance was read at 540 nm and the liberated reducing sugars were estimated from the absorbance (for FPase, endoglucanase and β – glucosidase) Enzyme activities were defined in International Units (IU). One unit of enzyme activity (IU) is defined as the amount of enzyme that released 1µmole reducing sugars (glucose) equivalent per minute, expressed as µmole/mL/min under the above assay conditions.

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) in 50 mM sodium tartarate buffer, pH 4.5 [14]. The reaction mixture contained 0.5 ml of 2mM ABTS in 50 mM sodium tartarate buffer and 0.5 ml enzyme extract. The oxidation of ABTS was measured by an increase in absorbance that was measured spectrophotometrically at 436 nm at 1 min interval. One unit of enzyme activity (U/L) is defined as the amount of enzyme that released 1µmole of oxidized product per minute, expressed as µmole/min/L.

### VII. Statistical analysis:

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

## RESULTS AND DISCUSSION:

### 1. Identification of fungal isolates:

Nine fungal isolates belonging to seven different fungal species were isolated from decomposing coconut coir pith. They were identified as *Aspergillus flavus* (C4, C6), *Curvularia pallescens* (C9), *Fusarium moniliforme* (C7), *Rhizopus arrhizus* (C1), *Trichoderma atroviride* (C8), *Trichoderma erinaceum* (C3, C10) and *Trichoderma sp*. aff., *T. piluliferum* (C2).

### 2. Qualitative screening:

**A. Congo red dye degradation for cellulolytic activity:**

Congo red stains binds with β-1-4 linked glycosidic bonds. Fungal strains producing cellulase hydrolyse all cellulose around their colonies in CBM-CMC plate and therefore Congo red cannot bind around these colonies. The carboxymethyl cellulose degradation appears as yellow opaque area against the red colour for un-degraded carboxymethyl cellulose. The diameter of yellow opaque area gives a direct qualitative estimate of the efficiency of cellulolytic activity of the test fungus.

*Aspergillus flavus, Fusarium moniliforme Trichoderma atroviride* and *T. erinaceum* showed maximum dye clearance. Moderate activity was shown by *Curvularia pallescens*. *Rhizopus arrhizus* degraded CMC very less, indicating low cellulase activity (Fig.1A). The change of the medium color may also be due to the organic acids secreted by the fungi that lowered the pH of the medium [22]. It was found that the colour of Congo red turned purplish blue to brown at pH 3.0 and red at pH 5.2.

**B. Azure-B agar clearance test:**

*Fusarium moniliforme, Trichoderma atroviride* and *T. erinaceum* showed clearance of Azure-B dye after a period of 15 days from the day of inoculation. The colorless zone formed around the colony due to the degradation of Azure – B dye indicated peroxidase activity. A*spergillus flavus, Curvularia pallescens, Rhizopus arrhizus*, and *Trichoderma piluliferum* did not show Azure B dye degradation, thus indicating no peroxidase activity (Fig.1B).

**C. ABTS Plate screen test :**

The chromogen ABTS is a very sensitive substrate that allows rapid screening of laccase producing fungal strains by means of a color reaction [14]. The formation of a green halo in the ABTS supplemented plates indicated laccase production by *Trichoderma atroviride, T. piluliferum,T. erinaceum and Fusarium moniliforme* (Fig.1C).The intensity and diameter of the halo indicates the level of enzymes secreted. Azure B dye degradation by Fusarium oxysporum (GU724514.1) has been reported [5].

Trichoderma strains have been reported to produce polyphenol oxidases and recently *Trichoderma atroviride* and *T. harzianum* have showed positive results for laccase [9,10].

### 3. Quantitative estimation of enzyme activities:

**Cellulase enzyme production:** *Trichoderma atroviride* and *T. erinaceum* showed significantly high FPAse and endoglucanase enzyme activities as compared to Aspergillus flavus. The production of FPAse and endoglucanase enzymes by *Curvularia pallescens* was moderately significant, higher than *Fusarium moniliforme*. Similar results have been reported by Okunowo et al., [15]. However, A. flavus produced high titres of β – glucosidase enzyme activity.

No significant β – glucosidase enzyme activity was observed between *Curvularia pallescens* and *Trichoderma atroviride*. Similarly no significant difference observed in *Trichoderma erinaceum* and *T. piluliferum*. *Rhizopus arrhizus* showed minimal production of cellulase enzyme production(Table1).

**Laccase enzyme production:**

Although *Fusarium moniliforme* and *Trichoderma piluliferum* showed positive tests for ABTS Plate assay, these fungi failed to show significant laccase production during submerged fermentation. Laccase enzyme activities were reported by *Trichoderma atroviride* (0.8685 U/L) and *T. erinaceum* ( 0.7234 U/L). Earlier studies also reported similar results[6].

Laccases are generally produced in low concentrations by laccase producing fungi and are governed by the cultural conditions provided to the fungus [21,8].

## CONCLUSION

*Trichoderma atroviride* and *T. erinaceum* showed highest FPAse and endoglucanase enzymes activities. However, the β-glucosidase enzyme activity in the selected *Trichoderma sp.*, was comparatively very low compared to A. flavus. β-glucosidase enzyme, being the bottle neck enzyme in cellulose degradation, thus becomes a rate limiting step in *Trichoderma sp*.

It is noteworthy that for degradation of lignocellulose both laccase and cellulase play important roles. A mixed consortia of *Trichoderma sp.* and *Aspergillus sp*. would give a better degradation of lignocellulose.

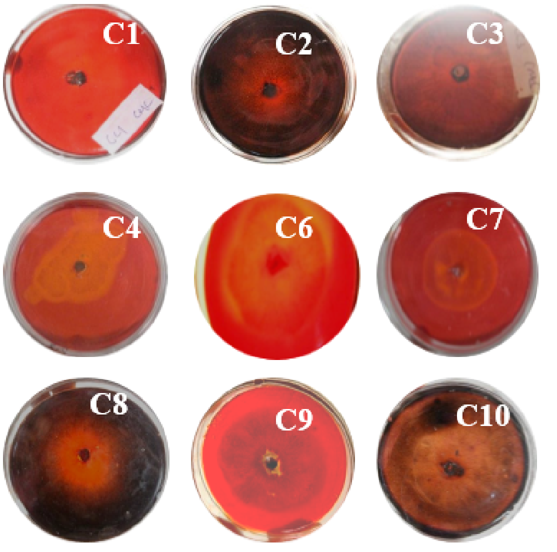


**Fig. 1 A. Congo red dye degradation by fungal isolates -C1: *Rhizopus arrhizus;***

C2: *Trichoderma piluliferum*;
C3: *T. erinaceum*; C4: *Aspergillus flavus*; C6: *A. flavus C7: Fusarium moniliforme*; C8: *T. atroviride*; C9: *Curvularia pallescens*; C10: *Trichoderma erinaceum.*


**Fig.1 B. Azure B dye degradation for peroxidase activity- C3: Trichoderma erinaceum;**

C7: *Fusarium moniliforme*; C8: *Trichoderma atroviride*

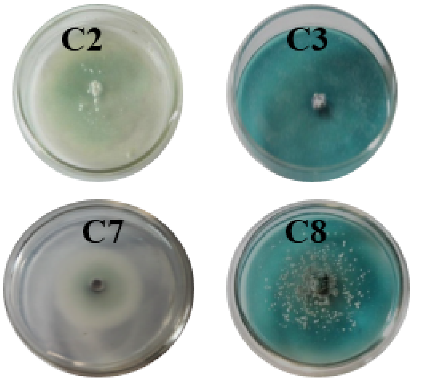


**Fig.1. C: ABTS Plate assay**

C2: *Trichoderma piluliferum* C3: *T. erinaceum;* C7: *Fusarium moniliforme*; C8: *T. atroviride*

**Table 1. Lignocellulolytic enzyme production by fungal isolates**

| Sr. No. | Fungal strain | Cellulase enzyme activities (U/mL) | | | |
|---|---|---|---|---|---|
| | | FPAse | Endoglucanase | β --glucosidase | Laccase (U/L) |
| 1 | *Aspergillus flavus* | 45.836 | 50.840 | 75.863** | ---- |
| 5 | *Curvularia pallescens* | 44.459 | 48.088 | 33.574 | ---- |
| 6 | *Fusarium moniliforme* | 38.204 | 40.205 | 21.313 | ---- |
| 7 | *Rhizopus arrhizus* | 23.315 | 14.557 | 10.053 | ---- |
| 8 | *Trichoderma atroviride* | 54.343** | 58.973** | 32.323 | 0.8685 |
| 9 | *Trichoderma erinaceum* | 50.715 | 53.092 | 25.317 | 0.7234 |
| 10 | *Trichoderma sp.aff., T. piluliferum* | 43.959 | 46.086 | 24.941 | ---- |

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