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# Studies on Cellulolytic Enzyme Production by *Trichoderma erinaceum*

KEYWORDS: agro industrial, cellulase, laccase, lignocellulolytic, Trichoderma erinaceum

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**ABSTRACT** *Trichoderma erinaceum was isolated from decomposing coconut coir and was screened for its ability to produce lignocellulolytic enzymes. Production of extracellular cellulase enzymes was carried out by submerged fermentation for six days at 28° ± 2° C and at 150 rpm. Effect of varying pH of the culture medium on cellulase production was investigated and the optimum pH was reported to be 5. Of the different lignocellulosic agro industrial residues used as substrate for cellulase production, sugarcane bagasse was found to be the most efficient substrate (FPase 82.620U/ml, endoglucanase 102.513 U/ml and β-glucosidase 65.479 U/ml), followed by coconut coir and least was saw dust. Maximum production of cellulase enzyme has been noticed with regard to natural carbon sources than synthetic one.*

## INTRODUCTION:

Cellulose, the world's most abundant natural polymer present in the lignocellulosic biomass is a potential source for the production of fuels and chemicals. 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]. Cellulolytic enzymes find enormous application in food, animal feed, textile, fuel, chemical industries, pulp and paper industries, bioremediation etc [12].

Among microorganisms, fungi are the efficient producers of lignocellulolytic enzymes. Production of cellulolytic enzymes by *Trichoderma* species has been reported. One of the most commercially important and extensively studied cellulolytic species is *T. reesei* [12]. Studies on the production and characterization of cellulases from *T. lignorum* have been carried out [3].

*Trichoderma erinaceum* is a filamentous fungus that produces chitinase and β-1,3- glucanase and can be used as an effective biocontrol agent [6]. However, to the best of our knowledge cellulase enzyme production by *Trichoderma erinaceum* have not been reported. In view of this, the present work has been carried out to evaluate the cellulase enzyme production by *Trichoderma erinaceum*.

## 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.

### Morphological and Molecular Identification:

The macroscopic characters colour, appearance, and diameter of colonies and microscopic (microstructures) characteristics were studied according to Barnett and Hunter [4] and the results were confirmed from Agharkar Research Institute, Pune, Maharashtra, India.

Molecular identification of *Trichoderma erinaceum* was carried out at Agharkar Research Institute, Pune, Maharashtra, India. Isolation and extraction of genomic DNA was carried out as per the methodology adapted by Aljanabi *et al.*, (1997) [2]. The PCR amplification of ITS-rDNA was performed with universal primer pairs ITS4/ITS5 [14]. The sequencing PCR was set up with ABI-BigDye® Terminatorv 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) as per the manufacturer's instructions on an ABI 3100 Avant Prism automated DNA sequencer (Applied Biosystems).The raw DNA sequences were edited and combined using ChromasLite v. 2.01. The obtained sequence data was aligned with online publicly available NCBI-BLAST tool. The final sequence is deposited in the NCBI nucleotide sequence database (Accession Number KP233741)

### Qualitative screening for cellulase production by Congo red dye degradation method:

The fungus was screened for its abilities to produce extracellular lignocellulolytic enzymes following the methods of Pointing [10] with slight modifications.

Petriplates containing the cellulolytic basal medium supplemented with 2% low viscosity Carboxymethyl cellulose were inoculated with fungal mycelial agar discs of 0.5 cm diameter, cut from the margins of the growing colonies on PDA plates. A control was also retained. The plates were incubated at 28°C in an incubator for five days. When the colony diameter reached approximately 4 cm, the plates were stained with 2% aqueous Congo red (C.I. 22120) for about 15 minutes followed by de-staining with 1M NaCl solution and then washed with distilled water. The carboxymethyl cellulose degradation by the fungal strains appears as yellow opaque area against the red colour for un-degraded carboxymethyl cellulose.

### V. 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 28° + 2°C

Volume : 6 | Issue : 6 | June 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50

on an orbital shaker at 150 rpm for 48 hrs. to obtain large quantity of active mycelia.

**Cultivation Media for cellulolytic Enzyme Production:**
Mandel and Weber's medium (modified) [8] was used for the production of cellulase enzyme by submerged fermentation which contained ($gL^{-1}$): 1.4 $(NH_4)_2SO_4$; 2.0 $KH_2PO_4$; 1.0 Proteose peptone; 0.3 Urea; 0.75 Yeast; 0.3 $CaCl_2$; 0.3 $MgSO_4 \cdot 7H_2O$; 0.0016 $MnSO_4 \cdot H_2O$; 0.005 $FeSO_4 \cdot 7H_2O$; 0.0014 $ZnSO_4 \cdot 7H_2O$; 0.002 $CoCl_2$; Tween 80 0.1% v/v and microcrystalline cellulose (1%) as carbon source.

**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 4ml of spore suspension 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 mins. The supernatant was used as the crude enzyme extract for further analysis.

**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 [5]. The reaction mixture tube contained 1ml of 0.05M Sodium citrate buffer (pH 4.8) and 1ml crude enzyme extract. For FPase 50 mg Whatman No.1 filter paper used as a substrate whereas endoglucanase activity, 0.5 ml 1% Carboxymethyl cellulose served as substrate.

The β – glucosidase assay was performed using the method of Tomaz and Roche [13]. The reaction mixture tube containing 1ml of 0.05M Sodium citrate buffer (pH 4.8), 1ml crude enzyme extract and 0.5 ml of substrate (1% Salicin). In all the above assays, the tubes were incubated at 50° C for 1 hour. After incubation the amount of reducing sugars released from the substrates was determined by Dinitrosalicylic acid method [9].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 using glucose as a standard curve.

**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 cellulase enzyme production the pH range of the culture medium used was 4,5,6,7 and 8.

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

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

**RESULTS AND DISCUSSION:**
**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 to8.0µm long), conidia 1.3- 1.5 (L/W) ellipsoidal to broadly ellipsoidal , smooth (Fig.1a and b ).

**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 isolate was thus designated as *Trichoderma erinaceum*. Sequence analysis with NCBI accession number KJ439115, *Trichoderma erinaceum* resulted in following alignment statistics: Query Length – 543, Score- 990 bits (536), Expect -0.0, Identities – 541-543 (99%), Gaps 2/543, Strand Plus/ Minus.

**Qualitative screening for cellulolytic activity:**
**Congo red dye degradation:**
The carboxymethyl cellulose degradation appears as yellow opaque area against the red colour for un-degraded carboxymethyl cellulose (Fig.1: c). 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 and a clear yellow opaque zone appears.

**Quantitative estimation of enzyme activities:**
**Effect of pH:**
Cellulase production by *Trichoderma erinaceum* appeared to be dependent of the initial pH of the culture medium. Cellulase production, expressed as enzyme activity increased as the pH increased from 4 to 5 but further with increasing pH enzyme activity decreased (Fig. 2:a). The enzyme activity was found to be maximum at pH of 5 being FPase (50. 715 U/ ml), endoglucanase ( 53.092 U/ ml), and β-glucosidase (25.317 U/ ml). However, the β-glucosidase production was comparatively lesser than FPase and CM-Case. Lee *et al.*, [7] reported that the cellulase production is affected by pH and the optimum pH for CMCase, Avicelase and FPase activities was pH 4 whereas pH optimum of β-glucosidase was between 5 and 6.

**Effect of lignocellulosic substrates:**
1% sugarcane bagasse served as an efficient carbon source for cellulase enzyme production (FPase 82.620U/ml, endoglucanase 102.513 U/ml and β-glucosidase 65.479 U/ml) followed by sawdust and coconut coir. (Fig. 2: b). It was observed that the natural lignocellulosic agro industrial residues induce more cellulase production compared to cellulose when used as a carbon source.

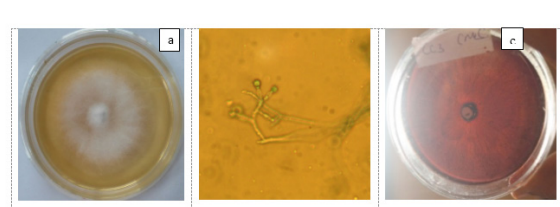
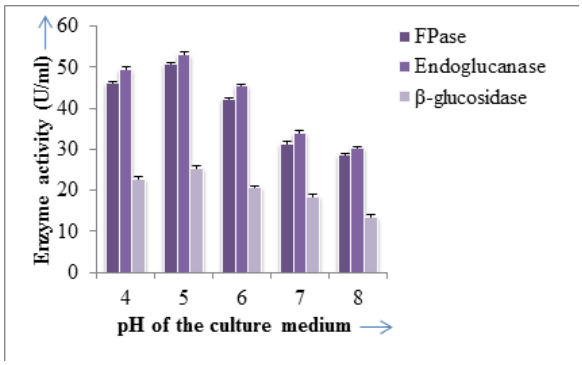



**Figure 1: T. erinaceum (a) Colony morphology (b) mycelia bearing conidiophores and conidiospores after staining and mounting with Lactophenol Cotton Blue**

Volume : 6 | Issue : 6 | June 2016 | ISSN - 2249-555X | IF : 3.919 | IC Value : 74.50



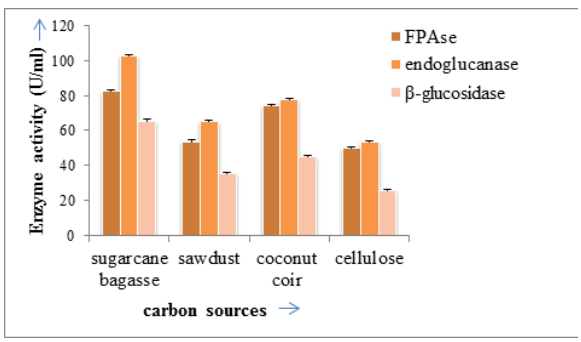




**Figure 2:** Optimization of parameters for cellulase production by *T. erinaceum*. (a) Effect of initial pH of culture medium on cellulase production (b) Cellulase production on different lignocellulosic substrates.; Error bars indicate the SD

**Conclusion:**

The results of the study clearly indicate that the isolate *Trichoderma erinaceum* has the ability to produce cellulase enzymes. However, more studies need to be carried out on optimization of various parameters.

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