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Trichoderma species Cellulases Produced by Solid State Fermentation

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Abstract

The main aim of this study was to analyze eight species of *Trichoderma* for cellulase enzyme production by solid state fermentation. Different carbon sources such as wheat bran, corn cob, sucrose, maltose and filter paper were used. Highest celluase enzyme production was achieved with *T. harzianum* on media supplemented with corn cob. The optimum pH, temperature and thermal stability of isolated enzymes were also analyzed. The best pH for enzyme production was found between 4-6. The optimum temperature range for cellulase production ranged between 30-40°C. Choosing the optimum pH, temperature and best carbon source are essential for the enzyme production. Compare to other fungal genera it has been found that *Trichoderma* spp. have the greater potential to synthesize cellulase enzyme.

Keywords: *Trichoderma;* Filter paper activity (FPase); Endoglucanase; Cellulase

Introduction

Cellulases are enzymes that degrade cellulose. These enzymes are produced by several microorganisms including bacteria and fungi. Fungi are the main source of cellulase enzyme.

The genus *Trichoderma* is well known for the production of cell wall degrading enzymes [1,2]. Out of the different cell wall degrading enzymes xylanase, chitinase and β-glucanases are associated with bio-control role while cellulases play a dual role, they are important for phyto-pathogenic effect as well as for industrial production also [3-5]. Todays, demand of cellulase derived from the microorganisms is gaining popularity all over the world. Solid state fermentation is gaining popularity for enzyme production as it is very simple and economical [6-8].

The filamentous fungi *Trichoderma* is an important fungus used to produce enzymes by fermentation process. This genus secretes large amounts of cellulase and hemicellulase enzymes capable of degrading carbohydrate polymer [9,10]. Solid state fermentation is a popular technique that is often employed for the enzymes due to some practical and economic advantages. The advantages associated with solid state fermentation are low capital costs for equipment and operating, high volumetric productivity, lower space requirements and easier downstream processing [11]. Fungi are able to degrade cellulose, hemicellulose and lignin by a complex set of excreted hydrolytic and oxidative enzymes which degrade plant tissues [12]. In *Trichoderma* it is very difficult to differentiate *Trichoderma* genus in cellulase producing and non cellulase producing taxa. Fungi belong to one of the five kingdoms in ecosystem, which was defined by Robert Whittaker based on the way of taking nutrients into the cells [13]. *Trichoderma* strains have been used in cellulase production for two decades. The rich cellulase-producing strains were soon isolated, including the strain *Trichoderma viride QM6a* first selected from a soil sample at Bougainville Island. Cellulases are distinguished from other glycoside hydrolases by their ability to hydrolyze specifically the β-1,4 glucosidic bonds [14]. The enzymatic degradation of β-1,4 linkages in cellulose polymer result by acid hydrolysis. Natural cellulosic substrates (especially of plant cell walls) are polysaccharide chains composed of different degrees of crystalinity and microfiber morphology [15]. To degrade these materials, organisms produce a number of enzymes, generally called enzymatic systems [16]. Cellulase systems are not just a cluster of enzymes (endoglucanases, exoglucanases and β-glucosidase with or without carbohydrate-binding module), each one acting in a coordinated manner to a more efficient hydrolysis of cellulose [17] from different substrates such as cotton fiber or textiles. The aim of our study was to prepare a cellulase crude enzyme from *Trichoderma* using solid state fermentation.

Fungal genera like *Trichoderma* and *Aspergillus* are thought to be the good cellulase producers [18]. Crude enzymes produced by these microorganisms are commercially available for agricultural and industrial uses [19].

Materials and methods

Isolation and maintenance of cultures

Trichoderma species were isolated from the different locations of Uttar Pradesh. It was grown and maintained on PDA thereafter submitted to the Indian Type Culture Collection at IARI (Pusa, New Delhi) and allotted with specific ITCC numbers.

Enzyme production

The cultures were grown in 250 ml Erlenmeyer flask that contained 50 ml of basal salt medium [20]. The pH of the medium was adjusted to 6.5 prior to sterilization. The flasks were inoculated with 2 agar discs (2 mm in diameter) of 7 days old culture from PDA plates and were incubated under stationary condition at 28°C up to 7 days. Mycelium was separated from the culture broth through filtration and the obtained filtrate was centrifuged at 11000 x g for 10 min to remove mycelium. The obtained supernatant is served as a crude enzyme source.

Enzyme assay

Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method of Hankin and Anagnostakis [21]. Aliquots of approximately diluted culture

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filtrate as enzyme source was added to whatman no. 1 filter paper strip (1 x 6 cm; 50 mg) immersed in one milliliter of 0.05 M Sodium citrate buffer of pH 5.0. After incubation at 50 ± 2°C for 1 hrs, the reducing sugar released was determined by dinitrosalicylic acid (DNS) method [22]. One unit of filter paper (FPU) activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar from filter paper per ml per min. Endoglucanase activity (CMCase) was measured using a reaction mixture containing 1 ml of 1% carboxymethyl cellulose (CMC) in 0.5 M citrate acetate buffer (pH 5.0) and aliquots of suitably diluted filtrate. The reaction mixture was incubated at 50 ± 2°C for 1 h and the reducing sugar produced was determined by DNS method [23]. One unit (IU) of endoglucanase activity was defined as the amount of enzyme releasing 1 µmole of reducing sugar per min.

Endoglucanase assay

0.5 ml of the enzyme solution was added into test tubes. The enzyme and substrate solution were equilibrated at 50°C. 0.5 ml of the CMC solution was taken into the test tubes and mixed well. Incubated at 50°C for 30 min. 3.0 ml of DNS solution was added and mixed well, boiled for exactly 5.0 min in vigorously boiling water. Place the tubes in an ice-cooled water bath to quench the reaction. Add 20 ml of distilled water. Mix by inverting the tubes several times. Absorbance was taken at 540 nm. Enzyme activity is expressed as IU/ml/min.

Optimization of culture conditions for enzyme production

Thermal stability of enzymes: For thermal stability study enzymes were incubated at 50°C for the time period (40 min-72 h). Then determine enzymatic activity by DNS method.

Effect of pH and temperature on enzyme production: The most suitable pH for the enzyme production was determined by adjusting the pH of the culture medium at different levels in the range of pH 3 to 9 using different buffers. In order to determine the effective temperature for cellulase production by the *Trichoderma* fermentation was carried out at 10°C intervals in the range of 20 to 80 ± 2°C.

Effect of carbon sources on enzyme production: Effect of various carbon compounds viz., cellulose, CMC, glucose, sucrose and maltose were studied for cellulase production. The broth was distributed into different flasks and 1% of each carbon sources were then added and incubated for 7 days at 45 ± 2°C.

Results and Discussion

Isolation and identification of cellulytic *Trichoderma* species (Table 1 and Figure 1).

Effect of carbon sources on enzyme production: Data presented in Table 2, Figure 2, showed that cellulase production by *Trichoderma* species was significantly influenced by the type of carbon source in the basal salt medium. Corn cob was the most effective carbon source for cellulase enzyme production followed by wheat bran, filter paper, sucrose and maltose Table 3 and Figure 3.

Effect of pH and temperature on enzyme production: The best pH for enzyme production was recorded between 4-6. Effect of pH on cellulase production by these fungi supports the findings of [24] who reported that CMCase and FPase activities exhibit a pH optimum between 4-7. The optimum temperature range was found between 30-40°C. Many workers have reported different temperatures for maximum cellulase production either in flask or in fermenter studies using *Trichoderma* sp. suggesting that the optimum temperature for cellulase production also depends on the strain variation of the microorganism [25,26].

Name of Bioagent	Culture No.	Source/ District	Id. No.
T. harzianum	*Th* azad	CSA Kanpur Nagar	6796
T. viride	01PP	Hardoi	8315
T. asperellum	T_{asp}/CSAU	CSA Kanpur Nagar	8940
T. koningii	T_K (CSAU)	CSA Kanpur Nagar	5201
T. atroviride	71 L	Hardoi	7445
T. longibrachiatum	21 PP	Kaushambi	7437
T. virens	T_{vi} (CSAU)	CSA Kanpur Nagar	4177
T. reesei	Tr(CSAU)	CSA Kanpur Nagar	7284

Table1: Identification of potential *Trichoderma* sp.

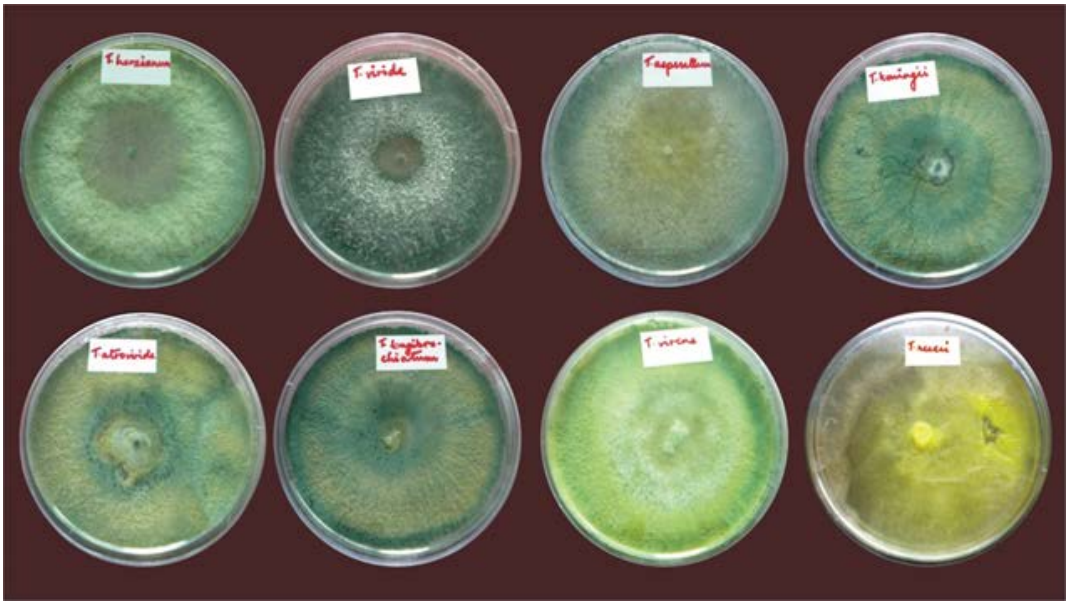

Figure 1: Eight different isolated strains of *Trichoderma*.

Carbon source (1%)	*T. harzianum*	*T.reesei*	*T. viride*	*T. koningii*	*T. atroviride*	*T. longibrachiatum*	*T. virens*	*T. asperellum*	CD at 5%
Maltose	0.19	0.17	0.14	0.11	0.08	0.1	0.06	0.08	**0.2408**
Corn Cob	1.21	1.1	1.04	0.65	0.39	0.38	0.39	0.45	**0.2693**
Wheat Bran	0.89	0.7	0.73	0.6	0.35	0.3	0.32	0.4	**0.2154**
Sucrose	0.45	0.3	0.3	0.25	0.12	0.13	0.1	0.16	**0.2693**
Filter Paper	0.56	0.44	0.34	0.26	0.1	0.09	0.16	0.18	**0.0762**

Table2: Cellulase filter paper activity produced by *Trichoderma* species grown on different carbon sources.

Carbon source (1%)	*T. harzianum*	*T.reesei*	*T. viride*	*T. koningii*	*T. atroviride*	*T. longibrachiatum*	*T. virens*	*T. asperellum*	CD at 5%
Maltose	0.23	0.19	0.16	0.15	0.1	0.07	0.07	0.09	**0.1408**
Corn Cob	1.15	1.04	1	0.76	0.45	0.45	0.37	0.31	**0.2930**
Wheat Bran	0.87	0.76	0.73	0.56	0.34	0.38	0.30	0.27	**0.1540**
Sucrose	0.24	0.14	0.18	0.1	0.09	0.08	0.1	0.09	**0.2193**
Filter Paper	0.56	0.44	0.34	0.3	0.25	0.2	0.18	0.17	**0.0462**

Table3: Endoglucanase activity produced by *Trichoderma* species grown on different carbon sources.

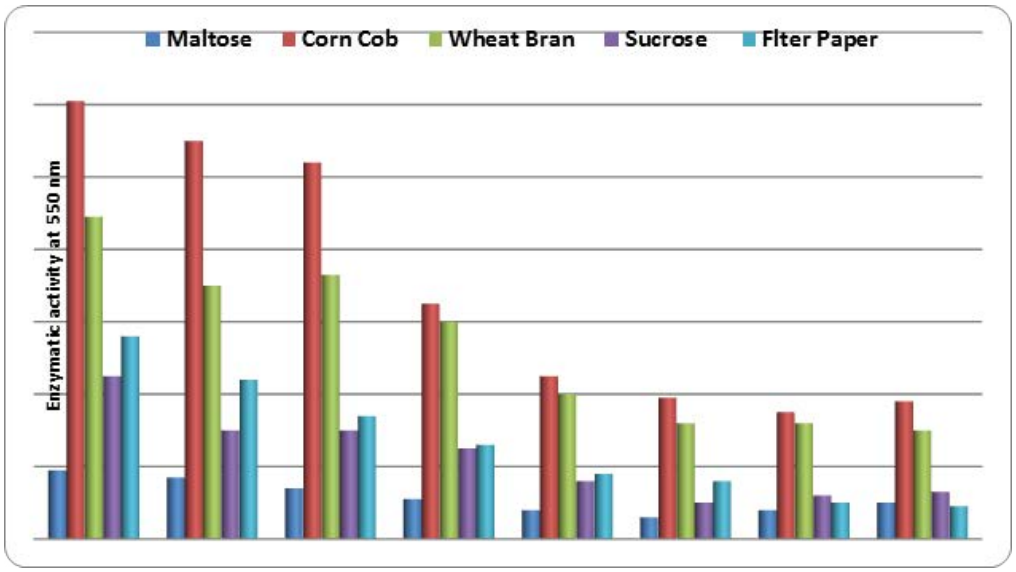

Figure 2: Effect of different carbon sources on FP- activity.

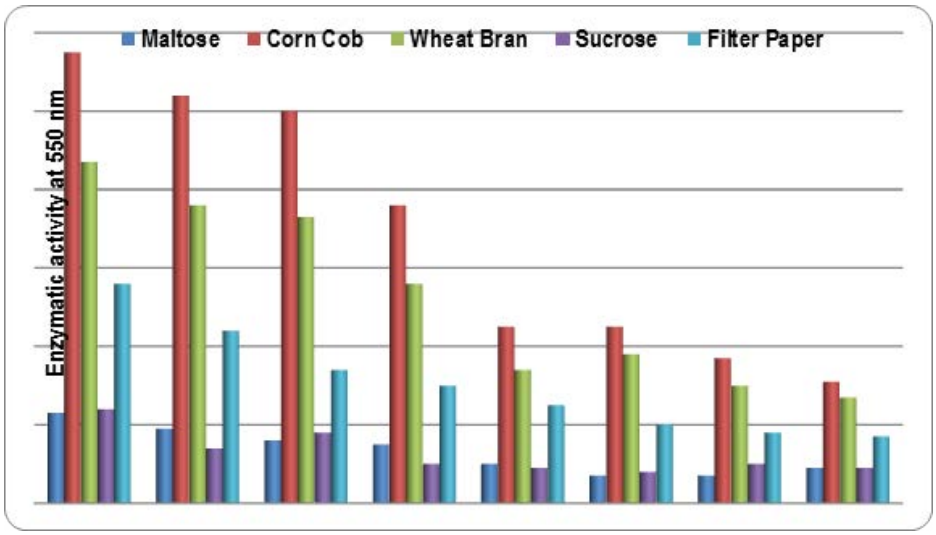

Figure 3: Effect of different carbon sources on endoglucanase activity.

Conclusion

A high cellulase yield was produced by *Trichoderma* spp. using corn cob as carbon source. This crude cellulase enzyme obtained by using the cheaper carbon source (Corn cob) represents an alternative for industrial applications. The main aim of this study was to analyze the *Trichoderma* spp. for cellulase production. To our knowledge this is the first report which shows the potential of eight *Trichoderma* species for cellulase production.

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