

Production of cellulases by fungal cultures isolated from forest litter soil

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Abstract. The aims of this study were the isolation and screening of fungal cultures from forest litter soil for cellulases production. Four fungal cultures were isolated and identified. Among these fungal cultures, three belonged to the genus *Aspergillus* and one belonged to the genus *Penicillium*. These fungal cultures were tested to find their ability to produce cellulases, that catalyze the degradation of cellulose, which is a linear polymer made of glucose subunits linked by β -1, 4 glycosidic bonds. The fungal isolate 3 (*Aspergillus* sp.) was noticed to show maximum zone of hydrolysis of carboxy-methyl cellulose and produce higher titers of cellulases including exoglucanase, endoglucanase and β -D-glucosidase. The activities of the cellulases were determined by Filter paper assay (FPA), Carboxy-methyl cellulase assay (CM-Case) and β -D-glucosidase assay respectively. The total soluble sugar and extracellular protein contents of the fungal filtrates were also determined.

Keywords forest litter soil, fungal cultures, screening of cellulases, protein content.

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Introduction

Forest ecosystem produces a lot of organic matter in the form of leaves, twigs, branches, fruits and reproductive parts, such as flowers, seeds, spores (Tandel et al. 2009). Plant residues added to the soil are transformed into CO₂, microbial material and relatively stable humus components (Shields et al. 1973). Cellulose is the abundant component of plant bio-

mass. It is found in nature almost exclusively in plant cell walls and also produced by some bacterial species (Lynd et al. 2002). Perpetual renewal of plant biomass via the process of photosynthesis ensures an inexhaustible supply of such organic matter. Plant biomass rich in cellulose is one of the foreseeable and sustainable sources of fuel, animal feed and feed stock for chemical synthesis (Bhat 2000). Cellulose has enormous potential as a renewable

source of energy (Coral et al. 2002). Therefore, the degradation of cellulosic biomass represents an important part of the carbon cycle within the biosphere (Beguin & Aubert et al. 1994). Bioconversion of cellulosic biomass to fermentable sugars through biocatalyst cellulases derived from cellulolytic organisms has been suggested as a feasible process and offers potential to reduce the use of fossil fuels and reduce environmental pollution (Dale 1999, Lynd et al. 1999). Any process which could efficiently and economically convert cellulosic material to glucose would be of important industrial significance (Walsh 2002). Cellulases provide a key opportunity for achieving tremendous benefits of biomass utilization (Wen et al. 2005). Fungi are well known agents of decomposition of organic matter, in general, and of cellulosic substrate in particular (Lynd et al. 2002). Even though there are many reports on fungi producing cellulases (Shin et al. 2000), only a few have proved high activities for commercial success (Kang et al. 1994, Elad 2000). A wide range of *Aspergillus* sp. have been identified to possess all components of cellulases complex (Vries & Visser 2001). Cellulases have been used for several years in food processing, feed preparation, waste-water treatment, detergent formulation, textile production and other areas. Additional potential applications include the production of wine, beer and fruit juice. Nevertheless, all these uses are of rather small magnitude compared with cellulase requirements for bioconversion of lignocelluloses to fuel ethanol (Philippidis 1994). In view of biotechnological importance of cellulases, the present study emphasizes on screening of fungal cultures for cellulases production.

Materials and methods

In the present study, *Aspergillus* sps. and *Penicillium* sp. were isolated from soil (collected at SVU Forest, Tirupati, Andhra Pradesh, In-

dia) by serial dilution method. 1 g of soil was transferred to 10 ml of distilled water in test tubes. Dilutions were made up to 10^{-6} and 0.1 ml of soil suspension was spread on to the sterilized Czapek-Dox agar medium with following composition (g/l): sucrose – 30, NaNO_3 – 2, K_2HPO_4 – 1, MgSO_4 – 0.05, KCl – 0.5, FeSO_4 – 0.01, Agar agar - 20. pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lbs pressure, 20 ml of sterile medium were transferred to sterile Petri plates and allowed for solidification. After solidification of the medium 0.1 ml of soil suspension was spread with the help of spreader and incubated at 28°C for 7 days. The fungal cultures grown on the medium were transferred on to the Potato dextrose agar slants and maintained at 4°C for further studies.

Preparation of fungal spore inocula. The isolated fungal cultures were grown on potato-dextrose agar slants. They were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for 7 days. After incubation, 3 ml of sterile distilled water were added to each slant. Fungal spore concentration was determined by haemocytometer. An Inoculum density of $2 \cdot 10^6$ spores (Narasimha et al. 2006) was used for screening of cellulolytic fungi as well as cellulase production on Czapek-Dox broth medium amended with 1% cellulose.

Screening of cellulolytic fungi. The isolated fungal cultures were screened for their ability to produce cellulases complex following the method of Teather & Wood (1982). Czapek-Dox medium used in this method contained (g/l): sucrose – 30, NaNO_3 – 2, K_2HPO_4 – 1, MgSO_4 – 0.05, KCl – 0.5, FeSO_4 – 0.01, carboxy- methyl cellulose – 1%, Agar agar - 20. pH of the medium was adjusted to 5. After autoclaving at 121°C and 15 lbs. pressure, the medium was poured into Petri plates and allowed to solidify. Cavities of 6 mm size were made in the solidified medium and inoculated with 0.1 ml of spore suspension prepared from 7 day old slants. The plates were incubated at room temperature ($28 \pm 2^\circ\text{C}$) for three days to

allow fungal growth, then again incubated for 18 h at 50 °C which is the optimum temperature for cellulases activity. After incubation, 10 ml of 1% Congo - Red staining solution was added to the plates that were shaken at 50 rev/min for 15 min. The Congo - Red staining solution was then discarded, 10 ml of 1 N NaOH was added to the plates and shaken again at 50 rev/min for 15 minutes. Finally 1 N NaOH was also discarded and the staining of the plates was analyzed by noticing the formation of yellow zones around the fungal spore inoculated wells.

Identification of fungal cultures. The pure fungal cultures were obtained by repeatedly transferring on to fresh potato dextrose agar (PDA) plates and each pure culture was inoculated separately on to PDA slants and maintained at 4 °C in a refrigerator. Cultural characteristics such as color and size of colonies during the growth was monitored and recorded. A little amount of mycelial mat was taken with a sterilized needle and placed on clean glass slide and then stained with lactophenol cotton-blue and covered by cover slip and mounted with nail polish. This mounted slide was microscopically analyzed for morphological characteristics of mycelia, conidiophores/fruiting bodies and conidia. Size of conidiophores/fruiting bodies and conidia were measured by using micrometer and shape of spores was recorded. Fungal isolates were identified by these characteristics with those listed in standard reference books (Domsch et al. 1980).

Production of cellulases from fungal isolates. The isolated fungal cultures, *Aspergillus* sps. and *Penicillium* sp. were used to know their potential for cellulases production. A volume of 100 ml of Czapek-Dox broth medium amended with 1% cellulose was distributed into separate 250 ml Erlenmeyer conical flasks. pH of the medium was adjusted to 5. After autoclaving at 121 °C and 15 lb. pressure, the flasks were inoculated with the fungal spore suspensions. The flasks were in-

culated at 32 °C on a rotary shaker at 120 rpm for 7 days. After incubation, the contents of the flasks were passed through Whatman filter paper No.1 to separate mycelial mat from culture filtrate. The filtrate thus obtained was used for the estimation of biomass, extracellular protein content, total soluble sugar and total activity of cellulases such as Filter paper activity, endoglucanase and β -glucosidase.

Analytical methods

Extracellular protein content. After 7 days of incubation, the contents of the flasks were aseptically passed through Whatman No.1 filter paper to separate mycelial mat from culture filtrates. An aliquot of this culture filtrates was used for estimation of extracellular protein content according to the method of Lowry et al. (1951). Bovine serum albumin was used as protein standard. Suitable aliquots of filtrates were mixed with 5 ml of alkaline solution. After 30 min, 0.5 ml of appropriately diluted Folin-Ciocalteu reagent was added. The color developed was read at 550 nm by using the spectrophotometer (ELICO, 171).

Estimation of total soluble sugar. The total soluble sugar content in the culture filtrates was determined according to the method of Miller (1959). Glucose was used as sugar standard. Suitable aliquots of culture filtrates were mixed with 3 ml of DNS reagent. The contents were boiled vigorously in a boiling water bath for exactly five minutes and the color developed was read at 540 nm by using the spectrophotometer (ELICO, SL 171).

Determination of fungal biomass. After appropriate incubation period (7 days) the contents of the flasks were aseptically passed through pre-weighed Whatman No.1 filter paper to separate mycelial mat from culture filtrates. The filter papers along with mycelial mat were dried at 70 °C in an oven until constant weight and the weight was recorded. Difference between the weight of the

filter paper bearing mycelial mat and weight of pre-weighed filter paper represented fungal biomass, which was expressed in terms of dry weight of mycelial mat (mg/flask).

Cellulases assay. Flasks containing growing culture of fungal isolates were withdrawn at every 7-day interval for processing. The filtrates obtained after removal of mycelial mat by filtration through Whatman No.1 filter paper were used as an enzyme source.

Filter paper assay (FPA). Filter paper activity of the culture filtrates was determined according to the method of Mandels and Weber (1969). Whatman filter paper strips containing 50 mg weight was suspended in 1 ml of 0.05 M sodium citrate buffer (pH 4.8) at 50 °C in a water bath. Suitable aliquots of enzyme source were added to the above mixture and incubated for 60 minutes at 50 °C. After incubation, the liberated reducing sugar was estimated by the addition of 3, 5-dinitrosalicylic acid (DNS). After cooling, the color developed in tubes was read at 540 nm by using the spectrophotometer (ELICO, SL 171). Appropriate control without enzyme was simultaneously run. Activity of cellulases was expressed in filter paper units. One filter paper unit (FPU) was defined as the amount of enzyme releasing 1 μ mole of reducing sugar from filter paper /ml /h.

Endoglucanase assay. Activity of endoglucanase in the culture filtrates was quantified by carboxy-methyl cellulase method (Ghosh 1987). The reaction mixture with 1.0 ml of 1% carboxymethyl cellulose in 0.2 M acetate buffer (pH 5.0) was pre-incubated at 50 °C in a water bath for 20 minutes. An aliquot of 0.5 ml of culture filtrate with appropriate dilution was added to the reaction mixture and incubated at 50 °C in water bath for one h. Appropriate control without enzyme was simultaneously run. The reducing sugar produced in the reaction mixture was determined by Dinitro-salicylic acid (DNS) method (Miller 1959). 3, 5-dinitro-salicylic acid reagent was added to aliquots of the reaction mixture and the color developed was read at wavelength 540 nm

by using the spectrophotometer (ELICO, SL 171). One unit of endoglucanase activity was defined as the amount of enzyme releasing one μ mole of reducing sugar /ml /h.

β - D-Glucosidase assay. Activity of β - glucosidase in the culture filtrates was quantified based on the method of Herr (1979). For the determination of β - D-glucosidase activity the assay mixture contained 0.2 ml of 5mM p-nitrophenyl β - D-glucopyranoside (PNGP) in 0.05 M citrate buffer pH 4.8 and 0.2 ml of diluted enzyme solution with appropriate controls. After incubation for 30 min at 50 °C, the reaction was stopped by adding 4 ml of 0.05 M NaOH-Glycine buffer (pH 10.6) and the yellow colored p - nitrophenol liberated was determined at 420 nm by using the spectrophotometer (ELICO, SL 171). One unit of β - glucosidase activity was defined as the amount of enzyme liberating 1 μ mole of p - nitrophenol /ml /h under standard assay conditions.

Results

The fungal cultures *Aspergillus* sps. and *Penicillium* sp. isolated from forest litter soil were screened for their cellulolytic activity. All the fungal cultures produced zones of hydrolysis in CMC agar plates within 3 days and results were represented (fig.1, table.1). Among the four fungal isolates, *Aspergillus* sp. (isolate 3) was detected to produce maximum zone of hydrolysis (4.2 cm) of carboxy- methyl cellulose whereas isolates 1, 2 and 4 produced zone diameters of 2.7, 2.3 and 4.0 cm respectively. The isolated fungal cultures were identified up to genus level based on their morphological and microscopic features and the results were represented in table. 2. Isolates 1, 3, 4 were identified as *Aspergillus* sps. and isolate 2 as *Penicillium* sp.. The isolated fungal cultures were grown on Czapek-Dox broth medium amended with 1% cellulose as substrate (Narasimha et al. 2006) for the production of cellulases and the results were shown

in figures 2,3and table.3. In the present study, *Aspergillus* sp. (Isolate 3) produced high titers of Filter paper assay (FPase)(14.16 U/ml) Carboxy-methyl cellulase assay (CMCase)(64 U/ml) β - glucosidase (0.014 U/ml) total soluble sugar (7.11 mg/ml), biomass (1560 mg/ml) and extracellular protein content (1.65 mg/ml) in comparison to the remaining fungal isolates. FPase activity of 12.77U/ml and CMCase of 43.32 U/ml were exhibited by isolate 2, which showed its efficiency next to isolate 3 (*Aspergillus* sp.). When compared with cellulases activity of isolate 3, isolates 1 and 4 exhibited lower activities of FPase (0.44-0.72 U/ml), CMCase (5.55-5.66 U/ml) and β - glucosidase (0.006-0.014 U/ml), as well as lower contents of total soluble sugar (0.06-0.33 mg/ml), extracellular protein (0.35-0.38 mg/ml) whereas least dry mass of fungal mat (1240 mg/flask) was recorded with isolate 4.

Discussion

The cellulase activity of fungal cultures, *Aspergillus* sp. and *Penicillium* sp. was confirmed by Congo Red Dye decoloration and also quantitatively with dinitro-salicylic acid reagent method. The results thus obtained by the above methods were very much the same and matched with earlier reports of Sazci et al. (1986). Isolates 1, 3, 4 were identified as *Aspergillus* sps. and isolate 2 as *Penicillium* sp. Narasimha et al. (1998) isolated *Aspergillus* and *Penicillium* sps.from soil contaminated with cotton ginning effluents and made similar identification reports. In the present investigation, *Aspergillus* sp. was shown to posses higher cellulose degrading ability. Similarly reports were made by other authors, Sadaf Jahangeer et al. (2005) reported that majority of *Aspergillus* and *Penicillium* sps. were found to posses cellulolytic activity. The most ac-

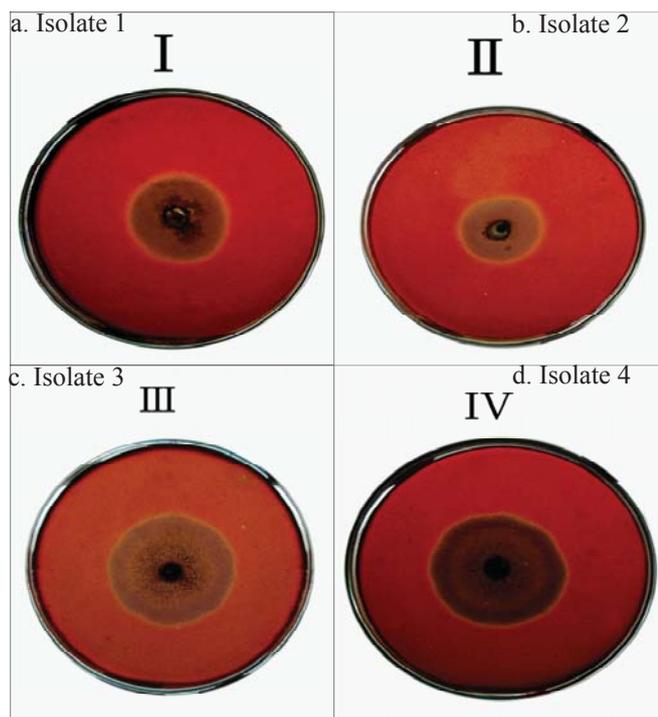


Figure 1 Screening of cellulolytic fungal cultures on CMC agar plate

Table 1 Biometric features of cellulolytic fungal cultures

S.No.	Fungal isolates	Colony Color	Colony diameter (cm) on CMC agar plates	Zone of hydrolysis on CMC Agar plates (in cm)
1.	1	Pale Yellow	1.0	2.7
2.	2	Bluish Green	0.8	2.3
3.	3	Brown	2.8	4.2
4.	4	Black	2.8	4.0

Table 2 Macroscopic features and microscopic characteristics of fungal cultures isolated from forest litter soil

Fungal isolate	Macroscopic features		Microscopic characteristics		
	Colony color	Colony diameter (cm)	Conidiophores length (mm)	Conidia diameter (μ m)	Conidia Shape
1.	Pale yellow	3.3	1.50	2.2	Slightly ellipsoidal
2.	Bluish green	2.2	0.84	2.8	globose
3.	Brown	2.8	1.80	4.6	Globose
4.	Black	1.9	2.42	4.2	Globose

Table 3 Cellulases production of fungal isolates

S.No	Fungal Isolate	FPase ^a (U/ml/hr)	CMCase ^b (U/ml/hr)	β -glucosidase ^c (U/ml/hr)
1.	1	0.722	5.55	0.006
2.	2	12.770	43.32	0.006
3.	3	14.160	64.00	0.014
4.	4	0.440	5.66	0.014

Note: Values represented in the table are averages of results of two separately conducted experiments: a. FPase was expressed in terms of filter paper units (U/ml). One filter paper unit is defined as the amount of enzyme releasing one μ mole of reducing sugar from filter paper per ml per hour; b. One unit of CMCase activity was defined as amount of enzyme releasing one μ mole of reducing sugar from filter paper per ml per hour; c. One unit of β -glucosidase was defined as amount of enzyme liberating one μ mole of *p*-nitro phenol per ml per hour.

tive producer of cellulolytic enzymes was *A. terreus* compared to *P. tigrinus*, *P. ostreatus*, *F. fomentarius* (Mirzaakhmedov et al. 2007). *Aspergillus niger* isolated from soil contaminated with effluents of cotton ginning industry showed highest cellulase activity (Narasimha et al. 1998). A wide range of *Aspergillus* sp. has been identified to possess all components of cellulases complex (Vries & Visser 2001).

Conclusion

In the present study, it could be concluded that the fungal cultures isolated from forest litter soil possess cellulolytic activity. Among these fungal cultures, *Aspergillus* sp. (isolate 3) was noticed to show maximum zone of hydrolysis (4.2 cm) of carboxy - methyl cellulose. It also produced high titers of Filter paper assay (FPase) (14.16 U/ml) Carboxy-methyl cellu-

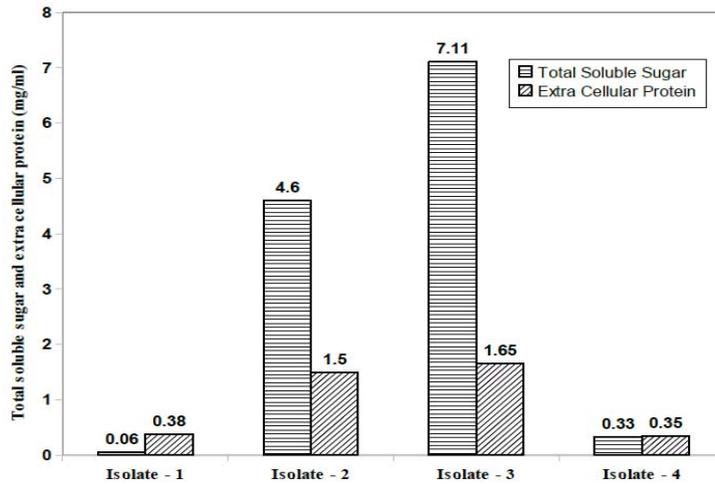


Figure 2 The total soluble sugar and extracellular protein contents of fungal filtrates

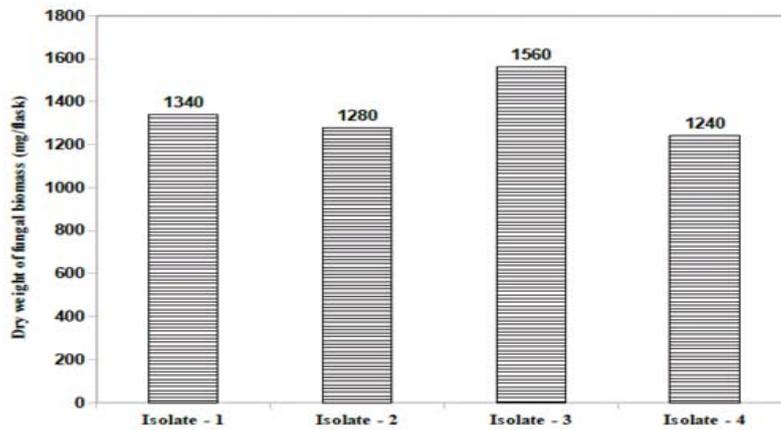


Figure 3 Dry weight biomass of fungal isolates grown in Czapek-Dox broth

Note: Values represented in the fig 2, 3 are averages of results of two separately conducted experiments

lase assay (CMCase (64 U/ml) β - glucosidase (0.014 U/ml), total soluble sugar (7.11 mg/ml), biomass (1560 mg/ml) and extracellular protein content (1.65 mg/ml). The fungal cultures isolated in the present investigation need to be further studied in depth for their cellulolytic potential for conversion of cellulosic waste material into useful products.

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