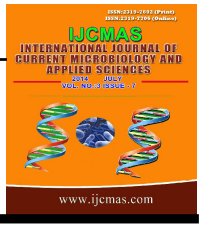
International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 3 Number 7 (2014) pp. 761-771**
http://www.ijcmas.com



Original Research Article

Screening, Identification and Isolation of Cellulolytic fungi from soils of Chittoor District, India

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A B S T R A C T

This study focused on the evaluation of cellulolytic fungi isolated from different soil samples collected in selected sites of chittoor district and Twenty three fungal strains were isolated and among which nine isolates found to have cellulolytic activity (βG, FPase, and CMCase). Identified strains are *Aspergillus niger, Aspergillus flavus, Nigrospora sphaerica, Chaetomium globosum, Cladosporium cladosporides, Trichoderma sp, Penicillium sp. Fusarium oxysporium, Acremonium sp.* All the selected strains were studied by cultural morphological characteristics and microscopic examinations. Preliminary quantification of Fungal load and physico-chemical properties of soils at selected sites was screened. To evaluate enzyme production and to test the functional abilities of screened fungal strains, isolates were inoculated onto PDA and measured after 7 days of incubation at different and highest growth of *Aspergillus niger* was found to be optimum at 30°C and pH 6. This study showed that the fungal isolates with appreciable cellulose degradation property. The highest cellulase producing isolate was *Aspergillus niger*, *Aspergillus flavus* and the least was *Trichoderma sp.* The best cellulolytic activity (βG, FPase, and CMCase) from the crude extract were measured at different pH and temperature values for all three enzymes were obtained at the same conditions with 5.0 g of substrate at 25 - 30°C and pH 5.0 - 6.0 with 72 hr of incubation. The enzyme production optimization demonstrated clearly the impact of the process parameters on the yield of the cellulolytic enzymes.

Keywords

Cellulolytic, Fungal strains, Chittoor District

Introduction

Fungi constitute a group of microorganisms that are widely distributed in environment especially in soil (Boer *et al*., 2005). Since they produce wide variety of hydrolytic enzymes and hence exist in nature in saprophytic mode (Ng TB, 2004).

Fungi are one of the dominant groups present in soil which strongly influence ecosystem structure and functioning and thus playing a key role in many ecological services.(Orgiazzi A *et al*., 2012). Therefore, there is a growing interest in assessing soil biodiversity and its biological functioning (Barrios E, 2007). At the ecosystem scale, extracellular enzyme activity is influenced by organic matter abundance and composition (Sinsabaugh RL *et al*., 2008).

Cellulose is the world's most abundant natural biopolymer and a potentially important source for the production of industrially useful materials such as fuels and chemicals. Degradation of the cellulosic materials is achieved chemically, enzymatically or by the combination of both chemical and enzymatic methods (Bailey MJ and Poutanen K, 1987; Christov LP *et al.*, 1999; Spreint A and Antranikian ,1990 and Xia L and Cen P, 1999).

Cellulases bring about the hydrolysis of cellulose, a homopolymer of β-1,4 linked glucose units that comprises amorphous and crystalline regions, by synergistic action of its constituent enzymes (Bhat M and Bhat S,1997). Cellulases are group of extracellular enzymes commonly employed in many industries for the hydrolysis of cellulolytic material.(Mahmood RT *et al.*, 2013).

β-glucosidase is generally responsible for the regulation of the whole cellulolytic process and is a rate-limiting factor during enzymatic hydrolysis of cellulose (Harhangi HR *et al.*, 2002). Klyosov (1990) described three major types of cellulases *viz.*, endoglucanase (endo-cellulase), which breaks down internal bonds to disrupt the crystalline structure of cellulose and expose individual cellulose polysaccharide chains; cellobiohydrolase (exo-cellulase) which cleaves 2-4 units from the ends of the exposed chains produced by endocellulase and cellobiase (Beta-extracellulase) which either hydrolyses cellobiose into individual monosaccharides such as glucose or depolymerizes cellulose by radical reactions.

Adverse spectrum of extensively studied mesophilic fungi degrades organic material aerobically (Falcon MA *et al*., 1995). Nearly, all the fungi that have been reported for the production of cellulases are mesophilic fungi and the best known cellulase producers include *Trichoderma* sp., *Aspergillus* sp., *Acremonium* sp., *Penicillium* sp., *Rhizopus* sp., *F. solani* and *Chaetomium* sp., among other mesophiles (Kuzmanova S *et al.*, 1991; Teeri T and Koivula A, 1995; Bhat M and Bhat S, 1997; Schulein M, 1997). Cellulose enzymes have a wide range of applications,economic importance in agriculture, biotechnology, and bioenergy (Nathan VK *et al.*,,2014; Phitsuwan P *et al*., 2012 & Herculano PN *et al*., 2011). Cellulases have been hailed as candidates for the production of pharmaceutically relevant proteins for therapeutic use. (Nevalainen H and Peterson R, 2014).

Hence research was attempted in this way in order to find more efficient cellulase producing strain capable of degrading. As cellulases have different important industrial applications it is imperative to screen new strains for efficient cellulase production. Thus, the present study was carried out as a first step in screening different fungal strains for cellulolytic enzyme production.

Materials and Methods

Selection of sample sites

Sampling sites of the present study in Suitable sites of Chittoor District were selected within the study area. Nine sites were chosen *viz.*,Srikalahasthi(L:1),Horsley Hills(L:2),Kuppam(L:3), Chittoor(L:4), Nagiri(L:5), Satyavedu(L:6),Punganur(L:7), Nagapatla Reserve Forest(L:8) and Mamandur (L:9) in Chittoor District of Andhra Pradesh.(Figure:1).

Collection of soil sample

Soil samples were collected at different sample points from the undisturbed localities employing sterile soil augers, hand

trowel and polythene bags (Akinyanju JA and Fadayomi O,1989). The soil was dug out using augers up to 20cm depth and was immediately scooped into sterile polythene bags using the hand trowel. The samples were collected from 2 spots in each site and then mixed together in order to obtain a representative sample (Mareckova SM *et al*., 2008). Isolation Of microorganisms and primary screening was done according to the method followed by Vega K *et al*., 2012). Field moist soil samples were composited, 2 mm sieved, and then stored at −10°C for a maximum of 1 week until enzyme analyses could be performed.

Primary Screening for Cellulolytic Activity

All the samples were moistened (1:1 ratio) with distilled water (sterilized in autoclave at 15 lb/in2 and 121°C for 15 minutes) and mixed to confine homogeneity (Figure:2). These samples were packed in polythene bags and incubated at 50±1°C for about three days. The isolated fungus was grown on basal salt media supplemented with 5% carboxymethylcellulose (Lingappa Y and Lockwood JL, 1962). The pure cultures were inoculated in the centre with almost equal amounts and incubated at 30 ± 2°C until substantial growth was recorded. The Petri plates were flooded with Congo red solution (0.1%), and after 5min the Congo red solution was discarded, and the plates were washed with 1N NaCl solution, allowed to stand for 15–20 minutes. The clear zone was observed around the colony when the enzyme had utilized the cellulose. Cellulolytic fungi were screened on the basis of their ability to hydrolyze cellulose by forming diameter zone of clearance around the fungal colony (Hankin L and Anagnostaksis L, 1975) (Table 1).

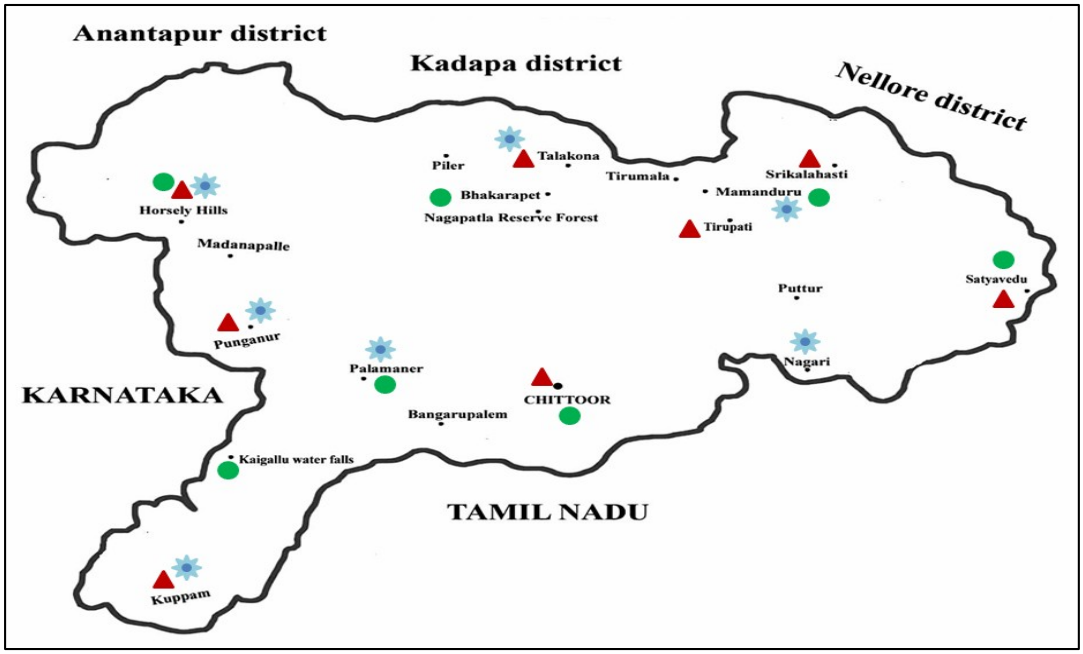
Characterization and Identification of fungal Isolates

Fungal colonies were isolated form soil samples enriched for cellulase producing microorganisms by serial dilution method wherein, PDA (potato dextrose agar) media was prepared, autoclaved and poured in sterile petriplates. 50µl of soil samples diluted upto 10^{-5} dilutions were spread on respective solidified PDA plates. The inoculated petriplates were incubated at 28°C for 48 hours. The isolates were further inoculated on sterile PDA plates by point inoculation and incubated at 28°C for 48 hours in order to obtain pure fungal plates. Colonial morphology and microscopic examinations of the various isolates of pure cultures were used to determine the reproductive and vegetative structures. Consequently, identification was done using Onion AHS *et al.*, (1981). Spore identification was achieved by reference to Spore atlases of Gregory PH (1973) and Anna LS (1990) (Table 2).

Cellulase assay for Enzyme production

The activity of β-glucosidase (βG), total cellulase (FPase) and endoglucanase (CMCase) was studied as cellulolytic activity. Filter paper activity (FPase) for total cellulase activity in the culture filtrate was determined according to the standard method (Eveleigh DE *et al*.2009). CMCase (carboxy methyl cellulase) activity was assayed using Dinitrosalicylic acid (DNS) method (Mandels and Weber, 1969). One unit of enzyme activity was defined as the amount of enzyme required to release 1 µ mol reducing sugars per ml under standard assay condition (Gilna and Khaleel 2011).

Figure.1 Selection of Sample Sites In Chittoor District



After incubation, the FPase and CMCase activities were measured for their values of pH and temperature optimum. For determination of the thermal stability, the crude enzymatic extract was incubated at temperatures ranging from 30 to 90 °C for 90 min. After that, the FPase and CMCase activities were determined following the method of Herculano PN *et al.*,(2011).

Results and Discussion

In this investigation, we have navigated the cellulolytic fungi from different localities of Chittoor district. Cellulase producing strains isolated from different soil samples were subjected to optimization of media and cultivation parameters for cellulose production. Optimizing of conditions was performed to attain the maximum cellulase enzyme activity with the fungal isolate.

Cellulase activity was quantified from 3rd day to 7th day to understand the enzyme production. The fungal strains were identified for their cellulolytic ability on plate clearing assay by using carboxy methyl cellulose and Congo red dye and by the formation of clear zone diameter. Plates having agar medium without any carbon source were treated as control, which showed no signs of growth and there was no clear zone formation (0 mm) observed, indicating no fungal growth and no enzyme activity. Based on visual observance, three isolates gave clear zones of cellulase activity having diameter 5 mm to 15mm with the enzyme reaction is shown in Figure 1.

During optimization studies, the enzyme activity was analysed only after 3rd day of incubation to allow the optimal fungal growth to be achieved. It was reported earlier that the enzyme production by the fungi started after a lag period of 24 hr or more, and the activities reached to

maximal levels within 5–7 days of incubation (Gomes I *et al*., 2006).

Maximum FPase activity was achieved by utilizing CMC as carbon source following 3 days of incubation. But there was a decline in enzyme activity following the 5th day in case of both CMCase and FPase. The decline trend of enzyme activity is likely to be due to the protease production into the medium. CMCase activity and FPase activity was high during incubation at 30°C for 72 hours of incubation (Figure 5).

At neutral pH 7, CMCase exhibited highest activity on 3rd to 5th day of incubation followed by a rapid decline (Figure 6). FPase showed maximum activity at pH 6 on 3rd day of incubation. It was concluded that under submerged fermentation, a medium with CMC as carbon source at pH 5 and 6 incubated at 30°C was suitable for FPase enzyme production.

Figure.2 Distribution of Fungal Diversity

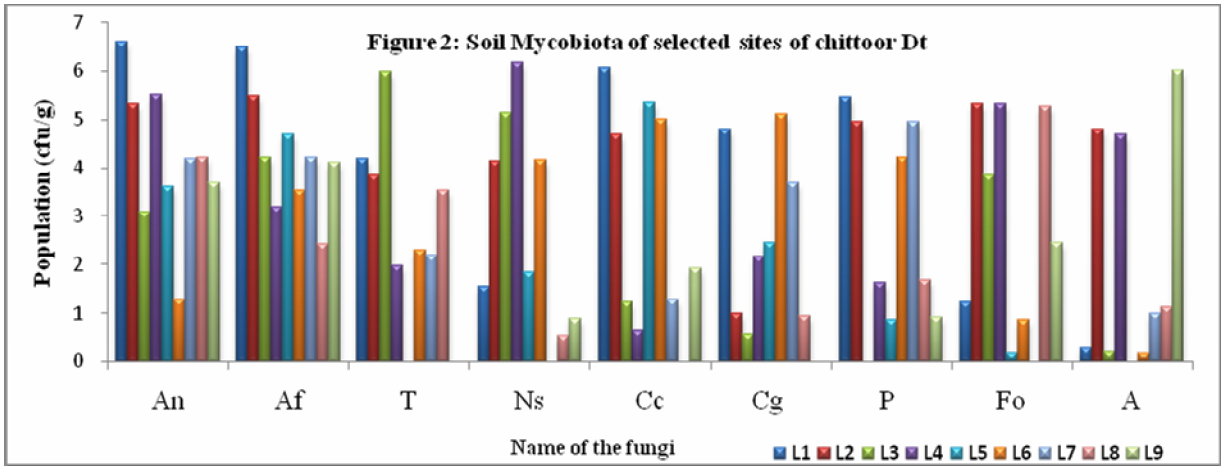


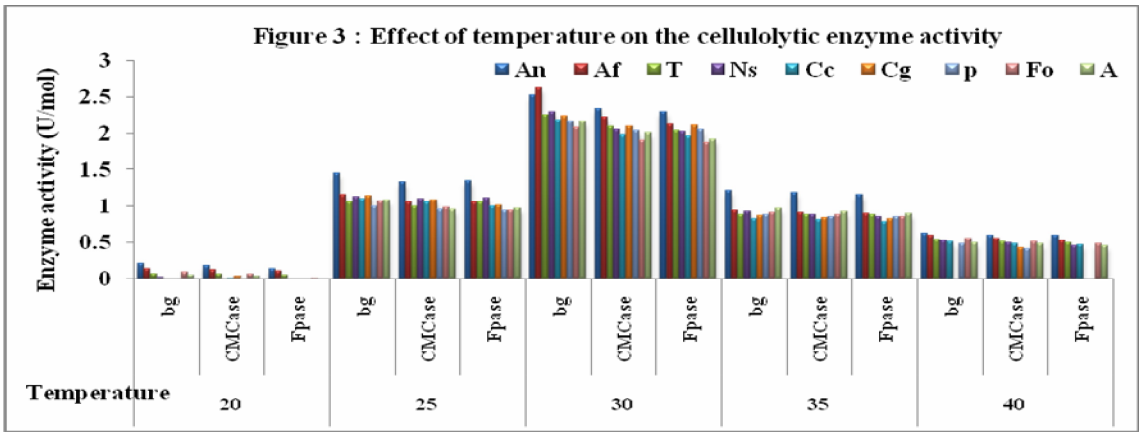
Table.1 Physico-chemical Properties of the soil at selected sites

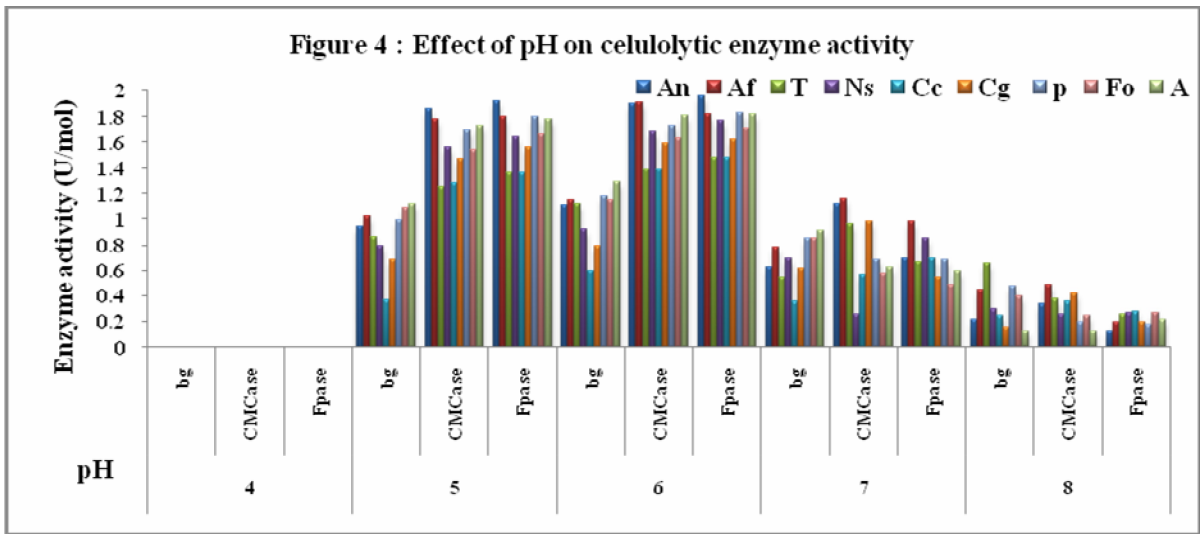
Locality	Organic matter	total Organic C	Total N (Kg/ha)	Total P (pm	K (Kg/ha)	Ca	Mg	Na	pH	EC (µS/cm)
Kalahasthi	2.35	0.97	0.151	51	12.58	1.9	0.26	3.5	6.19	1.25
Horsley hills	1.59	0.84	0.129	57	12.95	2.1	0.27	3.24	7.59	1.99
Kuppam	1.84	1.45	0.143	49	13.55	1.5	0.18	3.84	8.24	3.47
Nagapatla	2.87	1.28	0.157	55	12.97	2.7	0.19	3.49	6.62	0.91
Tirumala	2.22	0.73	0.146	63	11.98	2.3	0.15	3.53	6.44	0.59
Nagiri	2.04	1.51	0.137	61	12.46	2.4	0.22	3.04	7.22	1.12
Satyavedu	1.42	1.13	0.121	43	13.14	1.8	0.27	3.13	6.24	0.96
Mamandur	1.91	1.36	0.111	52	13.44	2.6	0.2	2.98	6.31	2.94
Tirupati	2.73	1.47	0.133	47	11.62	2.2	0.23	3.72	8.69	2.33

Table.2 Physico-chemical Properties of the soil at selected sites

Fungi name	Strain	Locality isolated	population CFU/g or fungal Load
A. niger	AN-K/1021	Kalahasthi	6.59
A. flavus	AF-HH/1022	Horsley hills	5.48
Trichoderma	T-K/1023	Kuppam	5.99
Nigrospora sphaerica	*NS-NRF/1024*	Nagapatla reserve forest	6.17
Cladosporium cladosporides	*CC-TML/1025*	Tirumala	5.35
Chaetomium globosum	*CG-N/1026*	Nagiri	5.12
Penicillium sp.	*P-S/1027*	Satyavedu	4.96
Fusarium oxysporium,	FO-MN/1028	Mamandur	5.27
Acremonium sp.	A-TPT/1029	Tirupati	6.01

#Population at the time of isolation ;
Bg,CMCase and FPase production optimization
at different medium pH and temperature.





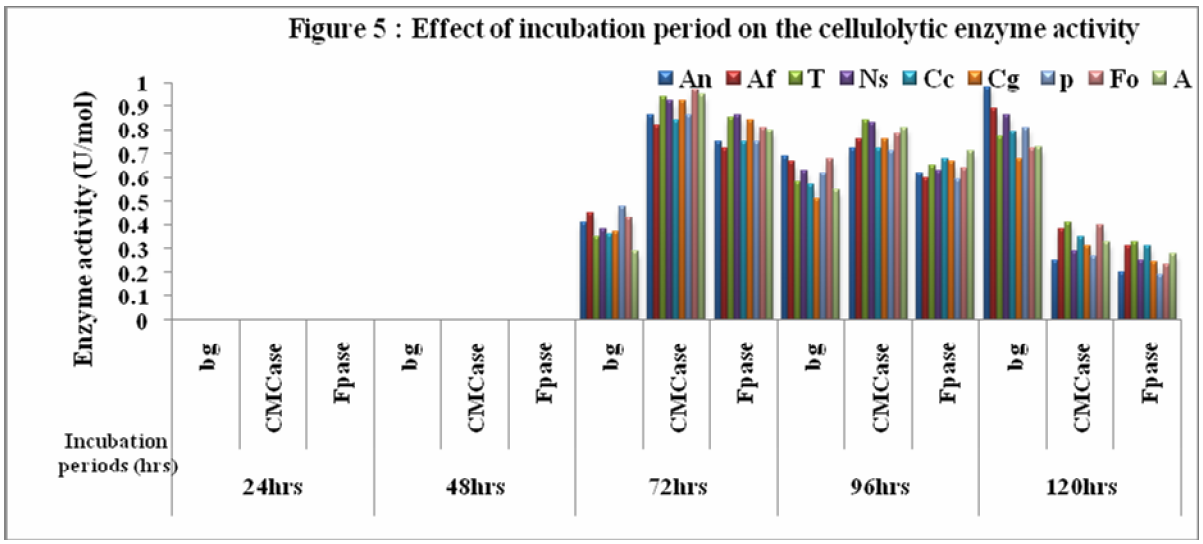
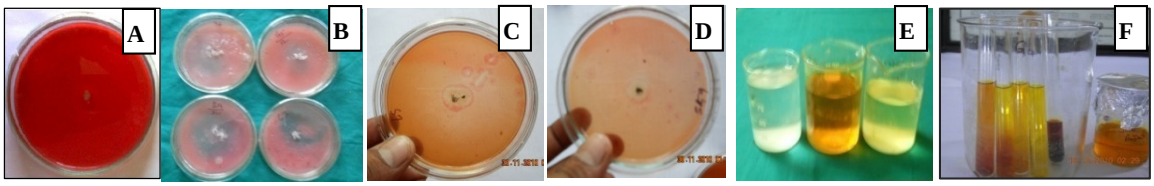


Figure.6 Plates with clear zone formed by fungal strain



(Fig A: Control plate; Fig B: *Chaetomium globosum*; Fig C & Fig D: *Aspergillus niger*; Fig E & Fig F: cellulase assay

Similarly medium supplemented with dextrose as carbon source and nitrogen source respectively with pH-6 incubated at 30°C achieved maximum βgase activity beyond 2nd day of incubation. However, all the isolates was able to produce cellulase in a better pH range from pH 5 to 7. βgase activity was high on 3rd day of incubation and enzyme recovery was optimal during this period beyond which there was a decline in its activity. Maximum FPase and CMCase were produced by all the selected isolates after 72 hours of incubation. The enzyme activities decrease by further incubation. Maximum cellulolytic activity was identified 72 hours of incubation. Optimum enzyme recovery period was identified between 3rd to 5th days of incubation beyond which the enzyme activity was reduced.

Trichoderma and *Aspergillus* are thought to be cellulase producers, and crude enzymes produced by these microorganisms are commercially available for agricultural use (Li X *et al*., 2010). *Trichoderma spp.* and *Aspergillus spp.* are two potential cellulase producers (Lynd LR *et al*. 2002). *T. viride*and *T. reesei* are two fungal strains extensively studied for their cellulase producing capability (Domigues FC *et al*. 2000, Gadgil NJ *et al*. 1995).

Most fungi can be adapted to anthropogenic substrates such as natural or waste cellulose or to substrates containing high amounts of cellulose. The cellulase enzyme produced extracellularly and harvested easily for determination. In preliminary screening, isolated fungal strains from different soil samples were

screened for their cellulolytic activity by plating them on carboxy methyl cellulose agar (CMC-agar) medium and looking for CMC clearing zones. Zone of clearance was observed for eight fungal isolates among 23 isolates tested. The positive isolates were picked up and inoculated into fungal basal medium and incubated at 120 rpm at $28 \pm 2°C$ for 3 to 5 days. Following the fifth incubation day, CMCase and FPase assay was performed for the same. Identification of fungi was done based on colony characters and microscopic examination and it was concluded that *A. niger* was the dominant fungal strain in soil samples. Besides *A. niger* the other organisms like *Aspergillus flavus, Nigrospora sphaerica, Chaetomium globosum, Cladosporium cladosporides, Trichoderma sp, Penicillium sp. Fusarium oxysporium, Acremonium sp.* The fungal load varied from 4.96×10^3 to 6.59×10^3 CFU (Figure 3,4,5).The variables analyzed (Physico-chemical analysis, pH and temperature) showed significant effects on βgase, FPase, and CMCase production. The cellulases were stable in the range of pH 5.0 – 6.0 and temperature 30°C. The viscosity of CMC solutions decreased rapidly and was followed by an increase in reducing compounds.

Each fungal isolate posses a pH,temparature range for its growth and activity with an optimum value within the range. Filamentous fungi have reasonably good growth with an optimal range of 3.8 to 6.0 (Gowthaman, MK., 2011).

Cellulase is a complex of three types of enzymatic complexes namely endoglucanase also called carboxymethyl cellulase, exoglucanase and β-glucosidase (Iqbal HMN *et al.* 2011).

Production of cellulases by the fungal isolates requires optimal conditions for their growth which leads to the release of extracellular enzymes. The growth conditions as well as extracellular enzyme production conditions is likely to vary among isolates. The major components of production medium like carbon and nitrogen sources and physical parameters like temperature, pH and incubation time were found to be critically affecting the cellulase production hence need to be optimized for every isolate (Kathiresan K and Manivannan S, 2006).

The most important factor among all the physical variables affecting the production of enzymes and metabolites is usually the incubation temperature because the enzymatic activities are sensitive to temperature (Krishna C, 2005).

Recently the interest in cellulases and hemicellulases has increased because of many potential applications of these enzymes. Various microorganisms under different cultural conditions produce cellulolytic enzymes.

Data suggest that investigation and Datasets provided in this study, *Aspergillus niger, Aspergillus flavus, Nigrospora sphaerica, Chaetomium globosum, Cladosporium cladosporides, Trichoderma sp, Penicillium sp., Fusarium oxysporium, Acremonium sp.* may contribute to future searches for fungal bio-indicators as biodiversity markers of a specific site or a land-use grown under dynamic carbon, pH and temperature of the medium to draw out the optimum conditions for cellulase production. The optimization of the enzyme production clearly demonstrated the impact of the process parameters on the yield of the cellulolytic enzymes and beneficial to be utilized for many industrial applications.

Acknowledgements

The authors are thankful to the Department of Botany for providing facilities and support for the work.

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