
Cellulase Induction in Three *Aspergillus* Species Isolated From *Artemisia annua* L. Plantation Soil Using Different Cellulose Substrates

A. I. Ogbonna (Corresponding author)

Department of Plant Science and Technology, University of Jos, Plateau State, Nigeria

Tel: 234-803-355-5188. E-mail: ogbonabi@yahoo.co.uk

P. O. Nwadiaro

Department of Plant Science and Technology, University of Jos, Plateau State, Nigeria

Tel: 234-803-703-3206. E-mail: patnwadiaro@yahoo.com

A. Chuku

Department of Microbiology, Federal University Lafia, Nasarawa State, Nigeria

Tel: 234-803-710-0101. E-mail: aleruchichuku@yahoo.com

C. I. C. Ogbonna

Department of Plant Science and Technology, University of Jos, Plateau State, Nigeria

Tel: 234-803-381-7606. E-mail: afriheb@yahoo.co.uk

F. C. Onwuliri

Department of Plant Science and Technology, University of Jos, Plateau State, Nigeria

Tel: 234-806-530-2804. E-mail: fconwuliri@yahoo.com

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Abstract

This study was aimed at isolation and screening of fungal species associated with *Artemisia annua* Plantation soils from one of the under studied areas in Plateau state, Nigeria for cellulase activity. A total of thirteen fungal species were isolated from various locations within the *A. annua* Plantation and were screened for cellulase production. Agar plate assay was carried out using basal medium supplemented with 1% Carboxymethylcellulose (CMC) powder and staining with 0.1% Congo red solution after the incubation period. Among these species, *Aspergillus fumigatus*, *A. niger* and *A. terreus* were more predominant and were recorded as cellulase producing species. They have shown to possess cellulose degrading ability and exhibited maximum zones of hydrolysis on Carboxymethylcellulose medium and were selected for enzyme assay using submerged fermentation (SmF). Enzyme production was analyzed by Dinitrosalicylic acid (DNSA) methods and the enzymes assayed for were CMCase (β -1,4-endoglucanase), β -glucosidase and FPase (total cellulose) using Carboxymethylcellulose, cellulose acetate and Filter paper as substrates respectively. The highest cellulase activity was observed on the 3rd day in *A. niger* with enzyme production of 0.045IU/ml and 0.040IU/ml on CMC and filter paper media respectively. *A. fumigatus* had high enzyme activity of 0.037IU/ml and 0.025IU/ml on filter paper and cellulose acetate media respectively. Highest enzyme production of 0.034IU/ml was recorded for *A. terreus* on the 3rd day on cellulose acetate medium. These fungal species could be employed specially to perform in situ environmental applications involving cellulose biodegradation of wastes.

Keywords: Cellulolytic, *Artemisia annua*, Carboxymethylcellulose, *Aspergillus* species, Soil, Congo red solution

1. Introduction

Cellulose, a renewable source of energy is one of the most abundant organic compounds on earth (Hong et al., 2001). It is a potential raw material for the microbial production of food, fuel and some chemicals (Coral et al., 2002). Several microorganisms utilize it as a source of carbon and it is a major polysaccharide constituent of plant cell walls (Hong et al., 2001). Cellulose is an unbranched glucose polymer and is composed of β -1, 4 glucose units linked by a β -1, 4-D-glycosidic bond (Gielkens et al., 1999). Cellulases belong to a group of hydrolytic enzymes which catalyze the hydrolysis of cellulose and cellooligosaccharide derivatives into glucose units. Fungal cellulases are extracellular and inducible and consist of three components namely exoglucanase, endoglucanase and β -glucosidase (Akiba et al., 1995; Wilson & Irwin, 1999) that act in a synergistic manner to hydrolyze the β -1,4-D-glycosidic bonds within the cellulose molecules. The enzymes play important role in natural biodegradation processes in which plant lignocellulosic materials are degraded by a wide variety of microorganisms (Khalid et al., 2006; Peciulyte, 2007; Chinedu et al., 2008). Various bacteria, actinomycetes, protozoa and filamentous fungi produce extra cellular cellulases when grown on cellulosic substrates though many actinomycetes have been reported to have less cellulase activity than fungi (Kluepfel et al., 1986). *Streptomyces* species have already been identified as good cellulase producers with considerable commercial interests (Chellapandi & Himanshu, 2008). *Aspergillus sp.* and *Penicillium sp.* are generally used for the production of β -glucosidase, an enzyme necessary for the

conversion of cellobiose to glucose because these fungi produced only low amounts of endoglucanase (Hamilton & Was, 1991; Steiner et al., 1994). Enzymatic hydrolysis of cellulose in industrial scales is hindered by the high cost of the enzyme and the low rates of conversion. There have been many researches directed towards finding new microbial sources with efficient endoglucanase synthesis, especially among fungi (Rosgaard et al., 2006). The present study therefore looks at enhancing the extracellular endoglucanase, β -glucosidase and FPase synthesis under submerged fermentation conditions using three *Aspergillus* species isolated from *Artemisia annua* plantation soil.

2. Materials and Methods

2.1 Isolation of Fungi From the Soil Samples

Soil fungi were isolated from *Artemisia annua* Plantation in Gangnum, Langtang South Local Government Area of Plateau State, Nigeria using standard microbiological techniques. A weight of 0.03 g of soil was plated out on Potato Dextrose Agar medium using soil plate method described by Warcup (1950). The medium was supplemented with gentamycin (40 mg/ml) in order to suppress the growth of bacteria. Plates were incubated at room temperature ($25 \pm 2^\circ\text{C}$) for 5 days. After incubation, small portion of mycelium from each fungal colony was transferred into PDA slants and were kept for further work.

2.2 Identification of Fungi

Fungal isolates were identified on the basis of routine cultural and morphological characteristics by comparing them with those that are present in the identification reference manuals (Barnett & Hunter, 1972; Von Arx, 1974; Onion et al., 1981). Thirteen fungal species were isolated from the experimental soil samples. Most of the isolates, obtained were *Aspergillus* species which are very dominant in soil natural environment.

2.3 Plate Assay for Cellulase Enzyme Activity Using Carboxymethylcellulose (CMC) Powder

Carboxymethylcellulose (CMC) is a substrate for endoglucanase and so was used as a test for β -1,4-endoglucanase activity. Modified method of Anita et al. (2013) was employed for the assay. Cellulolytic basal medium (1.5 g; Diammonium tartrate ($\text{C}_4\text{H}_{12}\text{N}_2\text{O}_6$), 0.25 g; Potassium dihydrogen phosphate (KH_2PO_4), 0.02 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0002 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (g/250 mL in distilled water) was prepared, supplemented with 2% w/v of low viscosity CMC and 1.5 % w/v agar, autoclaved at 121°C at 15psi for 15minutes and then transferred aseptically into sterile Petri dishes. The fungal isolates were point inoculated and incubated at $25 \pm 2^\circ\text{C}$ in a stationary position for 7 days. Agar plates were stained with 0.1% Congo red for 15 minutes. Plates were decanted and agar surface was washed with distilled water. The plates were destained by flooding with 1 M NaCl for 15 minutes. Halo zones of hydrolysis (mm) were observed, measured and recorded.

2.4 Enzyme Production Through Submerged State Fermentation Using Filter Paper (Fpase) for Total Cellulase Activity

Whatman No1 filter paper used in this assay was almost 100% cellulose. Cellulolytic basal medium (1.5 g; Diammonium tartrate ($\text{C}_4\text{H}_{12}\text{N}_2\text{O}_6$), 0.25 g; Potassium dihydrogen phosphate (KH_2PO_4), 0.02 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.0002 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (g/250 mL in distilled water) was prepared. A volume of 10 ml aliquots was transferred to 20 ml glass culture bottles and were autoclaved at 121°C at 15 psi for 15 minutes. Filter paper strips (0.5 cm \times 0.1 cm) were added

aseptically to each bottle making sure that all filter paper strips were completely submerged. Samples of test fungi which were previously obtained as pure cultures were inoculated separately and uninoculated bottles were retained as controls (Pointing, 1999). Care was taken that bottle caps were loosely fitted to allow adequate exchange of air. The bottles were incubated at $25\pm 2^{\circ}\text{C}$ without agitation and were assayed at 3, 6 and 9 day intervals using cell free culture supernatant of each of the fungi. Boiled enzyme extract was used as blank and D-glucose as standard. The glucose concentration was determined by DNSA method, as described by Miller (1959). The color that developed was measured at 540 nm using UV spectrophotometer. The experiment was done in triplicates. One unit of filter paper (FPU) activity was defined as the amount of enzyme-releasing $1\ \mu\text{mol}$ of reducing sugar from filter paper per mL per min.

2.5 Enzyme Production Through Submerged State Fermentation Using Cellulose Acetate for β -glucosidase Activity

This assay uses microcrystalline cellulose acetate powder. Generally microcrystalline cellulose is degraded more slowly than ball-milled or acid swollen cellulose. Cellulose Basal Medium was prepared by incorporating 2% w/v cellulose acetate powder. A volume of 10 ml aliquots was transferred to 20 ml glass culture bottles and were autoclaved at 121°C at 15psi for 15minutes. Samples of test fungi which were previously obtained as pure cultures were inoculated separately and uninoculated bottles were retained as controls. The bottles were incubated at $25\pm 2^{\circ}\text{C}$ without agitation and were assayed at 3, 6 and 9 day intervals using cell free culture supernatant of each of organisms. Boiled enzyme extract was used as blank and D-glucose as standard. The glucose concentration was determined by DNSA method, as described by Miller (1959). The colour that developed was measured at 540 nm using UV spectrophotometer. The experiment was done in triplicates. One unit of cellulose (β -glucosidase) activity was defined as the amount of enzyme-releasing $1\ \mu\text{mol}$ of reducing sugar per ml per min.

2.6 Enzyme Production Through Submerged State Fermentation Using Carboxymethylcellulose For (Endoglucanase) Activity

A volume of 10 ml aliquots of prepared Carboxymethylcellulose medium was transferred to 20 ml glass culture bottles and were autoclaved at 121°C at 15psi for 15minutes. Samples of test fungi which were previously obtained as pure cultures were inoculated separately and uninoculated bottles were retained as controls. The bottles were incubated at $25\pm 2^{\circ}\text{C}$ without agitation. Carboxymethylcellulase (CMCase) hydrolysed Carboxymethylcellulose to produce free Carboxymethyl glucose units. The free Carboxymethyl glucose units reacted with 3, 5-dinitrosalicylic acid (DNSA) reagent to form coloured complex which was detected spectrophotometrically at 540 nm (Wood & Garcia-Campayo, 1990). This was assayed at 3, 6 and 9 day intervals using cell free culture supernatant of each of the test organisms. Boiled enzyme extract was used as blank and D-glucose as standard. One unit of cellulose (β -glucosidase) activity was defined as the amount of enzyme-releasing $1\ \mu\text{mol}$ of reducing sugar per ml per min.

2.7 Statistical Analysis

The analyses done using plate assay for cellulase enzyme activity were performed on at least

three replicates. All the data obtained were subjected to analysis of variance (ANOVA) using Statistical Package for Social Sciences (SPSS). Duncan multiple range test was used to compare the means at $P \leq 0.05$.

3. Results

3.1 Fungal Isolates

The selected fungi were isolated from soils of *Artemisia annua* Plantation using Potato Dextrose Agar medium and were identified as *Aspergillus fumigatus*, *A. niger* and *A. terreus* by morphology. Colonies of *A. fumigatus* appeared grayish green and the reverse light yellow. No pigmentation was observed. Growth rate was rapid. Microscopic view showed smooth walled conidiophores, uniseriate and pyriform vesicle, metulae covering upper 2/3 portion of the vesicle as shown in Figure 1a. The conidia looked small, smooth and the conidiation was strongly columnar.

A. niger colonies were predominantly black. The reverse was colourless to light yellow. Pigmentation was absent. The conidiophores were long and smooth. Vesicles were large in size, biseriate with metulae covering the entire surface forming radiate head. The conidia were very rough, spiny and globose. The results are shown in Figures 1b.

Colonies of *A. terreus* were beige to buff to cinnamon. The reverse was colourless to light brown. Growth rate is rapid. Colonies become finely granular with massive conidial production. These features with the microscopic observation of *A. terreus* are depicted in Figure 1c. Hyphae were septate and hyaline. Conidial heads were biseriate and columnar. Conidiophores were smooth-walled and hyaline terminating in mostly globose vesicles. Conidia were small, globose, and smooth.

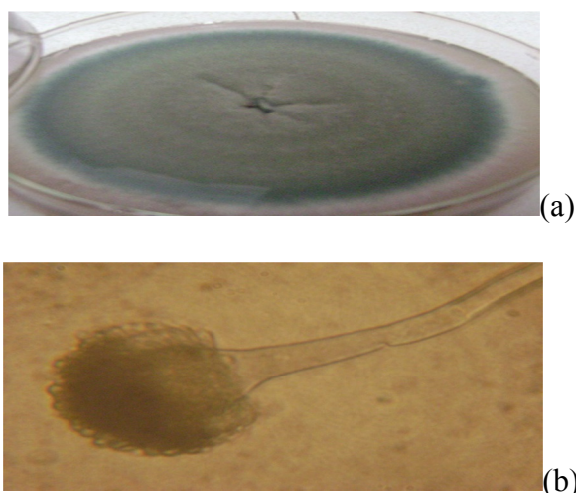


Figure 1a. *Aspergillus fumigatus* (a) colony (b) microscopic view

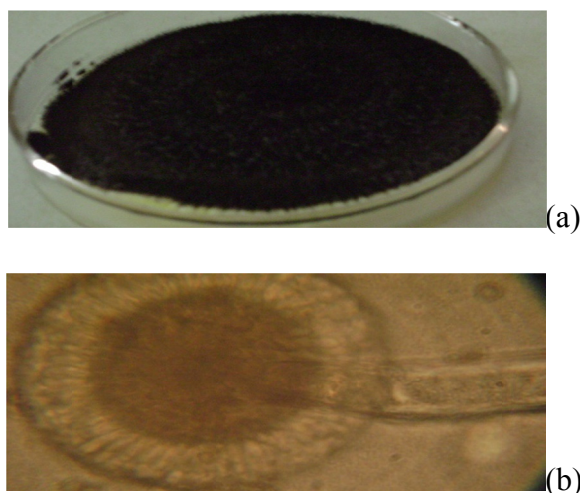


Figure 1b. *A. niger* (a) colony and (b) microscopic view

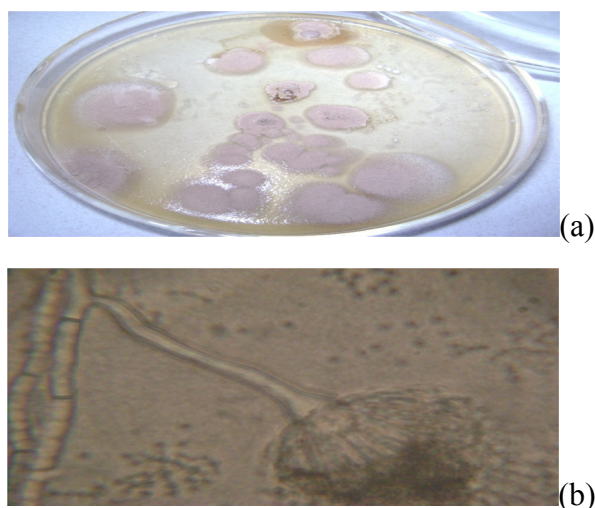


Figure 1c. *A. terreus* (a) colony and (b) microscopic view

3.2 Plate Screening for Cellulase Enzyme Activity

The screening of the isolated fungi for cellulose degrading activity was carried out on CMC medium. The appearance of the clear zone around the colony after the addition of Congo red solution was strong evidence for secretion of cellulase enzyme. Out of 13 fungal species (result not shown), only three fungi (*Aspergillus fumigatus*, *A. niger* and *A. terreus*) exhibited considerable activity to degrade the Carboxymethylcellulose substrate as depicted in Table 2. There was a significant difference on the mean diameter of clear zones produced by the three selected fungi. The highest zone of clearance was recorded for *Aspergillus fumigatus* (83.3 ± 3.06 mm) followed by *A. niger* (80.0 ± 4.00 mm) and then *A. terreus* (67.0 ± 4.58 mm). Therefore, they were selected for further experimentation.

Table1. Cellulolytic activity of the test fungi using CarboxymethylCellulose agar (Mean±SE)

| Test organisms | Mean diameter of Clearing (mm) | Activity Level |
|------------------------------|--------------------------------|--------------------------|
| <i>Aspergillus fumigatus</i> | *+++ | 83.3 ± 3.06 ^a |
| <i>A. niger</i> | +++ | 80.0 ± 4.00 ^a |
| <i>A. terreus</i> | +++ | 67.0 ± 4.58 ^b |

Figures in the same column having the same superscript are not significantly different ($p \leq 0.05$); +++ = strongly cellulolytic.

3.3 Enzyme Production Through Submerged State Fermentation

The selected fungal isolates were found to degrade various cellulosic substrates in liquid state fermentation. Maximal CarboxyMethylCellulase (CMCase) and FilterPaperase (FPase) production was obtained from *Aspergillus niger* (0.045 IU/ml and 0.040 IU/ml respectively) after the first three days of incubation (Figure 2), after which the activity reduced drastically on 6th and 9th days of incubation. *A. fumigatus* had a higher FPase production of 0.037 IU/ml to that of CMCase production (0.016 IU/ml) after the first three days. The activity declined after 6th day of incubation and remained steady up to the 9th day of incubation (Figure 3). The least enzyme production was observed in *A. terreus* for CMCase and FPase (0.018 IU/ml and 0.011 IU/ml) respectively after the 3rd day but showed a higher activity for β -glucosidase than *A. niger* and *A. fumigatus*. *A. terreus* had CMCase and FPase activity that was significantly lower than that of *A. niger* and *A. fumigatus*. As was observed, it took *A. terreus* 9 days to reach its peak of hydrolysis for CarboxyMethylCellulase (CMCase). The results are depicted in Figures 2-4.

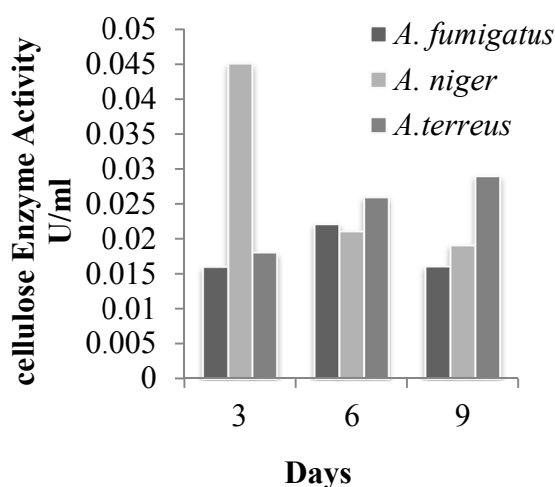


Figure 2. Cellulase production by *A. fumigatus*, *A. niger* and *A. terreus* at different periods of incubation on Carboxymethylcellulose medium

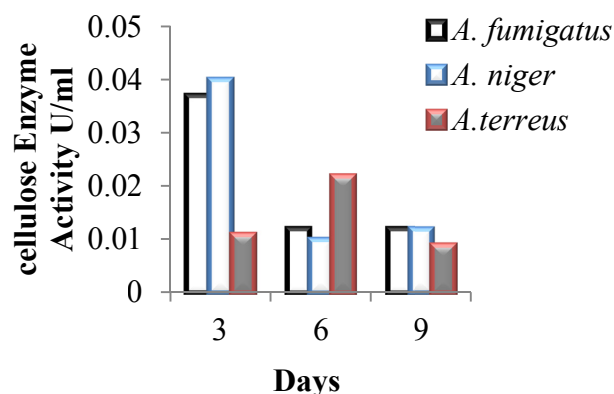


Figure 3. Cellulase production by *A. fumigatus*, *A. niger* and *A. terreus* at different periods of incubation on filter paper medium

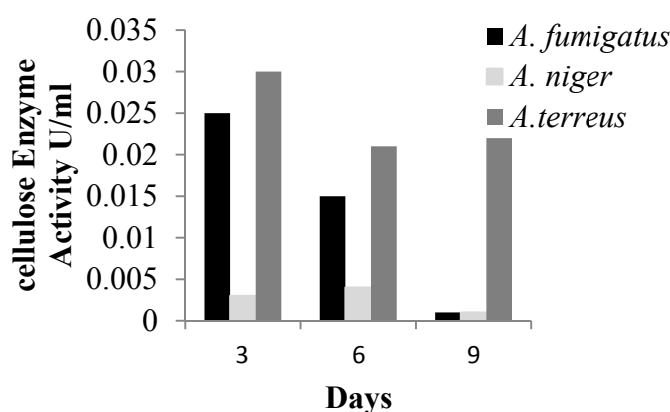


Figure 4. Cellulase production by *A. fumigatus*, *A. niger* and *A. terreus* at different periods of incubation on cellulose acetate medium

4. Discussion

4.1 Isolated Fungal Species

The three fungal species used in this research work were selected from various species that were isolated from soil of *A. annua* plantation. The selection stemmed from the fact that the rate and frequency of growth recorded for them were higher than those of other isolated species. The genus *Aspergillus* has been reported to be the most dominant among other species isolated from different natural habitats (Mukunda et al., 2012; Uttam et al., 2014). It is believed to be as a result of their abilities to grow fast on any available substrate due to their elaboration of hydrolytic enzymes that enable them breakdown the substrates they found themselves on.

5.2 Enzyme Activity of the Fungal Isolates on Plate Assay and Submerged State Fermentation (Smf)

The plate assay for the hydrolysis of Carboxymethylcellulose used as carbon source showed

that the three selected fungi hydrolysed the substrates at different rates (Table 1). *A. fumigatus* was found to be the best hydrolyzer of the CMC substrate on solid state with zone of clearance of 83.3 mm. *A. fumigatus* is a known cellulase producer. The results are in consonance with that of Sathyaprabha et al. (2011) who reported cellulase production in *A. fumigatus*, *A. versicolor*, *A. nidulans* and *A. niger*.

In submerged state fermentation for the production of cellulase, the selected fungi were inoculated separately in 100 mL fermentation media in 250 mL capacity flasks. The fungi utilized the media for their growth and secreted various secondary metabolites including cellulases into the culture flasks. The enzyme quantities were expected to increase with increase in fungal growth and incubation period. Therefore, the crude extract from the fermentation media was harvested at the interval of 3 days up to 9 days. The cultivation time allows for maximum growth of microorganisms and product formation to a certain degree in a fermentation system. In the submerged fermentation (SmF), *A. niger* was observed to have an enzyme activity (0.045 IU/ml) that was significantly higher than that of *A. fumigatus* (0.016 IU/ml) on the first three days of incubation and was observed as the peak for both species, after which enzyme activity dropped up to the ninth day of incubation (Figures 2 and 3). Explanation to this could be difficult since many factors including media composition, fermentation type and choice of substrate affect enzyme production and activity as reported by Sharma and Sreekantiah (1986). The results as presented in Figures 2 and 3 also indicated that *A. niger* showed a higher CMCase and FPase than *A. fumigatus* and *A. terreus*. The CMCase and FPase activity of *A. niger* and *A. fumigatus* were very rapid due to increase in cellulose utilization in the two fungal species and as such reached their peak enzyme activity on the 3rd day of fermentation. *Aspergillus* species especially *A. niger* have been reported as fast growing fungi (Oyeyiola & Hussein, 1992; Utamm et al., 2014).

A. terreus exhibited β -glucosidase activity that was significantly higher than that of *A. niger* and *A. fumigatus*. Cellulose acetate is known to have an additional acetyl group and requires the presence of esterases for its first step in biodegradation. The enzyme system of *A. terreus* probably could be equipped with acetyl esterase enzyme which performs the deacetylation step in cellulose acetate hydrolysis, which then allowed the polymer's cellulose backbone to be readily biodegraded by the selected test fungi. *A. terreus* had peak β -glucosidase enzyme activity of 0.030 IU/ml for cellulose acetate after the 3rd day of incubation. This was followed by *A. fumigatus* and *A. niger* with peak enzyme activity of 0.025 IU/ml and 0.003 IU/ml respectively after the 3rd day of incubation. The present finding indicates that the enzyme systems of these fungal forms contain enzymes complexes (β -glucosidase, CMCase (Endoglucanase) and FPase) for the effective hydrolysis of cellulose (Ali et al., 1991; Jahangeer et al., 2005). The cellulose degrading potential of *A. terreus* has been reported previously (Ali et al., 1991; Szakacs et al., 2001).

The results of the present research work showed that the selected test fungi had different cellulose degrading ability for the different cellulose substrates (Carboxymethylcellulose, Filter paper and cellulose acetate) used in the study. This result coincides with the findings of Sivakumaran, (2014) in his work on isolation of cellulolytic fungi and their degradation on cellulosic agricultural wastes. This result is relevant to the given physical and chemical conditions because, the activity of each fungal enzyme varies according to its individual

preference to the substrate, chemical nature of the medium, optimal temperature, and pH conditions. These factors are important in determining the survival of certain fungus on natural environment. If such factors are more suitable for a particular species, that species will ultimately win the competition on using all the resources more than the others and the later are eventually eliminated. However, some fungi can produce specialized structures to survive in such harsh conditions and will germinate when environmental conditions become favourable to them (Sivakumaran, 2014).

5. Conclusion

Conclusively, fungi generally are known agents of decomposition of cellulose and other substrate containing organic matter using their complex enzyme system. The enzyme system of the fungal species used in this research work could be employed in the decomposition of *Artemisia annua* process wastes in the plantation, thereby improving the humus content of the plantation soil.

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