

Screening for Lignocellulolytic Fungi From Biowastes Show Fungi with Hyper-Cellulase Producing Capability

Baturh Yarkwan^{1*} and Chukwudi U. Anyanwu²

1. Dept. Of Biochemistry, Federal University of Agriculture, P.M.B. 2373, Makurdi, Benue State, Nigeria.

2. Dept. Of Microbiology, University of Nigeria, Nsukka, Enugu state, Nigeria

Abstract

The decomposition of organic wastes, such as lignocellulosics to release the energy trapped therein has been remarked as one of the most important events of the carbon cycle. The organisms involved in these processes, if harnessed could be useful bio-resources for the production of value added products, such as cellulase. To screen for these organisms, decomposing waste materials were collected from different places in the environment. Screening was done following established methods. Four fungi species were obtained. These were *Aspergillus niger*, *Fusarium* spp, *Spadicoides* spp and *Aspergillus* spp. Of all these, *Aspergillus niger* had the highest (0.085 mg/ml) extracellular cellulase secreting ability, when cultivated on sodium carboxy-methyl cellulose (Na-CMC). All the organisms grew were on sugar cane bagasse (SCB) sore carbon sourced media. These organisms have been implicated in cellulase production previously, while *Spadicoides* spp has rarely, if at all ever been reported for having high extracellular cellulase secreting ability and is hereby reported for further studies.

Key words: *Aspergillus niger*, *Spadicoides* spp, cellulase, Sugar cane bagasse (SCB)

1.0 Introduction

Lignocellulose is the world's most abundant biomass (Pomeranz and Meloan, 1994, Perez *et al.*, 2002). It is the major component of woody and non-woody plants such as grass and represents the greatest source of renewable organic matter. It is produced chiefly by photosynthesis (Perez *et al.*, 2002; Howard *et al.*, 2003). Therefore, agricultural wastes (e.g. rice husks, corn stover, sugar cane bagasse, among others), forestry wastes (e.g. saw dust, mill wastes), municipal solid wastes (e.g. human excreta, paper waste) and several other industrial waste products contain essentially lignocellulose.

Lignocellulosics are composed of three basic components namely: lignin, hemicellulose and cellulose which are strongly intermeshed and chemically bonded together by non-covalent forces and covalent cross linkages, hydrogen bonding and van der Waals forces, thereby making them recalcitrant to enzymatic degradation (Perez *et al.*, 2002; Hetti, 2004; Kirubahar *et al.*, 2008).

By means of chemical, biological, and thermochemical processes, it is possible to transform this insoluble polymer to glucose (a major starting material for several industrial processes).

Chemical hydrolysis basically involves the use of acids in degrading lignocellulosics. In this method, lignin has little or no effect on the rate of acid hydrolysis (Seaman, 1981). Acid hydrolysis is probably the oldest and most studied method of lignocellulosics degradation. There are two types, dilute acid and concentrated acid (Seaman 1981; Conner *et al.*, 1985; Badger 2002).

The thermochemical method employs basically two approaches. Thermochemical and Biological Hybrid Systems: biomass are thermochemically gassified and the resultant synthesis gas, a mixture of hydrogen and carbondioxide passed through specially designed fermenters loaded with microorganism(s) which have the capability of fermenting the gases, under defined conditions (Badger, 2002). In the second thermochemical ethanol production process, biomass materials are thermochemically gasified and passed through a reactor containing catalysts, which cause the gas to be converted to ethanol (Badger, 2002).

The natural decomposition of lignocellulosics employ microbial enzymes in the exhaustive degradation of these materials (Kuwahara, 2000, Stepanova *et al.*, 2002). This process is one of the most important events of the carbon cycle (Bennet *et al.*, 2002). Lignocellulolytic enzymes are obtained from fungi and bacteria (Kader *et al.*, 1999; Perez *et al.*, 2002; Lee *et al.*, 2006; Okafogu and Nzelibe, 2006). However, filamentous fungi are the principal degraders of lignocellulose (Hammel, 2007). Of the microorganisms capable of producing cellulase, only a few produce significant amounts of extracellular cellulase, secreted in the growth medium (Kotchoni *et al.*, 2003).

Several families of extracellular enzymes have been implicated in lignocellulose breakdown. These include lignases (Biers 2003, Howard *et al.*, 2003), hemicellulases (Bennet *et al.*, 2002, Perez *et al.*, 2002) and cellulases (Kleman-layer *et al.*, 1996; Periera Junior *et al.*, 2003). The hydrolysis of cellulose to glucose requires three enzyme components which function synergistically and co-ordinately to produce glucose and these are exocellulases, endocellulases, and β -glucosidases (Han *et al.*, 1995; Kleman-layer *et al.*, 1996; Periera Junior *et al.*, 2003; Ray *et al.*, 2007).

Microorganisms capable of degrading cellulose include fungi, bacteria, eubacteria and actinomycetes (Kurasawa *et al.*, 1992; Bhat and Bhat 1997; Cai *et al.*, 1999; Howard *et al* 2003). Among these, fungal

cellulases are preferred for industrial applications because they are extracellular, inducible enzymes which can be produced in large quantities (Bennet *et al.*, 2002; Methuvelayudham and Viruthagiri, 2006; Immanuel *et al.*, 2007). Bacterial cellulases on the other hand are constitutive and cell wall bound, thus limiting their applications in industrial processes (Raimbault, 1998; Immanuel *et al.*, 2007). Even among fungal species, not many of them are capable of producing substantial quantities of cell free cellulase capable of completely hydrolysing cellulose in vitro (Methuvelayudham and Viruthagiri, 2006). This further lowers the ceiling on the number of cellulolytic fungi which are capable of producing cellulase on industrial level. Therefore, the aim of this study was to screen for cellulolytic micro-organisms in the local environment and test their adaptability on medium composed of SCB as the only carbon source.

2.0 Materials and Methods

2.1 Reagents

Ammonium chloride (NH_4Cl). (Fischer Scientific Company, USA). Agar-Agar. (Hopkins and Wichans England). Ammonium sulphate. (British Drug House (BDH) Chemicals Ltd. Poole, England). Dipotassium hydrogen orthophosphate trihydrate, Kermel, India Potassium chloride BDH chemicals Ltd Poole, England. Magnesium tetraoxosulphate vi heptahydrate Merck, Germany. Sodium potassium tartarate (Rochelle salt) Merck, Germany. Sodium acetate Vickers laboratories Ltd, West Yorkshire, London. Sodium hydroxide Merck, Germany. Standard buffers pH 4.01, 7.01 and 9.0 Qualigens Fine Chemicals, a division of Glaxosmithkline, London. 3,5-dinitrosalicylic acid (DNS) ($\text{C}_7\text{H}_4\text{N}_2\text{O}_7$) Lab. Tech Chemicals, Avighkar India. Hydrochloric acid (HCl) conc. Sabourand dextrose agar (SDA). Fluka chemical company, Germany. Calcium carbonate (CaCO_3). May and Baker Ltd. England. Sodium carboxymethyl cellulose (Na-CMC). BDH chemicals Ltd. Poole, England. Analytical grade. Cellulose powder. Whatman chemical ltd. England. Analytical grade. Copper tetraoxosulphate vi (CuSO_4). Merck, Germany. Analytical grade. Bovine Serum Albumin (BSA). D (+) Glucose (Industrial grade) M & B Ltd. Dagenham, England. Lacto phenol cotton staining blue. Kermel chemicals, China. Whatman No. 1 filter paper.

2.2. Apparatus

Weighing balance Ohaus Dial-O-Gram. Ohaus cooperation, N.J. USA. Autoclave UDAY BURDON'S Patent Autoclave, Made in India. Oven Gallenkamp Hotbox. Made in England. Water bath Grant instruments, (Cambridge) Ltd, Barrington, Cambridge. UV Spectrophotometer: UNICO UV 2102 PC spectrophotometer. pH meter: Ecoscan pH meter, Made in Singapore. Visible spectrophotometer : LKB Biochron Novaspec 4049, Camera for micrographs: Nikon cool PX 5000 Nikon Corporation, Japan. Made in Indonesia. Microscope: WESO microscope

2.3 Methods

2.3.1 Collection of and preparation of Sugar Cane Samples

Four improved varieties of sugar cane, namely, National Cereals Sugar (NCS) NCS-003, NCS-006, NCS-007 and Coimbatore (Co) Co 957, were collected from the National Cereals Research Institute, (NCRI) Badegi, Niger State. Besides these, one local variety was bought from farmers within Badegi. The improved varieties were two seasons old, and the local variety, one. The local variety grown in Fadama land, was more fresh than the improved varieties. Equal weights (6.00 kg) of all the samples were got using a top load weighing balance and transferred to the milling room.

The measured sugar samples were crushed using Jeffco Food and Fodder Cutter Grinder. After crushing, the bagasse was pressed using Apex Hydraulic Press, in order to remove the brix. The bagasse were then packaged in labelled polythene bags and carried to the Laboratory for further processing.

In the laboratory, each of the five sugar cane bagasse samples were divided into two parts of equal weights using Gallenkamp S/No. 588 top load weighing balance. One half was rinsed three times in running tap water and the water squeezed out with hands and marked washed (W). The other part was not washed and marked unwashed (UW). Both samples were dried in the oven at 105°C till constant weight according to Vermerris *et al* (2005). The dried SCB samples were milled using Thomas Willey Laboratory Mill. The milled SCB samples were then sieved with 0.5 mm^2 mesh size sieve. The fine sieved SCB was then packaged in polythene bags and kept in a shelf in the laboratory.

2.3.2 Isolation of Cellulolytic Microorganisms

Five soil samples were collected from different locations. These were: garden soil from the University of Nigeria Nsukka Zoological Park (GS ZOO), decomposing litter soil (DLS) near Continuous Education Centre (CEC), University of Nigeria Nsukka. Other sites were: sewage water (SW) and sewage sediment (SS) at the University Central Sewage Treatment Plant and decomposing saw dust (DSD) at the Nsukka timber shade, Isiakpu. Soil samples were collected early in the morning in clean dry plastic containers with tight lid and

immediately transported to the laboratory, according to the method of Alef and Nannipieri (1998).

2.3.3 Determination of Soil pH

The pH meter was standardized with standard buffers of pH 4.01, 7.01 and 10.1 according to Manufacturer's specifications. The air dried soil samples were sieved to fine particles using a 2 mm² mesh size sieve. 10 g of the sieved soil samples were weighed into a beaker and 25 ml of distilled water was added and stirred till a homogenous suspension was formed. The suspension was allowed to stand for one hour to allow the ions to properly equilibrate in the water. The suspension was stirred again after the one hour and the supernatant was used in measuring the pH. The probe was rinsed with distilled water both before and after reading pH of each soil sample.

2.3.4 Preparation of Soil Extract Enrichment and Isolation Mineral Salts Medium

Ten grams of fresh garden soil sample was weighed out into a clean, dry beaker and 10 ml of running tap water was added. After shaking vigorously, the suspension was filtered through Whatman No. 1 filter paper and the filtrate collected into a clean dry test tube. This was used to enrich the broth with the necessary mineral required by micro-organisms in their natural environment to grow.

0.5 g each of MgSO₄.7H₂O, NH₄Cl and K₂HPO₄.3H₂O were measured out into a beaker containing 500 ml of tap water. 2.5 ml of soil extract enrichment was added and mixed thoroughly. The pH was brought to 7.0 by drop-wise addition of 0.1 M sodium hydroxide solution. Five 250 ml Erlenmeyer flasks, labelled according to the soil samples were arranged. 100 ml of the isolation mineral salts prepared earlier was measured into the flasks. Small pieces of Whatman No. 1 filter paper were evenly distributed into the flasks and tightly stoppered. The flasks were autoclaved at 121°C for 15 min at 15 pounds per square inch (psi).

2.3.5 Preparation of Soil Sample Extracts for Microbial Isolation

Debris and stones were hand picked from the soil samples and 10 g weighed out into clean dry labelled beakers. Ten millilitres of tap water was added and shaken vigorously to form a mixture. Five millilitres from each mixture was transferred alone to an autoclaved flask. For the sewage water, 5 ml was transferred into an appropriate flask.

2.3.6 Incubation of the Inoculated Flasks

The inoculated flasks were covered with carbon paper to prevent light penetration in order to avoid algal growth. These were allowed to stand at the prevalent room temperature (29±1°C) for 14 days in a dark cupboard.

Another mineral salt broth was prepared just as described above. The 2.5 ml soil enrichment extract was now obtained from air dried garden soil sample, the remaining of the one which was used earlier. 95 ml of running tap water was measured into clean dry Erlenmeyer flask and prepared just as described above. After autoclaving and cooling, 5 ml of the earlier cultures were transferred under aseptic conditions into the newly prepared broth and incubated for another 14 days.

Sub-culturing unto media was done after these 14 days. The medium contained 0.1% MgSO₄.7H₂O, NH₄Cl and K₂HPO₄.3H₂O (w/v), 1.0% of cellulose powder (w/v) and 1.5% agar-agar (the gelling agent) (w/v). The medium was prepared following standard specifications. It was allowed to cool to about 45°C and then made to set in sterile petri dishes. The plates were then incubated in a B & T Trimline incubator at 37°C overnight to check for sterility.

2.3.7 Inoculation of the Plates.

A sterile inoculation loop was dipped into the incubated broth, then removed and streaked on the medium. The plates were thereafter incubated at room temperature till mycelia growths were observed. This was repeated till pure fungal cultures were obtained.

The pure fungal isolates were stored on SDA slants, prepared according to the Manufacturer's prescription.

2.3.8 Macroscopic Features of the Isolated Fungi.

Three days old pure cultures were examined. Colour, texture, nature of mycelia or spores and, growth patterns were observed. Photographs of the cultures were also taken.

2.3.9 Culturing of the Isolates on Sugar Cane Bagasse (SCB) Media.

The medium was prepared as described earlier (section 2.3.6), but cellulose powder was replaced with SCB. Since all the isolates grew well on this medium, after 3 successive cultures, the organisms were preserved on slants prepared using SCB as the only carbon source.

2.3.11 Fermentation Experiments

The fermentation broth contained (w/v): 1% SCB, 0.1% $MgSO_4 \cdot 7H_2O$, NH_4Cl , and K_2HPO_4 in distilled water. 50 ml of the broth was measured out into clean, dry 250 ml Erlenmeyer flasks, stoppered with aluminium foil and autoclaved at standard specifications. Other broths were prepared in a similar manner but SCB was replaced with Na-CMC.

From the slants, three day old fungal cultures were prepared and used to inoculate the tubes. A 6.0 mm² diameter cork borer was used to transfer the actively growing cultures into the autoclaved broth. Table top Incubation was done in the laboratory having average temperature of 29 ± 1 °C.

At each day of harvest, flasks were selected from the respective groups. The broth was filtered through Whatman No. 1 filter paper. The filtrate was collected and used for the respective assay.

2.3.12 Cellulase Assay

The assay substrate was a Whatman No. 1 filter paper strip measuring 1 cm X 3 cm. Each of these strips contain, by standard units, an equivalent of 25 mg of glucose (Jeffries, 1987).

The assay mixture contained 2 ml of 0.2 M NaOAc buffer, pH 5.5, 0.5 ml of the enzyme filtrate. A strip of the filter paper was then immersed wholly into the mixture and incubated in a water bath at 50°C for 6 hr. An enzyme blank was prepared in a similar manner, but no enzyme was added. A substrate blank was also prepared by mixing 0.5 ml of the enzyme and 2.0 ml of the buffer but no substrate was added into it. After incubation, the tubes were removed, 0.5 ml of DNS reagent was added, and the mixture placed in boiling water for 10 min. After cooling, the absorbance was read at 540 nm using a LKB Biochron Novaspec 4049 visible spectrophotometer. The blank was used to zero the instrument before taking the readings.

Reducing sugar formed per ml was calculated according to Wu *et al* (2006). One unit of activity was defined as the amount (in micro moles) of reducing sugar formed per minute per milligram protein under the assay conditions.

2.3.13 Procedure for Protein Determination

The method of Lowry *et al* (1951) was adopted.

Briefly, 10 test tubes were arranged in duplicate containing 0.0 – 1.0 ml of 0.2 mg of protein stock solution. The volume was made up to 1 ml with distilled water. 5 ml of the alkaline solution E was added and allowed to stand at room temperature for about 10 min. 0.5 ml of solution C (diluted Folin Ciocalteu Reagent) was added with rapid mixing. After standing at room temperature for 30 min, the absorbance was taken at 750 nm. A calibration curve of absorbance against standard BSA concentration was made. Concentration of protein per ml in each tube was determined.

2.3.14 Fungal Identification

Three-day old cultures were used in preparing the microscope slides. A tuft of the mycelia was dropped on the slide and a drop of lacto phenol cotton staining blue was added to it, just enough to cover the specimen. A cover slip was placed over it and examination was performed under the light microscope at X400.

Conclusive identification was carried out by relating the macroscopic features and the micrographs to “Atlas of Mycology” by Barnett and Hunter (1972).

3.0 Results and Discussion

The soil pH was lowest in decomposing saw dust (6.65) and highest in decomposing litter soil (7.43) as shown in Table 1.

Table 1: pH of soil samples used for isolating cellulolytic micro-organisms.

Soil Sample	pH
Decomposing saw dust (DSD)	6.65
Decomposing litter soil (DLS)	7.43
Sewage water (SW)	6.82
Sewage sediment (SS)	6.75
Garden soil from Zoological park (GS ZOO)	7.20

Extracellular protein secretion in *Aspergillus niger* was highest at day 2 (0.085 mg/ml) (Fig. 1). It then declined steadily to the lowest value of 0.05 at days 8 and 10. *Spadicoides spp* had its highest extracellular protein secretion at day 2 (0.080 mg/ml), and thereafter declined to the lowest value of 0.015 mg/ml on day 10.

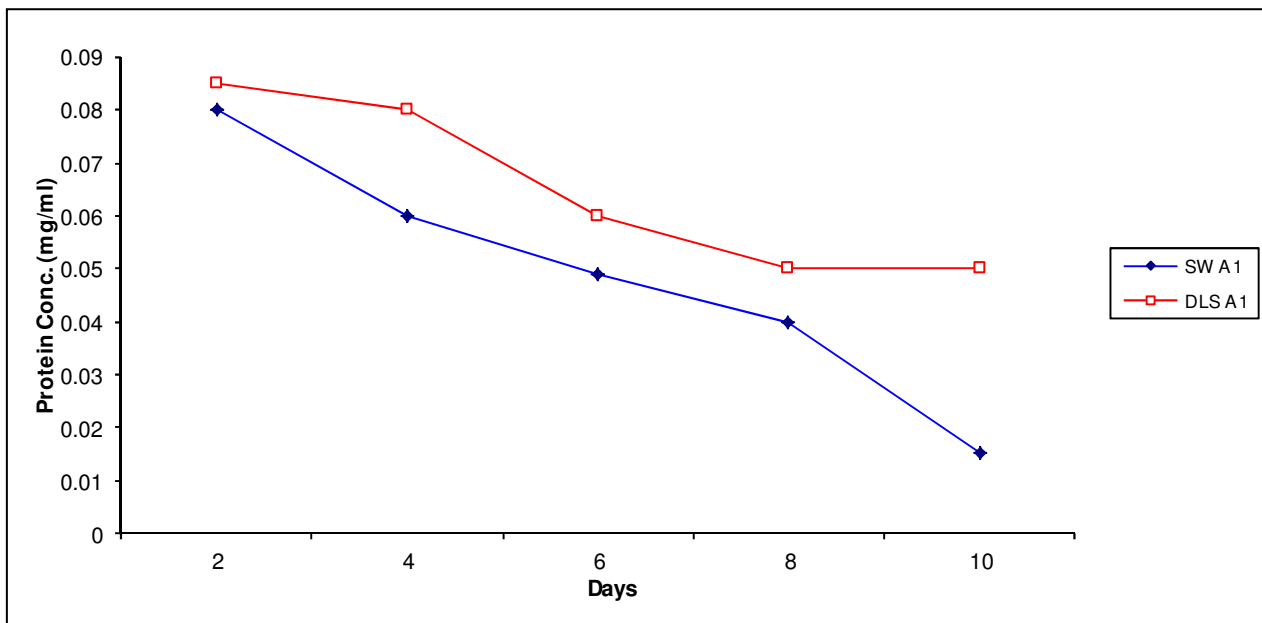


Fig.1: Extracellular protein concentration (mg/ml), of *Spadicoides* spp (SW A1) and *Aspergillus niger* (DLS A1) grown on Na-CMC over 10 days.

Several factors affect the extracellular secretion of cellulose (extracellular protein) during fermentation, accounting for the increase and decrease in extracellular enzyme activity. The depletion of micro and macro nutrients in the fermentation medium which affects the pH of the medium could result in a decrease of enzyme synthesis over time (Haq *et al.*, 2005). The decrease in cellulase (extracellular protein) content between the 6th and 10th day could be as a result of the accumulation of cellobiose, arising from the degradation of the cellulose. Cellobiose inhibits both endoglucanase and β -glucosidase (Ojumu *et al.*, 2003). Glucose, the end product of cellulase action, is a potent catabolite repressor of cellulase biosynthesis (Ilmen *et al.*, 1997; Zhang and Lynd, 2005).

Cellulase Activity was highest for both *Spadicoides* spp (58.33 units) and *Aspergillus niger* (54.83 units) at eighth day (Fig. 2). Activity decreased after day 8.

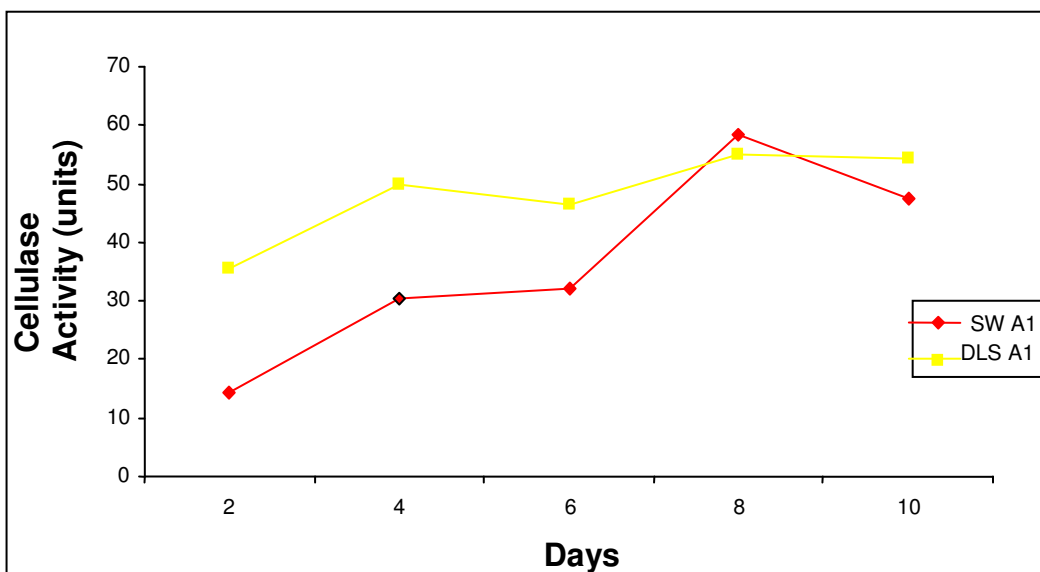


Fig. 2: Time-dependent cellulase activity of *Spadicoides* spp (SW A1) and *Aspergillus niger* (DLS A1) isolates grown on Na-CMC. The substrate for the assay was filter paper. Activity is given in units/min. (Unit = micromoles/min)

These organisms have shown cellulase activity. The activity was more than that reported by Milala *et al* (2000); Ojumu *et al* (2003) but less than the activity of some mutant strains of *T. reesei* (FAO, 1996), and the activities of *Bacillus subtilis* and *B. carculans* (Ray *et al.*, 2007). Averagely, the cellulase of *Aspergillus niger* is more active against filter paper than that of *Spadicoides* spp (fig.2).

The specific activity increased to a peak for *Spadicoides* spp (7.02) and *Aspergillus niger* (6.58) at the eighth day (Fig. 3). It decreased after day 8.

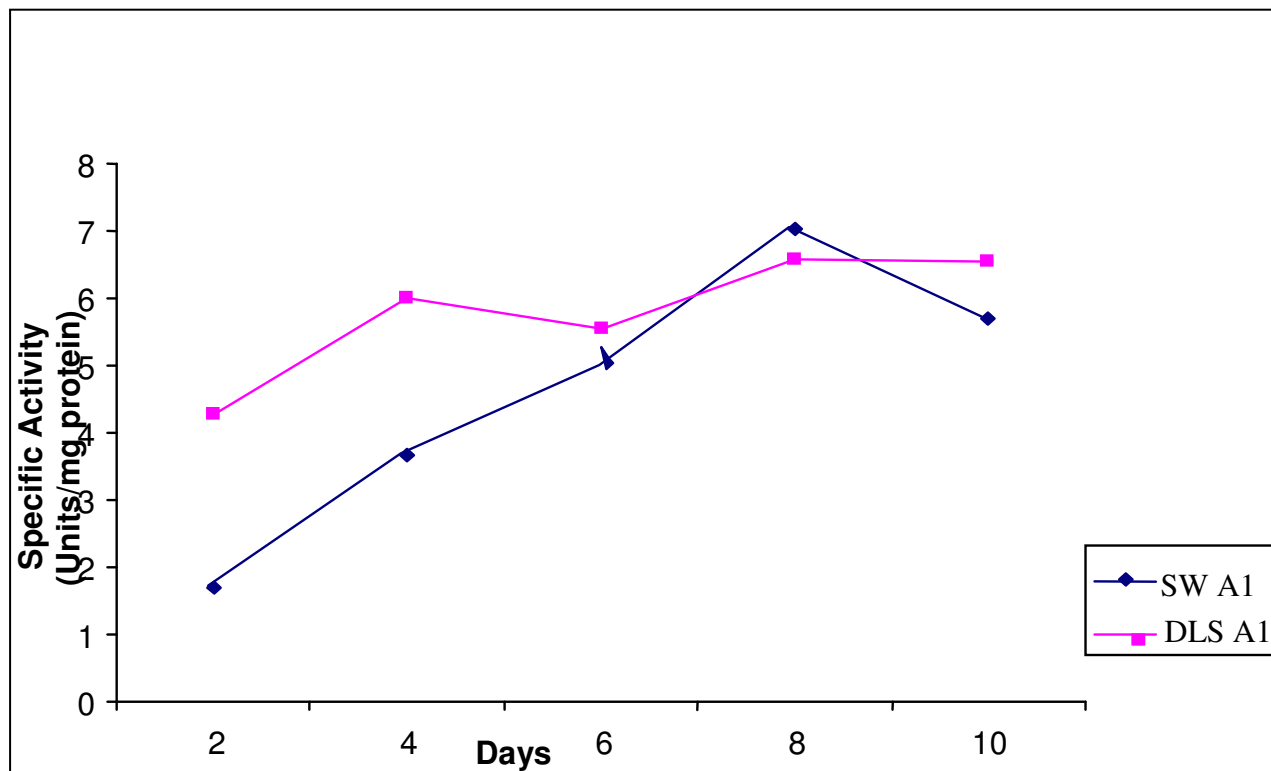


Fig. 3: Specific Activity (Units/mg protein) of *Spadicoides* spp (SW A1) and *Aspergillus niger* (DLS A1) isolates grown on Na-CMC. The substrate for the assay was filter paper. (Unit = micromoles/min).

Fungal identification was by observing both the macroscopic and microscopic features of three days old pure cultures. Colour, texture, nature of mycelia and/or spores produced, growth pattern in addition to microscopic features such as septation, spore shape, etc.

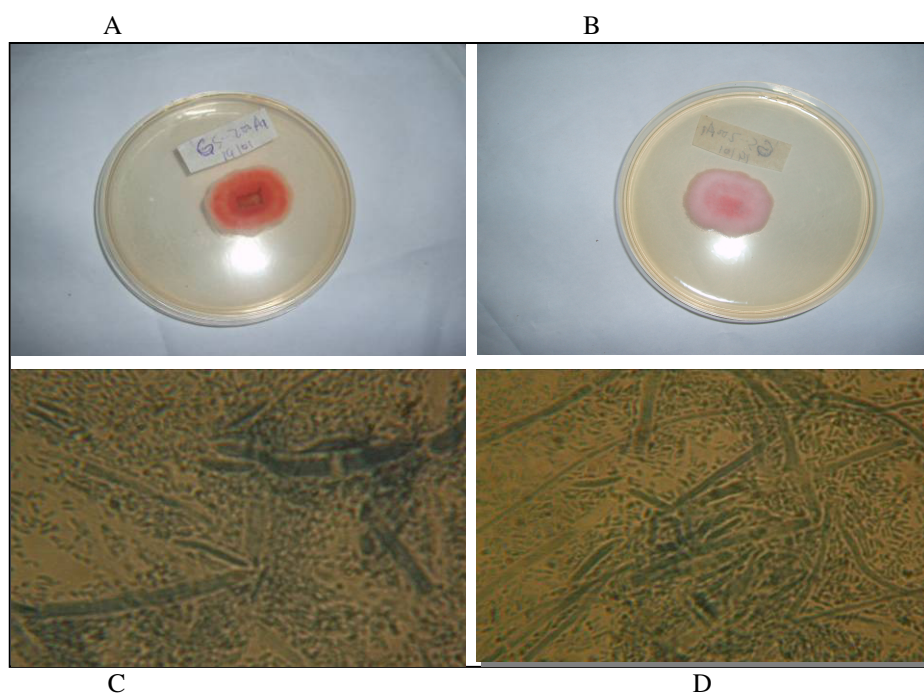


Fig. 4: *Fusarium* spp showing 3 days old culture grown on SDA plates. A is the reverse side view B is the top view while C and D shows the micrographs (X400) of lactophenol cotton blue stained structures showing hyphae and spores.

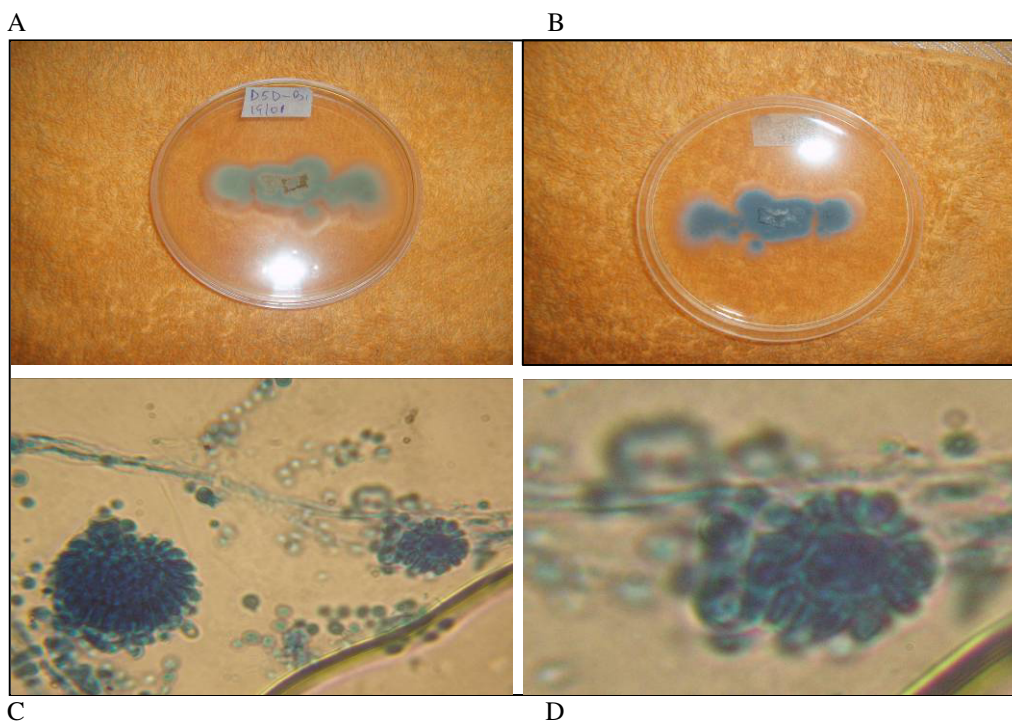


Fig. 5: *Aspergillus niger* showing 3 days old culture grown on SDA plates. A is the reverse side view B is the top view while C and D shows the micrographs (X400) of lactophenol cotton blue stained structures showing hyphae and spores.

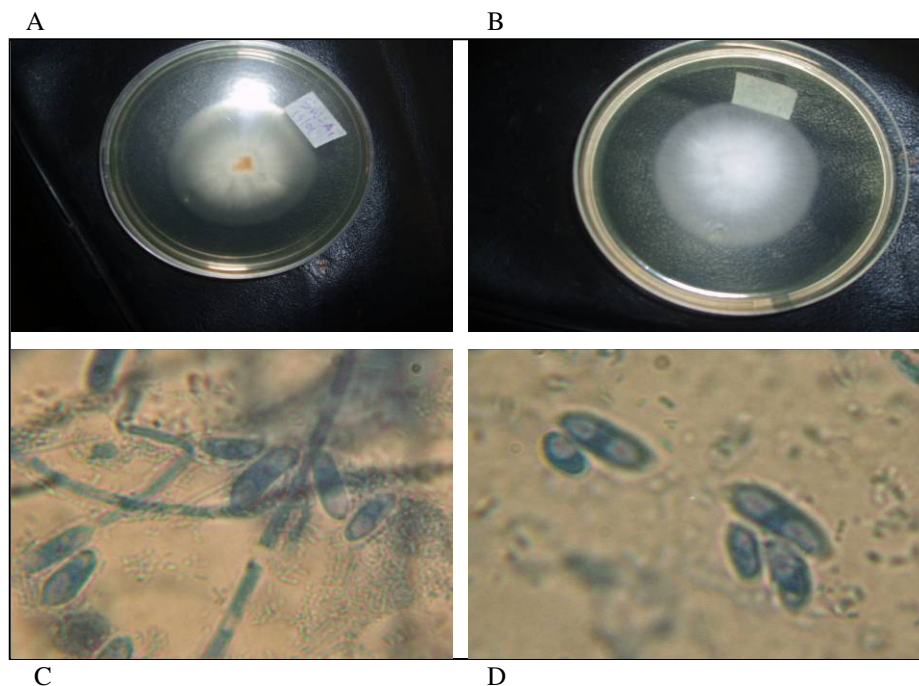


Fig. 6: *Spadicoides* spp showing 3 days old culture grown on SDA plates. A is the reverse side view, B is the top view while C and D shows the micrographs (X400) of lactophenol cotton blue stained structures showing septate hyphae bearing spores (C) and spores (D).

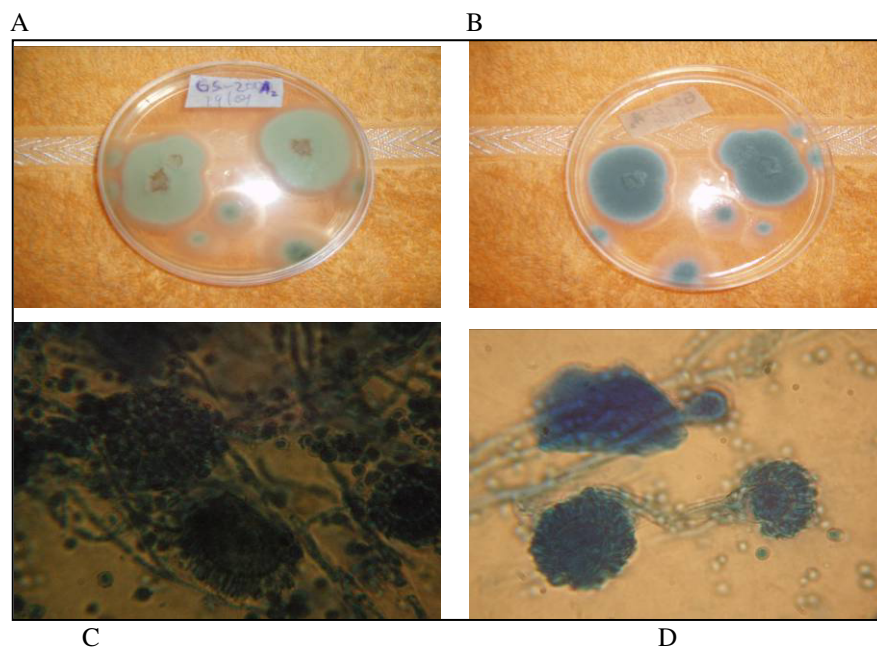


Fig. 7: *Aspergillus* spp showing 3 days old culture grown on SDA plates and micrographs. A is the reverse side view, B is the top view while C and D shows the micrographs (X400) of lactophenol cotton blue stained structures showing thick ascospores bearing spores (C) and ascospores borne on hyphae (D).

4.0 Conclusion and Recommendations

This study has demonstrated the possibility of isolating novel strains of cellulolytic organisms within our environment. A wild strain of *Aspergillus niger* was able to secrete as much as 0.085 mg/ml of extracellular protein.

SCB was used to isolate cellulolytic organisms. The organisms were capable of hydrolysing Na-CMC. The extracellular enzymes were collected in crude form and used in the degradation of cellulose, indicating

extracellular cellulase activity.

Aspergillus niger is a better cellulolytic organism than *Spadicoides* spp.

To improve on this study, genetic improvement of these organisms will make them produce more cellulase. *Spadicoides* spp was capable of producing large biomass within a short period of time. It would be important to explore this large biomass accumulation potential of this organism for protein enrichment of animal feeds.

5.0 References

- Alef, K. (1998). Enrichment of fungi. In: K. Alef and Nannieperi, P. (eds.). *Methods in Applied Soil Microbiology and Biochemistry*. Academic Press p.145.
- Badger, P.C. (2002). Ethanol From Cellulose: A General Review In Janick, J. and Whipkey, A. (eds). *Trends in New Crops and Uses*. ASHS Press, Alexandria, VA. pp 17 – 21
- Barnett, H.L., and Hunter, B.B. (1972). *Illustrated General of Imperfect Fungi* 3rd edn. Burgess, Minneapolis, Minnesota, U.S.A. pp 20 – 130.
- Bennet, J.W., Wunch, K.G. and Faison, B.D. (2002). Use of Fungi Biodegradation In: Hurst, C.J. *Manual of Environmental Microbiology* 7th edn. pp 960 – 971
- Bhat, M.K. and Bhat, S. (1997). Cellulose Degrading Enzymes and Their potential Industrial Applications. *Biotech. Adv.* **15**:583 - 620
- Biers, E.J. (2003) Microbial Interactions on decomposing Spartian Alterniflora: Use of Fungally Mediated Leachate by Bacterial Communities in an Experimental Salt Marsh Decomposition System. A Thesis Submitted to the Graduate Faculty, University of Georgia in Partial Fulfilment of the Requirements for the Degree of M.Sc.
- Cai, Y.J., Chapman, S.J., Buswell, J.A., and Chang, S. (1999). Production and Distribution of Endoglucanase, Cellobiohydrolase, and β -glucosidase Components of the Cellulytic System of *Volvariella volvacea*, the edible Straw Mushroom. *Appl. Environ. Microbiol.* **65**: 553 – 559.
- Conner, A.H., Wood B.F., Hill Jnr C.G., and Harris, J.F. (1985). Kinetic Modelling for the Dilute Sulfuric Acid Scharification of Lignocellulose. *J. Wood Chem. Tech.* **4**: 461-489
- FAO (1996). Cellulase From Natural Feedstocks. An Assessment of Environmental Impact of Cellulase Production from Feedstocks. www.fao.org Downloaded September, 2007
- Hammel, K. (2007). Research Summary. 1 p. www.bact.wisc.edu Downloaded June, 2007
- Han, S.J., Yoo, Y.J., and Kang, H.S. (1995). Characterization of a Bifunctional Cellulase and Its Structural Gene. *J. Biol. Chem.* **270**: 26012 – 26019
- Haq, I., Javed, M.M., Khan, T.S. and Siddiq, Z. (2005). Cotton Scharifying Activity of Cellulases Produced by Co-culture of *Aspergillus niger* and *Trichoderma viride*. *Res. J. Agric. Biol. Sci.* **1** :241 – 245
- Howard, R.L., Abotsi, E., Jansen Van Rensburg, E.L. and Howard, S. (2003). Lignocelulose Biotechnology: Issues of Bioconversion and Enzyme Production. *Afr. J. Biotech.* **2**: 602-619
- Howard, R.L., Musoko, P. and Abotsi, E. (2003). Enzyme Activity of *Phanerochaete chrysosporium* Cellobiohydrolase (CBH II) Expressed as a Heterogenous Protein from *Escherichia coli*. *Afr. J. Biotech.* **2**:296 – 300.
- Ilmen, M., Saloheimo, A., Onnela, M., and Penttila, M.E. (1997). Regulation of Cellulase gene Expression in the Filamentous Fungus *Trichoderma reesei*. *Appl. Environ. Microbiol.* **63**: 1298 – 1306
- Immanuel, G., Bhagavath, C.M.A., Raj, P.I., Esakkiraj P. and Palavesam, A. (2007). Production and Partial Purification of Cellulase by *Aspergillus niger* and *Aspergillus fumigatus* Fermented in Coir Waste and Sawdust. *The Inter. J. Microbiol.* **3**
- Jeffries, T. W. (1987). Production and Application of Cellulase Laboratory Procedures. USDA Forest Products Laboratory, Madison, Wisconsin. Available at <http://calvin.biotech.wisc.edu/jeffries/cellulase/mandel.html> Downloaded July, 2008
- Kader, A.J., Omar, O., and Feng, L.S. (1999). Isolation of Cellulolytic Fungi From the Bario Highlands, Sarawak. *ARBEC*. <http://www.arbec.com.my/pdf/art12sepoct99.pdf>
- Kirubahar, B., Sharma, C., Das, U. and Ghosh, D. Which Properties are Behind Fuzz Removal. Epygen Biotech Industries, Dubai, UAE. www.epygen.com Downloaded 08/2008.
- Kleman-Layer, K.M., Siika-Aho, M., Teeri, T.T., and Kirk, T.K. (1996). The Cellulase Endoglucanase I and Cellobiohydrolases II of *Trichoderma reesei* Act Synergistically to Solubilize Native Cotton Cellulose but Not to Decrease Its Molecular Size. *Appl. Environ. Microbiol.* **62**: 2883 – 2887
- Kotchoni, O.S., Shonukan, O.O. and Gachomo, W.E. (2003). *Bacillus pumilus* BpCRI 6, a promising Candidate for Cellulase Production under Conditions of Catabolite Repression. *Afr. J. Biotech.* **2**(6):140-146
- Kurasawa, T., Makoto, Y., Suto, M., Kamagata, Y., Takoa, S., and Tomita, F. (1992). Induction of Celulase by Gentiobiose and Its Sulfur-Containing Analog in *Penicillium purpurogenum*. *Appl. Environ. Microbiol.* **58**: 106 – 110

- Kuwahara, M. (2000). Effective Conversion of Renewable Plant Biomass to Energy by Using Molecular Improved Fungi. Research summary presentation, Kyoto University, Japan.
- Lee, M.J., Heitmann, J.A., and Pawlak. (2006). Rheology of Carboxymethyl Cellulose Solutions Treated With Cellulases. *Biores.* **2**: 20 – 33
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951). Determination of Protein. *J. Biol. Chem.* **193**: 265-275
- Methuvelayudham, R. and Viruthagiri T. (2006). Fermentative production and Kinetics of Cellulase Protein on *Trichoderma reesei* using Sugar Cane Bagasse and Rice Straw. *Afr. J. Biotech.* **5**: 1873 – 1881
- Milala, M.A., Shugaba, A.G., Ene, A.C. and Wafar, J.A. (2005). Studies on the Use of Agricultural Wastes for Cellulase Enzyme Production by *Aspergillus niger*. *Res. J. Agric. Biol. Sci.* **1**: 325 - 328
- Ojumu, T.V., Solomon, B.O., Betiku, E., Layokun, S.K. and Amigun, B. (2003). Cellulase Production by *Aspergillus flavus* Linn Isolate NSPR 101 Fermented in Sawdust, Bagasse and Corncob. *Afr. J. Biotech.* **2**: 150 – 152
- Okaforagu, C.U., and Nzelibe, H.C. (2006). Effect of Acid Hydrolysis of Garcinia Kola (Bitter Kola) Pulp Waste on the Production of CM-Cellulase and β -Glucosidase using *Aspergillus niger*. *Afr. J. Biotech.* **5**: 819 – 822
- Pereira Junior J.S.A., Correia, M.J., and Oliveira, N.T. (2003). Cellulase Activity of a *Lentinula edodes* (Berk.) Pegl. Strain Grown in Media Containing Carboxymethylcellulose or Microcrystalline Cellulose. *Braz. Arch. Bio. Tech.* **46**
- Perez, J., Munoz-Dorado, J., de la Rubia, T., and Martinez, J. (2002). Biodegradation and Biological Treatment of Cellulose, Hemicellulose and Lignin: an Overview. *Inter. Microbiol.* **5**: 53 - 63
- Pomeranz, Y., and Meloan, C.E. (1994). *Food Analysis: Theory and Practice* 3rd edn. Chapman and Hall Ltd. pp 625 - 677
- Raimbault, M. (1998). General and Microbiological Aspects of Solid Substrate Fermentation. *Elect. J. Biotech.* **1**: 1-9
- Ray, A. K., Bairagi, A., Ghosh, K.S. and Sen, S.K. (2007). Optimization of Fermentation Conditions for Cellulase Production by *Bacillus subtilis* CY5 and *Bacillus circulans* TP3 Isolated from Fish Gut. *ACTA Ichthia Pisca*, **37**: 47 - 53
- Seaman, J.F. (1979). Key Factors in Hydrolysis of Cellulose In: Klass, D.L. (ed) Biomass as a Non fossil Fuel Source. American Chemical Society Symposium, Series No. 144.
- Stepanova, E.V., Koroleva, O.V., Balischenko, L.G., Karapentyan, K.N., Landesman, E.O., Yavmetdinov, I.S., Kozlov, Y.P. and Rabinovich, M.L. (2003). Fungal Decomposition of Oat Straw During Liquid and Solid-State Fermentation. *Appl. Biochem. Microbiol.* **39**: 65 – 73
- Wu, B. Zhao, Y., and Gao. P.J. (2006). A New Approach to Measurement of Saccharifying Capacities of Crude Cellulases. *Biores.* **1**: 189 – 200
- Zhang, Y.P., and Lynd, L.R. (2005). Regulation of Cellulase Synthesis in Batch and Continuous Cultures of *Clostridium thermocellum*. *J. Bacteriol.* **187**: 99 – 106