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PRODUCTION OF CELLULOSIC ENZYMES BY *Aspergillus niger* AND HYDROLYSIS OF CELLULOSIC MATERIALS

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ABSTRACT

Microorganisms such as fungi can fragment carbon compounds by hydrolytic enzymes. The filamentous fungus, *Aspergillus niger* is now mostly considered because of its ubiquitous nature, non-fastidious nutritional requirements and it is classified generally as safe. This study was aimed at the production of cellulosic enzymes by *A. niger* and hydrolytic degradation of cellulosic materials by these enzymes. Standard methods were employed in soil samples collection, isolation of *A. niger* from the soils and their screening for enzyme production. Results showed that the *A. niger* isolates exhibited considerable activities of degrading and hydrolyzing cellulose in the agar media. The highest FPase, cellulase and xylanase activities were obtained from white saw dust with concentrations of 0.4059 U/ml, 0.7695U/ml and 1.3488 U/ml respectively. Also, results showed high enzyme activity at pH 6 (0.52U/ml) and temperature of 30ºC (0.72U/ml). Acid hydrolysis of the cellulosic substrates resulted to the release of 6.5% total sugar from white sawdust. The findings of this study revealed that the enzymes produced by *A. niger* hydrolyzed cellulosic materials but acid is more efficient than the enzymes in the hydrolysis and release of total sugar from cellulosic materials. This study recommends that cellulolytic enzymes used in the industries should be produced locally using filamentous fungus such as *Aspergillus niger* and cellulosic materials as carbon source.

KEYWORDS: *Aspergillus niger*, Cellulose, Enzymes, Hydrolysis

INTRODUCTION

Biomasses are major sources of renewable energy that could supplement or replace the depleting petroleum resources. Biofuel like bioethanol can be produced from plant biomass such as lignocellulosic materials (Bakare *et al*., 2019). Lignocellulose consists mainly of lignin, cellulose and hemicellulose (Sa-Pereira *et al*., 2003). Lignocellulosic materials among other alternative energy sources are easy to come by, but their biochemical conversion requires several processing. Several useful compounds can be produced from the degradation of lignocellulosic materials. As such, there is need to properly evaluate efficient processes (chemical and biological processes) to fully convert structural carbohydrates, such as cellulose, hemicellulose and lignin into simple sugars like glucose, galactose, xylose, arabinose, mannose, among others.

Thereafter, sugars could be subjected to fermentation so as to produce high biofuel and other valuable products to varying degrees of effectiveness by the action of modified microbial strains. The degradation of cellulosic materials into sugar is a process which is only possible by the action of chemical agents and/or microorganisms. The potential of using microorganisms as biological sources of industrially economic enzymes has stimulated interest in the exploitation of extracellular enzymatic activity in several microorganisms (Pandey *et al*., 2000). Recently, increased attention has been paid towards the use of agricultural waste for the large-scale production of various industrial enzymes using microorganisms. A great variety of microorganisms such as bacteria, fungi and yeasts can fragment carbon compound by hydrolytic enzymes (Pandey *et al.*, 1999). Various microorganisms have the ability to produce enzymes for hydrolyzing carbon compounds but the use

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of filamentous fungi like *Aspergillus niger* is now mostly considered because it is classified generally as safe by the United States Food and Drug Administration (De-Vries and Visser, 2001). Also, due to the ubiquitous nature and non-fastidious nutritional requirements of *Aspergillus niger*, there is great need to carry out studies on the production of extracellular enzymes by this organism and in which case, can be exploited for the degradation of cellulosic materials.

Cellulases are enzymes that have the ability to degrade cellulose. Cellulases can be divided into endoglucanase (carboxymethyl cellulase) and exoglucanase (cellobiohydrolase) which work synergistically in the hydrolysis of cellulose (Rabinovich *et al.,* 2002; Malherb and Clote, 2003). Hemicellulose, which is one of the major components of lignocellulosic materials, is degraded by xylanase. Xylanases have been produced by various fungi from lignocellulosic materials via solid state fermentation (Park *et al.,* 2002).

The degradation of cellulosic materials such as wood, grasses, agricultural waste, leaf litter, municipal solid waste, leads to the release of nutrients (Ndibe *et al*., 2017), production of biofuel (Bakare *et al*., 2019) and many other important products (Onwumere and Ndibe, 2017). More so, the degradation of plant biomass could help in reducing environmental pollution caused by these wastes. Chemical and biological treatments are the most common methods employed for the degradation of biomass; therefore, there is need to evaluate the degradation of cellulosic materials by means of acid hydrolysis and enzymatic hydrolysis.

The aim of this study was to determine the effectiveness of *Aspergillus niger* in the production of cellulosic enzymes and subsequent hydrolysis of cellulosic materials by the enzymes.

MATERIALS AND METHODS

Soil Sample Collection

Soil samples were collected from compost soil, garden soil and walk-way soil of the Nigerian Defence Academy (NDA), Kaduna in accordance with the standard method described by Razak *et al.* (1999). Sterile hand trowels were used to collect soil samples from 20 points from each soil type location at a depth of 5-10 cm. The samples were then bulked per site and a representative sample of 5 kg each was put into clean polyethylene bags and labelled appropriately. The samples were taken to Microbiology Laboratory of the Biological Sciences Department, NDA, for analyses.

Screening of the Soil Sample

One gramme (1g) of the soil sample was put into test tube containing 10ml of sterilized distilled water and mixed by shaking vigorously to make stocked suspensions. Three-fold serial dilutions of the soil sample were prepared by taking 9ml each of sterilized distilled water into separate clean test tubes. Dilute suspensions of 0.1ml from samples were aseptically taken using micro-pipette onto the plates and evenly spread using sterile spreader over Potato Dextrose Agar (PDA) medium plates which was amended with 30ug/ml of streptomycin to suppress bacterial growth. The Petri plates were incubated at room temperature of about 28± 1ºC for 7 days. Pure cultures of the isolates were obtained by sub-culturing on Czapek Dox Agar (CDA). The presumptive isolates were again maintained on Potato Dextrose Agar (PDA) slants and stored at 4ºC in the laboratory refrigerator for subsequent use.

Morphological Identification of *Aspergillus niger* Isolates.

The isolates were identified following the guide given by Reanprayoon and Pathomsiriwong (2012). Scraps of the pure isolates were taken from the slants and teased on clean grease-free slides, then stained with lactophenol-in-cotton blue solution. The slides were covered with clean grease-free cover slips and were examined under low and high power of the light microscope.

Qualitative Screening for Enzyme Producing Ability of *Aspergillus niger* Isolates

The *Aspergillus niger* isolates obtained were screened for cellulase activity by culturing on modified Mandels minerals agar medium (Mandels *et al.,* 1981). The medium was dispensed aseptically into Petri plates and then inoculated with mycelia disc of *Aspergillus niger* isolates from the stored slant bottles. The plate culture was incubated at 32ºC for 72 hours, and thereafter transferred to an incubator at 50ºC for 24 hours, followed by de-staining with 1M NaCl solution for about 30 minutes at room temperature. Cellulase activity of the carboxymethyl cellulose agar was recorded as the Index of Relative Enzyme Activity, which is equal to clear zone diameter divided by colony diameter (Eriksson, 2002). *A. niger* which exhibited widest zone of inhibition was considered to have the highest cellulase activity and was selected for crude enzyme production in submerged fermentation.

The *A. niger* isolates were also screened for xylanase-producing activity using Remazol Brilliant Blue xylan (RBB) plates screening method incorporated into Czapek-Dox medium. The mycelia disc of *A. niger* isolates from the slant bottles were inoculated into the plates. The culture plates were incubated at 32ºC for 72 hours and were observed for clear zones around the *A. niger* isolates. Thereafter, plates were flooded with iodine stain for an hour. The diameters of the colonies were measured, recorded and documented as positive reaction and the *A. niger* isolate that exhibited highest xylanase activity was selected for enzyme production in submerged state fermentation (Hahn-Hagardal *et al.,* 2007).

Enzyme Production by *Aspergillus niger*

Extracellular enzyme production was carried out using the selected *A. niger* isolates grown separately on 2g each of different cellulosic substrates such as white sawdust, red sawdust, black sawdust, corn bran, rice bran, sorghum bran and commercial cellulose as carbon sources in submerged fermentation state. Each substrate was placed in 250ml Erlenmeyer flasks containing 100ml of the Mandel's fermentation medium (Mandels *et al.,* 1981). The flasks were cotton-plugged and the media were sterilized at 121ºC for 15 minutes and then were allowed to cool to room temperature. Each medium was aseptically inoculated with 3ml of spore suspension of the selected *A. niger*. A 100ml of sterilized distilled water was inoculated with 3ml *A. niger* that was set up as control. The submerged cultures

were incubated at room temperature on an orbital shaker with a stirring rate of 200rpm for 5days; after fermentation, each of the culture media was filtered through Whatman filter paper No. 1 to remove mycelia growth. The culture filtrate was centrifuged at 10,000 rpm for 15 minutes at 10ºC in the laboratory centrifuge to remove undissolved biomass and fungal spores. The supernatant was carefully collected and stored aseptically under refrigerated temperature conditions of 4-10 ºC to prevent contamination. Thereafter, the different supernatant obtained were used as crude enzymes sources and the activity of different components in the crude enzymes source were measured in terms of FPase, CMCase and xylanase (Liming and Xueliang, 2004).

Quantitative Determination of Exoglucanase Activity

Exoglucanase activity was determined according to the method described by Lynd *et al.* (2002) using filter paper. It was assayed by mixing 1.0ml of the crude enzymes solution with a roll of '1cm by 6cm' (50mg) filter paper strip (Whatman filter paper No. 1) and 1.0ml of 0.05M sodium citrate buffer (pH 4.8) contained in 18 mm test-tube. The suspension was mixed on a vortex mixer to coil the paper and was incubated for 60 minutes at 50ºC in a water bath, the mixture was removed and was allowed to attain room temperature conditions. Four millilitres (4ml) mixture of Somogyi I and II was added to stop enzyme reaction in the mixture. The tubes were placed in boiling water bath to boil vigorously for 15 minutes and then cooled in a water bath. Two milliliters of Nelson reagent were added and the content was gently mixed. Each tube was diluted with 5ml of distilled water and mixed by inversion. The absorbance was determined using U.V spectrophotometer at 540nm. The activity was calculated and expressed in International Units (IU) as described by (Jannas *et al.,* 2002) using the following formula.

$$IU/ml/\min = \frac{\text{Activity of enzyme}}{\text{Molecular weight of glucose} \times \text{incubation time}} \times 100$$

One-unit exoglucanase corresponds to the amount necessary to release one milligram (1mg) of glucose per minute at 50ºC.

Quantitative Determination of Endoglucanase Activity (CMCase)

Endoglucanase activity was determined using carboxymethyl cellulose by the method of Lynd *et al*. (2002). The assay of endoglucanase involved the mixture of 1.0ml of 1% carboxymethyl cellulose added to 1.0ml of 0.05M citrate buffer, (pH 5.0) in a test tube. Thereafter, 1.0ml of crude enzyme solution was added and incubated at 37ºC for 6 hours. The test tubes were transferred to water bath and equilibrated at 50ºC for 10minutes. Somogyi I and II, and Nelson reagent were added and the content was gently mixed. The absorbance was measured at 540nm. The activity of the endoglucanase was then calculated.

Quantitative Determination of Endo-β-xylanase (Xylanase) Activity Assay

The assay of endo-β-xylanase was performed using Azo-xylan (Birchwood) as substrate. A 1.0ml of liquid crude enzyme was transferred to 49 ml of 100 mM sodium phosphate buffer (pH 6.0) using a displacement dispenser. The absorbance was measured at 590 nm; the activity of xylanase was calculated and expressed in international units (IU) as described by Megazyme (2007). One unit of xylanase activity was defined as the amount of enzyme necessary to releasing 1U mole of reducing sugar (glucose equivalent) from the substrate per minute

Temperature and pH Optimization

The effect of different temperatures (10ºC, 20ºC, 30ºC, 40ºC, 50ºC, 60ºC, 70ºC, 80ºC and 90ºC) were carried out using 1ml of the crude enzyme added to 1ml of 10% (w/v) carboxymethyl cellulose in 0.05M citrate buffer at optimum pH of 5.0 in each of the flasks. The mixture was incubated in water bath at different temperatures. Enzyme was assayed by measuring the reducing sugars released using 3, 5- dinitrosalicylic acid at 540 nm.

The effect of pH on the enzyme yield by *A. niger* was also carried out using carboxymethyl cellulose. The medium was adjusted to different pH values of (pH 1.0 – 12.0) using 0.1M NaOH or 0.05M H_2SO_4). The enzyme was assayed using 3, 5- dinitrosalicylic acid.

Enzymatic Hydrolysis of Cellulosic Substrates

The enzymatic hydrolysis experiment was carried out by the method of Dawson and Boopathy (2007), using the crude enzymes produced by *A. niger*. Five grammes (5g) of each substrate was placed in 100ml of 0.05M sodium citrate buffer at of pH 5.0 in 250ml Erlenmeyer flasks. The substrate and the buffer mixture were then autoclaved for 15minutes at 121ºC to prevent microbial contamination. Five millilitres (5ml) of the crude enzyme (Xylanase-1.35 U/ml, CMCase-0.77 U/ml, FPU-0.28 U/ml) were added to the reaction flasks and were incubated at 50ºC for 1 minute. After the incubation, the mixture was filtered through Whatman Filter Paper No. 1 in a Buchner funnel to removed residues. The volume for each hydrolyzed substrate was recorded and the percentage of sugar brix (the total sugar content in an aqueous solution) was taken using Atago portable refractor meter.

Acid Hydrolysis of Cellulosic Substrates

Five grams (5g) of substrate was separately mixed with 100ml of 1M H_2SO_4 in 250ml Erlenmeyer flasks and were left to soak for 24 hours (Dawson and Boopathy, 2007). The mixture was filtered using Whatman filter paper No.1 in a Buchner funnel to remove solid materials. The filtrates were neutralized using 2M NaOH, and 1g (w/v) of $Ca(OH)_2$ was added to detoxify the harmful content present and the filtrates were

centrifuged. The volume for each hydrolyzed substrate was recorded and the percentage of sugar brix was taken using Atago portable refractor meter.

RESULTS

Morphological Identification of *Aspergillusniger* Isolates

The cultural characteristics showed distinct conidiophores with a swollen vesicle bearing flask-shaped biserate phialides and large conidia head (Plate 1).

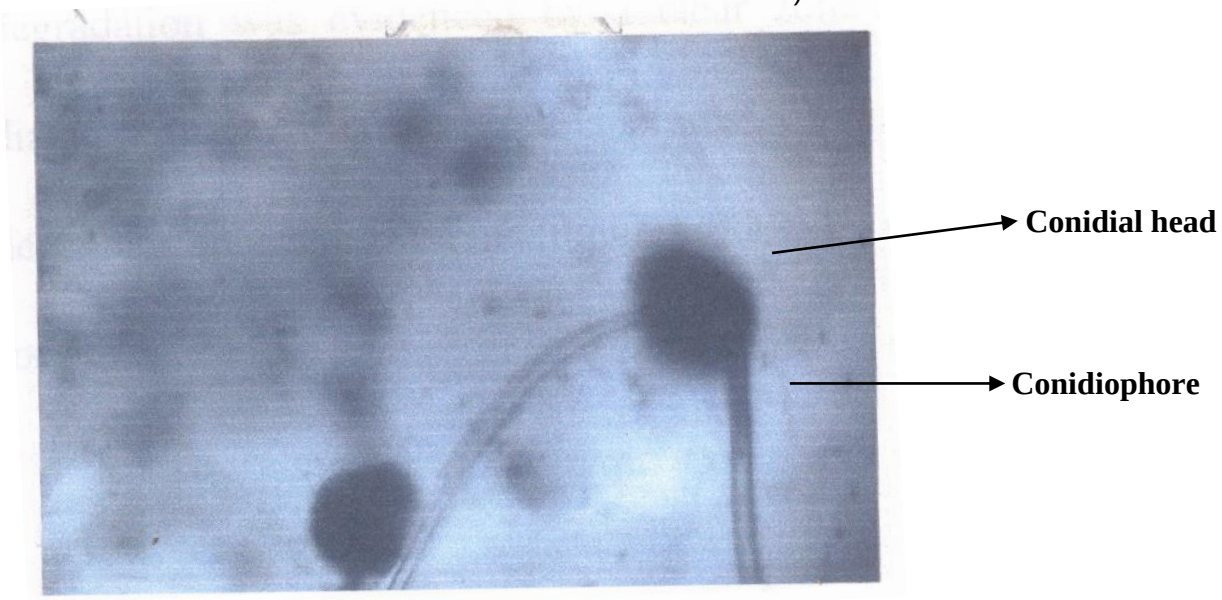

Plate 1. Microscopic view of *Aspergillus niger* (x 1000)

Qualitative Screening for Cellulase and Xylanase Activity

All the *A. niger* isolates screened exhibited considerable activities of degrading and hydrolyzing the cellulose present in the agar medium while some were unable to degrade and hydrolyzed cellulose in the medium. The highest zone of clearance was observed for compost soil *A. niger* isolate (37.0mm) followed by garden soil *A. niger* (23.0mm) and the walk way soil *A. niger* isolate (22.0mm). The results of xylanase activity showed that *A. niger* isolated from compost soil produced the largest zone of clearance (32.0 mm), followed by *A. niger* isolate from garden soil (30mm) and *A. niger* isolated from walk way soil (27.0 mm) while some could not show zone of inhibition. *A. niger* isolate which showed highest zone of clearest for both cellulase and xylanase activities were selected for production of crude enzyme (Fig. 1).

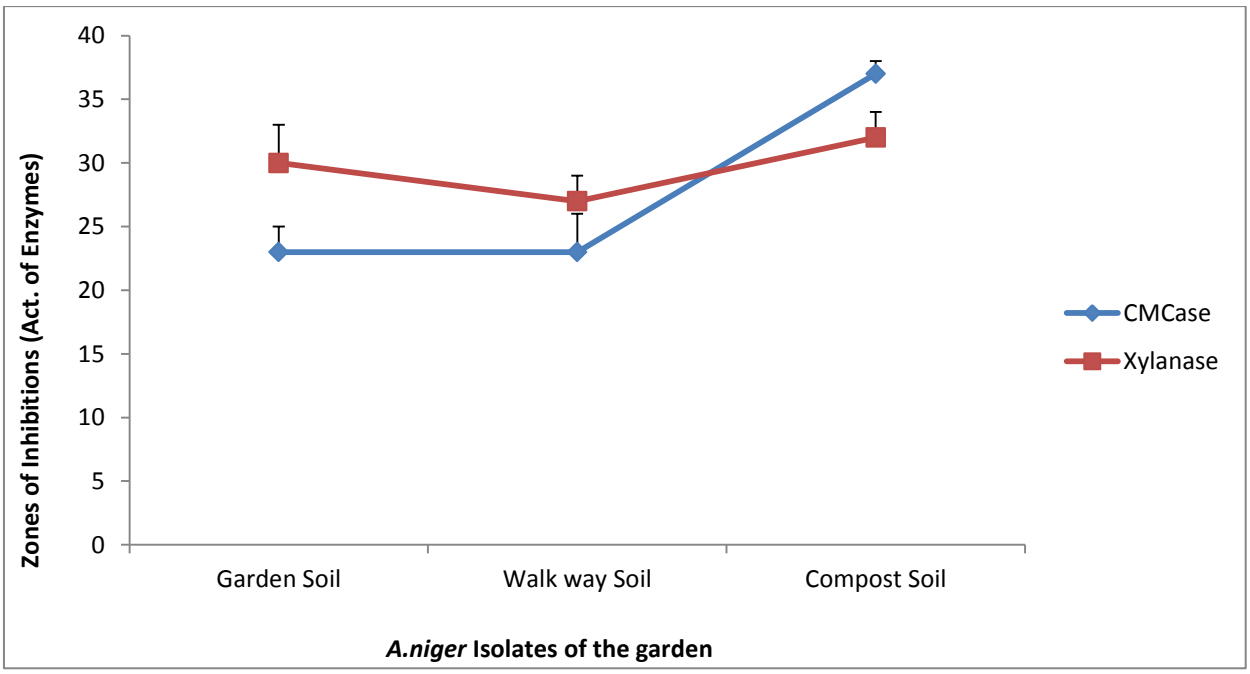

Fig. 1 Screening of *Aspergillus niger* isolates for CMCase and Xylanase Activities in Agar Plates Medium, with standard error bars.

Enzymes Production in Submerged State Fermentation.

The effect of different cellulosic substrates on the production of enzymes by *A. niger* during fermentation and the enzyme activities measured in one-unit mole (1U/ml) is shown in Fig. 2. The highest FPase activity was obtained from white saw dust (0.4059 U/ml) followed by red sawdust (0.3293 U/ml), black sawdust (0.2813 U/ml), the rice bran (0.1939 U/ml), corn bran (0.1008 U/ml) and sorghum bran which showed the lowest activity (0.0948U/ml).

The highest activity of cellulase was recorded for white sawdust (0.7695U/ml) followed by red sawdust (0.6283U/ml) and black sawdust (0.5900U/ml). The cellulase activities for rice bran, corn bran and sorghum bran had cellulase activity of 0.5242 U/ml, 0.3259 U/ml and 0.3111 U/ml respectively (Fig. 2).

The highest xylanase activity was also observed in white sawdust (1.3488 U/ml), followed by red sawdust (1.2505 U/ml) and then black sawdust (1.2167U/ml). Xylanase activities on rice bran, corn bran and sorghum bran were 0.9681 U/ml, 0.7317 U/ml and 0.6128 U/ml, respectively (Fig. 2).

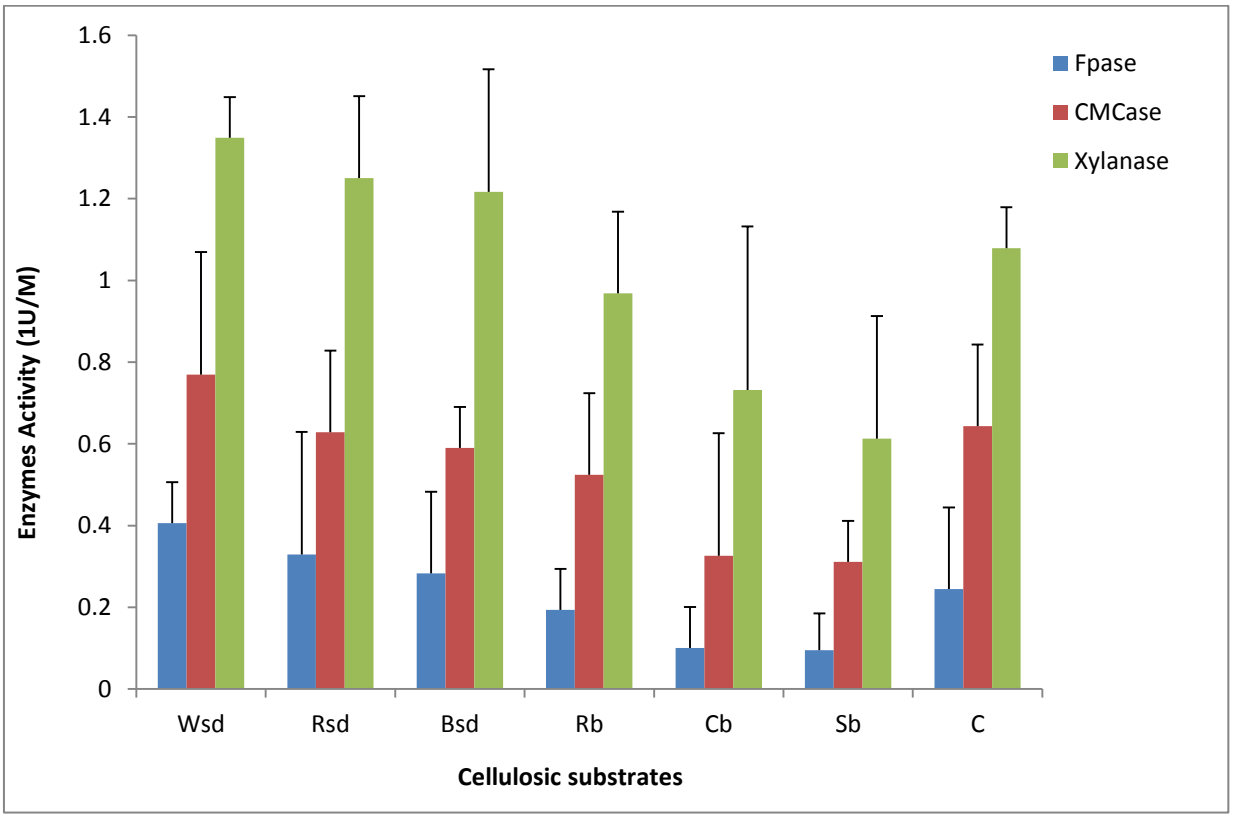

Fig. 2. Effect of substrates on extracellular production of FPase, CMCase and Xylanase by *A. niger*. Wsd - white sawdust, Rsd - red sawdust, Bsd - black sawdust, Cb - corn bran, Rb - rice bran, Sb - sorghum bran and C- commercial cellulose; with standard error bars.

Effect of pH and Temperature on Cellulosic Enzyme Produced by *Aspergillus niger*

The result on effect of pH revealed enzyme activity increased progressively from pH 1 to 6 and then declined. The highest enzyme activity of the crude enzyme from *Aspergillus niger* was 0.52U/ml at pH 6, indicating that cellulosic enzymes of the organism work best at slightly acidic medium (Fig. 3).

The results also showed high enzyme activity at a temperature of 30ºC (0.72U/ml). At high temperature range of 50 to 90ºC, the enzymes activities diminished, which means that the fungi that produced the enzyme are mesophilic in nature (Fig. 4).

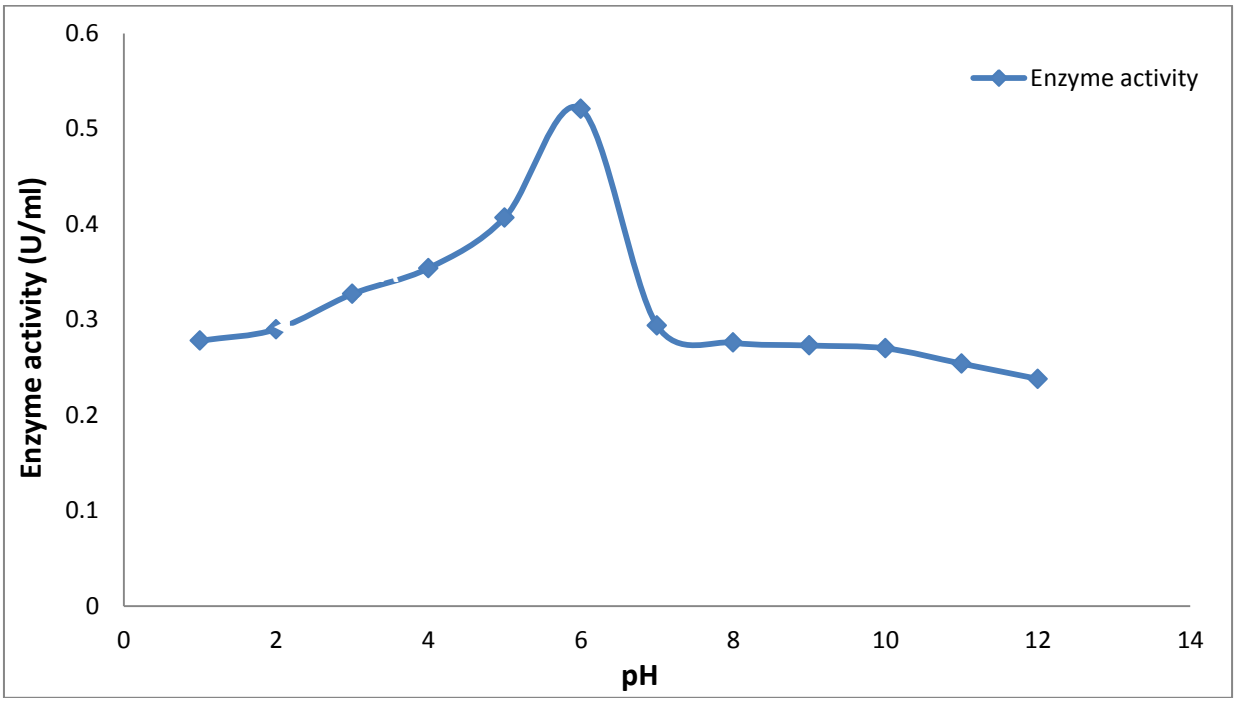

Fig. 3. Effect of pH on the activity of enzyme produced from white saw dust by *Aspergillusniger*

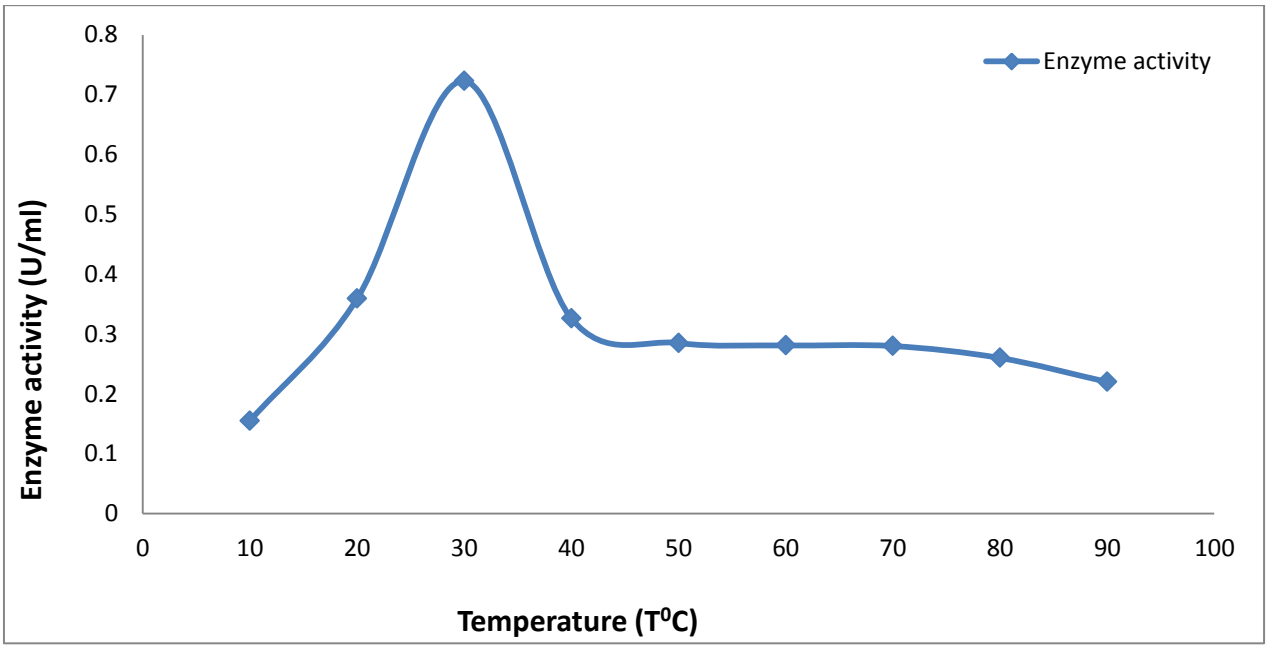

Fig. 4: Effect of temperature on the activity of enzyme produced from white saw dust by *Aspergillus niger*

Enzymatic and Acid Hydrolyses of the Substrates

The yields of sugars in hydrolyzed liquors upon incubations with crude cellulases at a loading of 0.4U/ml FPU/ml, 0.77U/ml of CMCase and 1.35 U/ml xylanase showed that white sawdust released the highest amount of sugar followed by red sawdust, rice bran, black sawdust, corn bran and sorghum bran being 5.0%, 4.0%, 3.7%, 3.5%, 2.5% and 2.5% respectively (Fig. 5).

Acid hydrolysis of the cellulosic substrates resulted to the release of 6.5% total sugar from white sawdust, whereas red sawdust, black sawdust, rice bran, corn bran and sorghum bran released 6.0%, 5.0%, 5.0%, 4.0% and 4.5% total sugar respectively (Fig. 5). As a way of comparison, results showed that acid is more efficient than cellulosic enzymes in the hydrolysis and release of total sugar from cellulosic materials (Fig. 5).

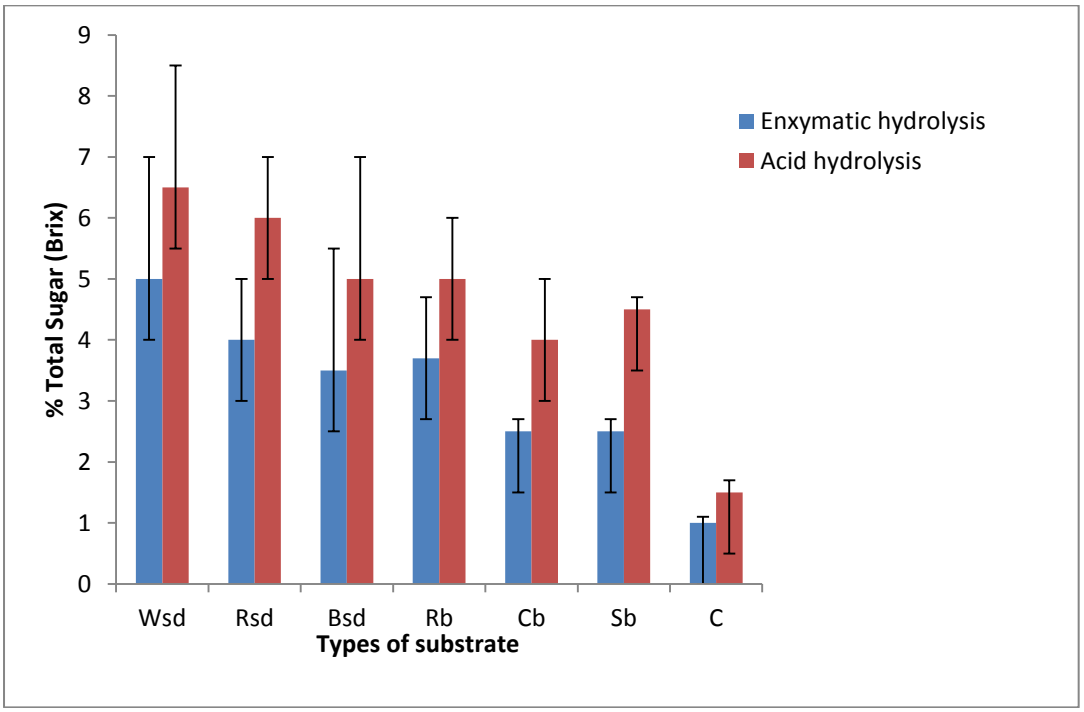

Fig. 5: Total sugar released during enzymatic and acid hydrolyses of the substrates. Wsd - white sawdust, Rsd - red sawdust, Bsd - black sawdust, Cb - corn bran, Rb - rice bran, Sb - sorghum bran and C- commercial cellulose.

DISCUSSION

Fungi are known to be agents of degradation of cellulosic substrates and organic matters (Haan–Darand Zyl-von, 2002). The filamentous fungus like *Aspergillus niger* is mostly used because of its predominance in the soil and also, it is generally regarded as safe. This is similar to the report of Reanprayoon and Pathomsiriwong (2012)who established that members of the genera *Aspergillus* and *Trichoderma* are dominant fungi in tropical soils and that the organisms are ubiquitous in nature. The strains of *A. niger* were randomly picked and were subjected for further studies because they showed efficiency of enzyme activity for biodegradation. However, it also showed that enzymes produced by *A. niger* strains could vary considerably and this type of variations in enzymes production were noted by the relative wide range of clearance zones. *A. niger* of compost soil has high value of xylanase and cellulase activity: 32.0mm and 37.0mm respectively as recorded from the measurement of the clear zone in diameter (mm). This is due to response on the limiting cellulose in growth medium. Any fungus that has the ability to degrade cellulose, hemicellulose and lignin simultaneously possesses cellulolytic activity (Bhat,2000). Enzyme activity of this kind was reported by De-Vries and Visser (2001) for *A. niger* strain which is believed to have the tendency of hydrolyzing celluloses content of the substrates and can be potent in enzymes production. The best produced clear zone by the *A. niger* strain was selected and then used for the production of extracellular enzymes like endoglucanase, exoglucanase and xylanase in the fermentation medium. This study showed that endoglucanase (CMCase), exoglucanase (FPase) and Xylanase were produced in the medium with different concentrations. The highest enzyme assay shows: CMCase 0.77U/ml, FPase 0.41U/ml and Xylanase 1.35U/ml were produced during degradation of white saw dust by *A. niger* (compost soil) strain in the fermentation medium. The least of the enzymes produced was found during degradation of sorghum bran by the same *A. niger* strain in fermentation medium which produced CMCase 0.31U/ml, FPase 0.09U/ml and xylanase 0.61U/ml. This is similar to the results obtained by De-Vries and Visser

(2001) who reported that different species of genus *Aspergillus* were identified to have different systems of producing enzymes.

Comparing the hydrolyses ability, the fungal crude enzyme produced by *A. niger* using white sawdust and 1.0M sulphuric acid were used for the hydrolysis of substrates. The results show highest hydrolysis efficiency of (6.5%), while the lowest hydrolysis efficiency of (4.0%) was recorded for acid hydrolysis with white saw dust and corn bran as against (6.0%) with enzymatic hydrolysis of white saw dust and lowest (2.5%) for corn and sorghum bran. The extent of hydrolysis in relation to different substrates could be as a result of biomass content in each lignocellulosic substrate since the hydrolysis effectiveness is highly dependent on the chemical and physical properties of the feedstock as reported by Berlin *et al.* (2006). It is also possible that enzyme hydrolysis on raw cellulosic materials at first stage can be inefficient, probably because of impeded access of the enzyme molecules to the amorphous sections of the carbohydrate chains (Valchev *et al.,*2009).

CONCLUSION

Aspergillus niger was isolated from different soil samples and in line with clear zone formation showed cellulase and xylanase activities of the fungal isolate. *A. niger* was found to produce enzymes CMCase, FPase and Xylanase, same were used as crude enzymes for hydrolysis. As cellulose is encased in a matrix of lignin and hemicellulose in lignocelluloses, it is clear that hydrolyzed lignocellulosic substrates can be tapped as sources for the production of fermentative sugars that can subsequently be used for different value-added products by microorganisms. Nevertheless, this study showed a potential use of locally produced crude enzymes which hydrolyzed lignocellulosic materials to an extent, as compared to the acid which hydrolyzed the substrates better. However, it is recommended that enzymes used in the industries should be produced locally using cellulolytic organisms like *Aspergillus niger* and cellulosic materials as carbon source.

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