Endoglucanase (EC3.2.1.4) Production by *Aspergillus niger, Trichoderma reesei* and *Rhizopus stolonifer* using agro industrial by-products

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ABSTRACT

Agro industrial by products (wheat offal, corn offal and groundnut pod) and cellulose were used as substrates for the production of endoglucanase (EC3.2.1.4) by *Aspergillus niger (A.niger), Trichoderma reesei (T. reesei)* and *Rhizopus stolonifer (R.stolonifer).* The optimum enzyme activity for *T. reesei* (0.75 unit mg protein^{-1}) was obtained at 72 h of cultivation, while *A. niger* (0.65 unit mg protein^{-1}) and *R. stolonifer* (0.51 unit mg protein^{-1}) gave their highest enzyme activities at 48 and 72 hours respectively when the fungi were incubated on cellulose for 96 hours. For the agro industrial by-products, maximum enzyme activity was obtained with groundnut pod where *A. niger*, *T. reesei* and *R. stolonifer* gave the maximum enzyme activity of 0.35, 0.30 and 0.29 units mg Protein^{-1} respectively after 144 h of growth. *A. niger* had the highest enzyme activity with any of the agro industrial by products followed by *T. reesei*. Hence, the study showed that the use of groundnut pod is the best among the agro industrial by-products for low-cost commercial production of endoglucanase using *A. niger*.

Keywords: agro industrial-by-products, fungi, endoglucanase, bioconversion

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INTRODUCTION

Owing to the ever increasing cost of poultry production in Nigeria because of increase in the cost of conventional feed ingredients due to the competition between humans and the monogastric, it becomes imperative for farmers to begin to consider alternatives to these conventional feed ingredients. Attention may therefore be paid to the availability and abundant production of agricultural and industrial byproducts (AIBs). They are renewable resource available in tremendous quantities as agricultural, industrial and possibly municipal wastes. Bioconversion of

the lignocelluloses into animal feed-stock, bulk chemicals and biofuel is being studied as a means of alleviating food and energy shortages and reduction of environmental pollution (Pandey et al., 2000; Porfiri et al., 2012). Nigeria, being an agricultural country, a large amount of agro wastes is accumulated annually. Cellulosic biomass constitutes the most abundant organic material on earth and is continually replenished by carbon dioxide fixation via photosynthesis. Cellulose occurs naturally in plants cell walls as basic fibrils in deep attachment with other polysaccharides most especially hemicelluloses and lignin. Lignocelluloses basically contains by weight 35-50% cellulose, 20-35% hemicellulose and 5-30% lignin (Lynd et al., 2002). All cellulosic materials can be transformed into commercially important products. Bioconversion of the AIBs via enzymatic hydrolysis has been a subject of intensive research. Utilization of cellulose and other cell wall polysaccharides is a function of their conversion into simple sugars(Lynd 2008).The relevance of the hydrolysis is chiefly acknowledged in the tendency to converting the "wastes" into "wealth" such as carbon and energy sources for fermentation, improved animal feed and human food (Belewu and Afolabi, 2000). No doubt, biological conversion of these AIBs into these viable products could enhance their quality and use thereby reducing feed, food and energy scarcity and also minimize pollution in our environment. No doubt, the emergence of an industrial process for cellulose bioconversion would reduce shortages in food and animal feeds and also reduce the problems of urban waste disposal. Full utilization of AIBs is a function of development of a process which would include the production of cellulases required for the enzymatic hydrolysis of cellulosic materials (Sahnoun et al., 2012; Subhedar and Gogate, 2014). Cellulose hydrolysis takes place by synergistic action of endoglucase (EC 3.2.1.4), exoglucanase (EC 3.2.1. 91) and β-glucosidase (EC 3.2.1. 21). Cellulases are extracellular enzyme mixtures produced by fungi, bacteria, insects and lower animals (they all use cellulose nutritionally as carbon source). Cellulases can break cellulose to smaller sugar parts like glucose. Notably, microorganisms produce three major cellulolytic enzymes which are: endoglucanase, exoglucanase and glycosidase and they all act synergistically on cellulose generating low molecular weight reducing sugars. Endoglucanases cut at random at internal amorphous sites in the cellulose polysaccharide chain producing oligosaccharides of different lengths and consequently new chain ends. Endoglucanase initiates depolymerization of cellulose by exposing the reducing and non-reducing ends and cellobiohydrolases acts upon these reducing and non-reducing ends to liberate cello-oligosaccharides and cellobiose units and β-glucosidase breaks the cellobiose to release glucose and this completes the hydrolysis process (Świątek et al., 2014). No doubt, the study of endoglucanase is important because of its prominent role in the conversion of cellulosic AIB substrates into feed and food materials. This work studied the possibility of using wheat offal, corn offal and groundnut pod as carbon sources for endoglucanase production using *Aspergillus niger, Trichoderma reesei* and *Rhizopus stolonifer*. In addition, the use of cellulose was explored in order to examine the relative and comparative influence of the AIBs on endoglucanase production by the microorganisms.

MATERIALS AND METHODS

Cellulosic Substrates

Wheat and corn offal were obtained from a feed mill in Ibadan, Nigeria while groundnut pod was also obtained from a groundnut oil producing company also in Ibadan, Nigeria. The pods were sun-dried for 7 days in order to minimize the water content and make the grinding possible. The

milled products were used as the substrates. Crystalline cellulose and Potato Dextrose Agar (PDA) were made by Merck, Germany. Other chemicals and reagents used were made by Sigma Chemicals Co. Ltd, England and they were of analytical grade.

Organisms

The organisms were obtained from the School of Biotechnology, Jawaharlal Nehru Technological University, Hyderabad, India. They were sub-cultured on PDA plates and incubated at 30^{o}C for 4-6 days in order to get the spores used for this work.

Mineral Culture Medium and Cultivation of Microorganisms for Endoglucanase Production

Fifty grams of milled samples of wheat offal, corn offal and groundnut pod was moistened with100ml of the mineral culture medium (KNO3, 5.0mg;KH_2PO_4, 2.0g; $MgSO_4.7H_2O$, 0.5g; Tryptone, 0.5g; FeSO4.4H2O, 3.5mg; Nicotinic acid, 0.5mg; Thiamine, 0.05mg and Biotin, 0.05mg per litre of distilled H_2O). The preparation was mixed thoroughly and the pH of 6.0 was obtained by using 0.IN HCl to effect the adjustment. The flasks were plugged with cotton wool and then autoclaved at 121^{o}C for 15 minutes. The autoclaved culture was cooled to room temperature. Autoclaving at was done before adding the mineral culture medium. The inoculum of each isolate was prepared by pouring 10ml of sterile distilled water onto spores of each agar slants and using sterile wire loop to wash the spores into the water. The filtrate of each isolate was subsequently diluted with more sterilized distilled water until a spore count of approximately 2.85 X 10^6 per ml was obtained using haemocytometer. Each flask was inoculated with 1.0ml of an aqueous spore suspension of each isolate. The flasks were incubated in an orbital cooled shaking incubator (JSSI 300C Gallenkamp) at 25 ^{o}C. 5.0ml of samples were taken from each of the flasks for the determination of crude protein concentration and endoglucanase activity.

Enzyme Assay

Endoglucanase (EC 3. 2. 1. 4) activity was assayed by a modification of the reducing-sugar method described by Khan (1980) using carboxymethyl-cellulose (CMC) as enzyme substrate. The reaction mixture had 2 mL of 1.0% (w/v) CMC in 0.1M solution of sodium acetate buffer, pH 5.0 and 2.0 mL of the cell-free culture supernatant. The mixture was incubated at 37^{O}C in water bath with shaking for 30 minutes. The reducing sugar released by the enzyme was measured as glucose equivalent using 3, 5 -dinitrosalicyclic acid reagent and read at 540nm using a spectrophotometer (Miller, 1959). The released reducing sugar was expressed in glucose equivalent and expressed in Units mL^{-1}. A unit of activity was defined as the amount of enzyme required to liberate 1μmol of glucose per minute under the assay conditions.

Protein Assay

The Protein content of the crude enzyme was determined using the method of Lowry *et al*., (1951) and adopting bovine serum albumin (BSA) as standard.

RESULTS

Figure 1 reveals the differences in cellulase activities of *A. niger*, *T. reesei* and *R. stolonifer* when grown in cellulose media incubated for 96 hours. Maximum enzyme activity was realized from the culture broth of *T. reesei* at 72h incubation and the enzyme activity was 0.75 Units mg protein^{-1}. Enzyme activity of *A. niger* was highest at 48 h with an activity of 0.65 units mg protein^{-1} but that of *R. stolonifer* got to the peak at 36 hours of incubation and the value was 0.5 units mg protein^{-1}. Enzyme activities show two prominent peaks with *A. niger* and *T. reesei* during the 48 and 72 hours incubation periods respectively (Fig. 1). Besides, for A. *niger*, the least activity was at 36 hours. The least activity for *T. reesei* (0.25 units mg protein^{-1}) occurred at 96hours. Table I shows the extracellular protein obtained from *A. niger*, *T. reesei* and *R. stolonifer* incubated with different agro industrial byproducts. More proteins were obtained from the use of AIBs than the pure crystalline cellulose. Figures 2, 3 and 4 show the graph of cellulase activities of *A.niger*, T. reesei and R. stolonifer incubated on the three agro industrial by-products (wheat offal, corn offal and groundnut pod) for a period of 192 hours. Figure 2 expresses the cellulase activity from *A. niger*. The maximum cellulase activity of 0.35 unit mg protein^{-1} was observed with groundnut pod during 144 hours of growth. The highest cellulase activities value for corn offal and wheat offal were 0.25 and 0.30 unit mg protein^{-1} respectively. Figure 3 shows cellulase activities of *T. reesei* incubated on wheat offal, corn offal and groundnut pod for 192 hours. Highest activity (0.29 unit mg protein^{-1} was obtained at 144hours when grown on groundnut pod while the least (0.05 unit mg protein$^{-1)}$ was recorded when T. reesei was cultivated on corn offal at 192hours. The cellulase activity recorded from the broth of *R. stolonifer is* shown in Fig. 4. Peak cellulase activity (0.33 unit mg protein^{-1}) was obtained with wheat offal at 120 hours. The maximum cellulase activity was followed by 0.29 unit mg protein^{-1} recorded at 144hours when *R. stolonifer* was incubated on groundnut pod. Cellulase activities of *A. niger*, *T. reesei* and *R. stolonifer* incubated on groundnut pod containing media for 192 hours are shown in figure 5. *T. reesei* produced the highest amount of cellulase at 144 hours. The least amount (0.05 unit mg protein^{-1}) was observed with the *T. reesei* during the 48 hour.

Table 1: Extracellular protein obtained from *A. niger, T. reesei* and *R. stolonifer* incubated with different agro industrial byproducts

Organisms	Average extracellular protein released (µgml^{-1})			
	Cellulose	Wheat offal	Corn offal	Groundnut pod
A. niger	172±34	447±52	418±81	341±64
T. reesei	147±21	482±64	395±75	289±30
R. stolonifer	122±41	430±92	364±51	325±65

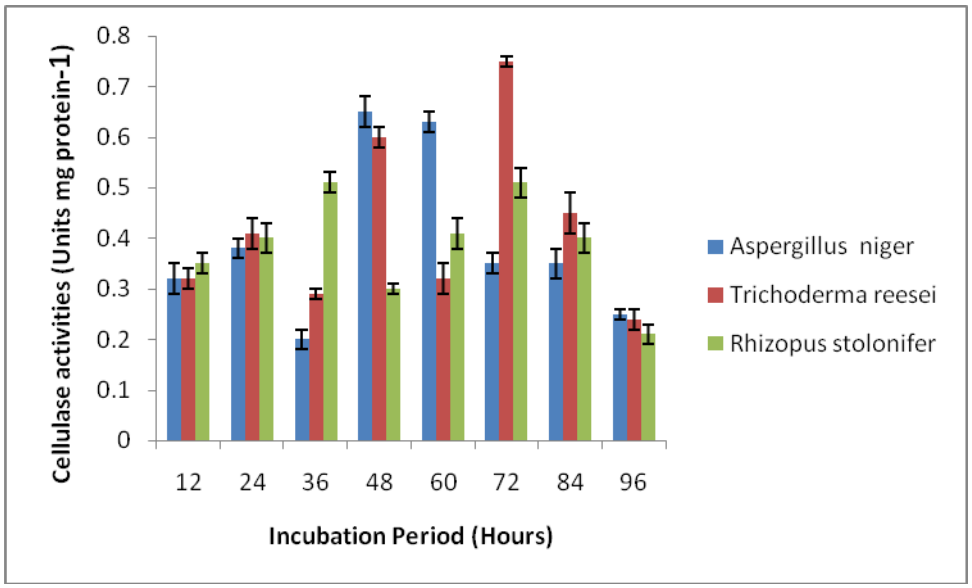

Fig. 1: Cellulase activities of *A. niger, T. reesei* and *R. stolonifer* incubated in cellulose media for 96 hours.

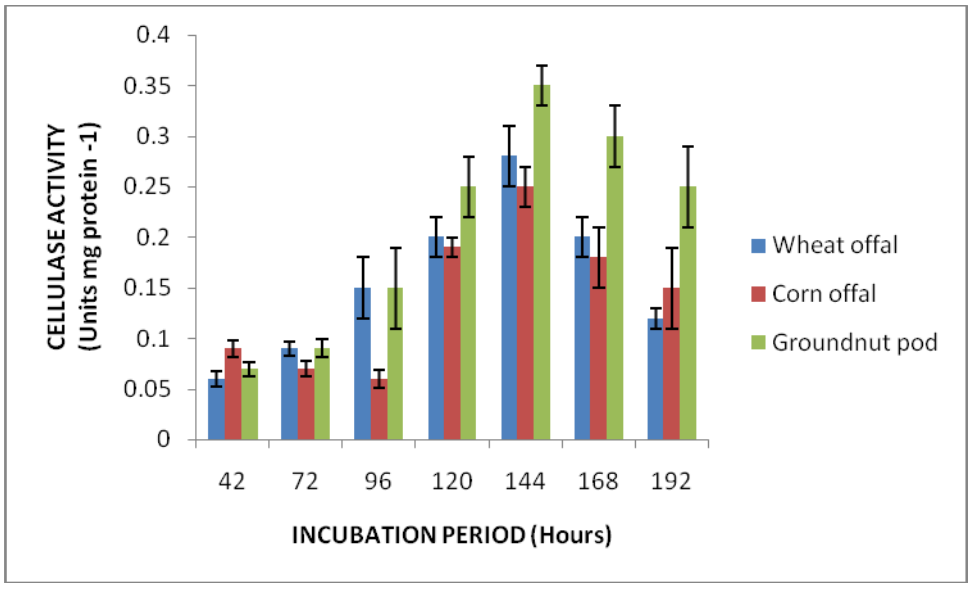

Fig. 2: Cellulase activities of *A. niger* incubated on wheat offal, corn offal and groundnut pod for 192 hours.

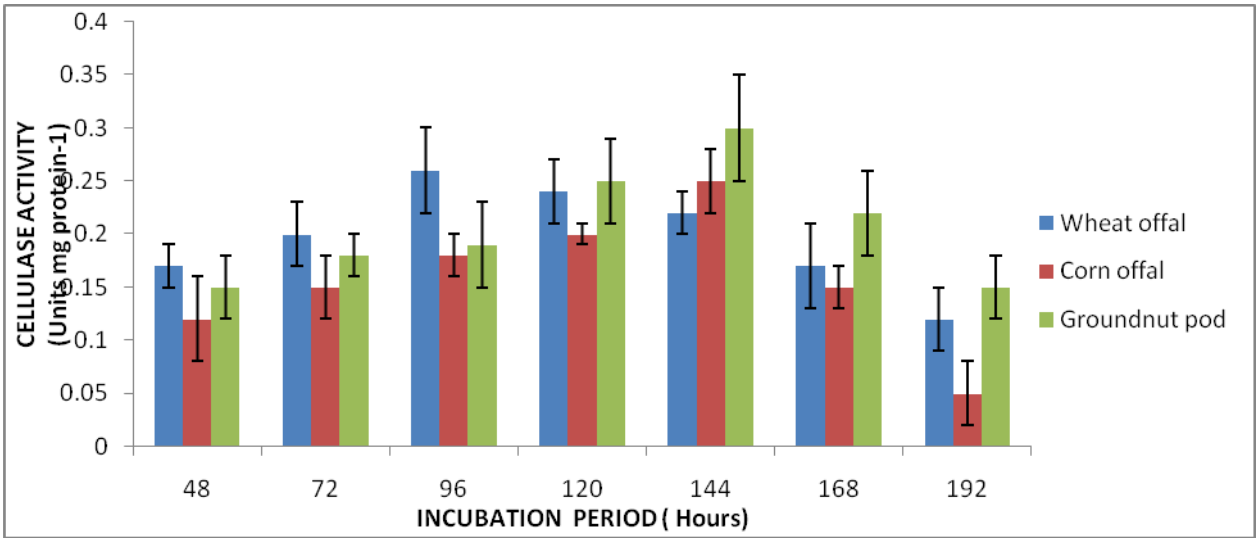

Fig. 3: Cellulase activities of *T. reesei* incubated on wheat offal, corn offal and groundnut pod for 192 hours.

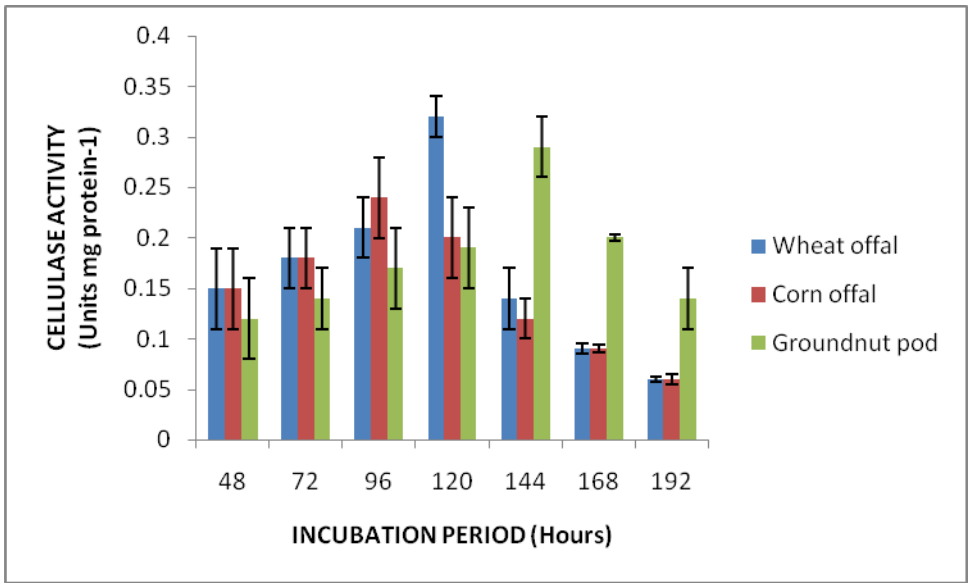

Fig. 4: Cellulase activities of *R. stolonifer* incubated on wheat offal, corn offal and groundnut pod for 192 hours.

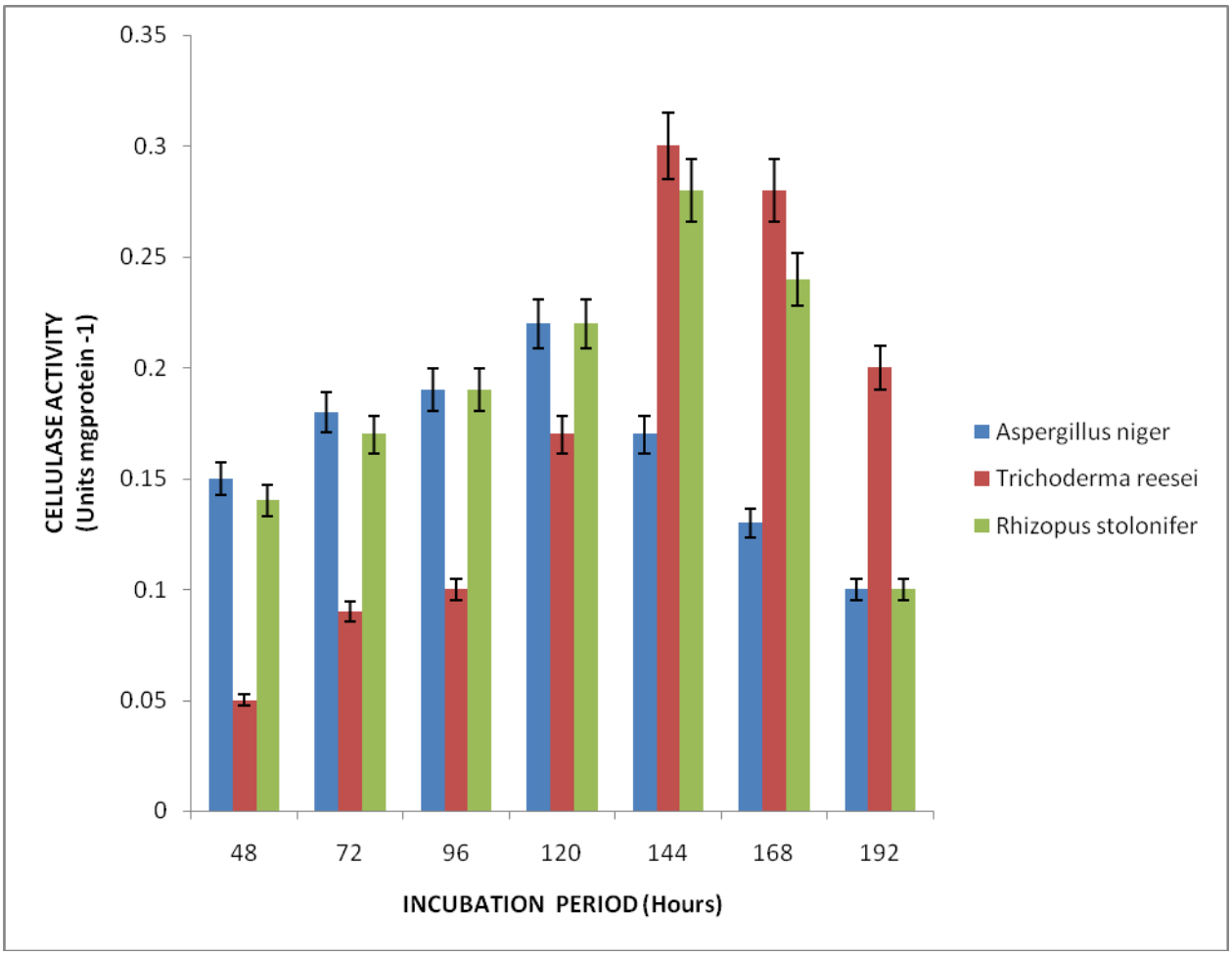

Fig. 5: Cellulase activities of *A. niger, T. reesei* and *R. stolonifer* incubated on groundnut pod containing media for 192 hours.

DISCUSSION

From the obtained results, it was observed that the fungi have various times for maximum cellulase production. Notably, different times for A. *niger* compared to *T. reesei* and *R. stolonifer* when grown on cellulose and different AIBs (Table 1). The differences in the complexity of the carbon sources could account for the disparity in the cellulase production. Cellulose is a polymer of β-D-glucose while wheat offal, corn offal and groundnut pod are composed of complex plant cell wall polymers which include cellulose, hemicelluloses and lignin (Ojumu et al., 2003; Vries and Visers 2001). For the organisms to obtain simple sugars from cellulose or the AIBs they have to synthesize cellulases and other enzymes required for the hydrolysis of the macromolecules. The extracellular protein released by the organisms when

cultured on cellulose is low when compared to the enzymes produced when incubated on the AIBs. This is likely to be like that because the fungi would not have to do much work to obtain simple sugars from the cellulose and therefore do not need to produce the hydrolytic enzymes (extracellular proteins). It is important to note that cellulases and most of the plant cell-wall hydrolyzing enzymes are inducible and are also influenced by catabolite repression in many fungi (Chinedu-Nwodo et al., 2005; Chinedu-Nwodo et al., 2007). In other words, availability of high concentration of glucose in the media will reduce the production of enzymes. This explains why the endoglucanase (EC3.2.1.4) was more produced in the AIBs than in the crystalline cellulose. In addition, the higher protein yield obtained in media containing AIBs suggests the availability of other proteins (apart from cellulase enzyme) which may include many other cell-wall hydrolyzing enzymes. Moreover, secondary plant cell wall apart from cellulose do contain other polymers particularly hemicelluloses and lignin and this may possibly induce the production of many enzymes. The pattern of cellulase activities of *A. niger, T. reesei* and *R. stolonifer* when cultured on cellulose for 96 hours is shown in figure 1. Maximum activity happened later and not from the beginning. This may be due to the fact that the organisms took time to initiate the hydrolysis process as it takes time for the fungi to establish the rhizoids on the substrates (Ashly et al., 2011; Okafor et al., 2007). In figures 2 and 3 groundnut pod appeared to be the most suitable AIB for the endoglucanase production. In fact, in figure 4, it ranked second after wheat offal. This may be due to better availability of secondary plant cell wall materials containing other polymers particularly hemicelluloses and lignin in addition to cellulose which would have induced better cellulase activities (Hashemi et al., 2013; Chinedu-Nwodo et al., 2007) . Owing to the possibility of producing more cellulase activities if grown on groundnut pods, the fungi (*A. niger, T. reesei* and *R. stolonifer)* were therefore cultured on it (Figure 5). It was discovered that *T. reesei* recorded the highest activity (0.33 unit mg protein^{-1} at 144hour).

CONCLUSION

In conclusion, *A. niger, T. reesei* and *R. stolonifer* have the ability to produce endoglucanase from wheat offal, corn offal and groundnut pod. Among the three AIBs, groundnut pod recorded the highest yield of enzyme when *A. niger* and *T. reesei* were grown on it and it came second when *R. stolonifer* was cultured on the AIBs. From the foregoing, it is evident that these AIBs may be considered for the commercial production of endoglucanase. Moreover, their use will lead to production of cheaper cellulase and reduction of environmental pollution.

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