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## Research Article

# Production of Mannanase Enzyme Using *Aspergillus* spp. Isolated from Decaying Palm Press Cake

**Agu K.C.[1]*, Okafor, F.C. [1], Amadi, O.C.[2], Mbachu A.E.[1], Awah N.S.[1] and Odili, L.C. [3]**
[1]Department of Applied Microbiology and Brewing, Nnamdi Azikiwe University, PMB 5025, Awka, Nigeria.
[2]Department of Microbiology, University of Nigeria, Nsukka, Nigeria
[3]Department of Applied Biochemistry, Nnamdi Azikiwe University, PMB 5025, Awka, Nigeria.

***Corresponding author**
Agu K.C.
**Email:** agukingsleyc@yahoo.com

**Abstract:** A total of three *Aspergillus* species were isolated from decaying palm press cake. These organisms were screened on Dox medium for mannanase production. All three isolates grew on the medium, thus indicating that the organisms were able to utilise the locust bean gum present in the medium. The *Aspergillus* isolates were used to produce mannanase enzyme under submerged fermentation. Cocoyam powder was used as the sole carbon source in the Dox medium and it served as a substitute to locust bean gum. The final pH values of the three isolates were in the acidic range. *Aspergillus tamari* had a growth rate of 0.17g/ml, while the *Aspergillus niger* had a growth rate of 0.25g/ml. The *Aspergillus niger* crude enzyme solution had a higher protein content of 0.83mg/ml when compared to *Aspergillus flavus* (0.75mg/ml) and *Aspergillus tamari* (0.73mg/ml). *Aspergillus niger* also produced a higher amount of mannanase (0.3489U/ml), when compared to *Aspergillus flavus* (0.1830U/ml) and *tamari* (0.1546U/ml) respectively.
**Keywords:** Mannanase, *Aspergillus* species, Palm-pressed cake, Submerged fermentation, Solid State Fermentation (SSF), pH

## INTRODUCTION

Mannanases are hydrolytic enzymes, which act on hemicellulose chains (called mannans) whose composition and structure chemistry is formed by units of D-mannose. Hemicellulose is not a homopolymer, consequently its chemical structure is not formed by only one type of sugar, but rather its monomeric units are two to four different residuals of sugar forming a heteropolysaccharide [1]. The mannanases or (1-4)-D-mannan hydrolases are those enzymes that produce oligosaccharides through the hydrolysis of the connections (β-1-4)-D-manopyranosil into hemicellulose chains. Carbohydrates are essentials for life on Earth. They play many important roles in nature, for example carbohydrates in the form of polysaccharides are the main structural elements in plants [2]. In terms of biomass, cellulose and hemicellulose are the most abundant polysaccharides on Earth and are synthesized in huge amounts. Hemicelluloses comprise a family of diverse polysaccharides. Generally hemicelluloses have a complex chemical structure and are often classified as mannans, xylans, galactans and arabinans on the basis of the predominant sugar type in the main chain [3]. One of the most common mannans is the O-acetyl-galactoglucomannan which comprises up to 25% of the dry weight in soft wood [2]. O-acetyl-galactoglucomannan is a major hemicellulose found in softwoods. Galactomannan is built up by a glucomannan main chain, which has (α-1,6)-linked galactosyl side-groups attached at some mannose residues. O-acetyl-galactoglucomannan can be divided into two fractions: One soluble in water, which has a galactose/glucose/mannose ratio of 1:1:3; and one soluble in alkali, which has a galactose/glucose/mannose ratio of 0.1:1:3 [4].Two major endo-acting enzymes involved in the degradation of hemicellulose are β-mannanase and β-xylanase. In the case of O-acetyl-galactoglucomannan, β-mannanse is the major depolymerising enzyme [2].

Mannanase have found several industrial applications including improving the quality of animal feeds, bio-leaching of pulp in the paper industry, bioconversion of biomass wastes to fermentable sugar and reducing the viscosity of coffee extracts [5, 6, 7 and 2]. Furthermore, the manno-oligosaccharides which are derived from the hydrolysis of mannanase and mannan have been reported to be used as no nutritional food additives, for selective growth of human-beneficial intestinal micro flora, *bifidobacterium species* [8].

Microbial production, purification and characterization of mannanases have been studied in

both Submerged Fermentation (SmF) and Solid State Fermentation (SSF). However most studies in Mannanase production has been carried out under submerged fermentation. In SSF, bacteria, yeasts or fungi are able to grow and utilize the solid moist substrate materials in the absence of free flowing water. In submerged fermentation, the bioactive compounds are secreted into the fermentation broth. Submerged Fermentation is primarily used in the extraction of secondary metabolites that need to be used in liquid form. More than 75% of the industrial enzymes are produced using submerged fermentation, because submerged fermentation supports the utilization of genetically modified organisms to a greater extent than SSF and purification of products is easier. An additional advantage of this technique is lack of paraphernalia regarding the production of various enzymes using SSF. This is highly critical due to the fact that the metabolism exhibited by microorganisms is different in SSF and SmF, and the influx of nutrients and efflux of waste materials needs to be carried out based on these metabolic parameters. Any slight deviation from the specified parameters will result in an undesirable product [9]. Youssef *et al.* [10] reported that *Aspergillus* species show the highest mannanase activity, protein content and growth among other isolated fungi in both static and shaken fermentation conditions

Some bacteria, actinomycetes, yeasts and fungi are known to be mannan-degraders. Fungal-mannanases have been described [11, 12 and 13]. Filamentous fungi belonging to the genus *Aspergillus* are commonly associated with biomass degradation and produce a wide range of secreted hydrolases, including native endo and exo-acting enzymes involved in the degradation of plant cell walls. *Aspergillus* species especially GRAS-designated strains (*Aspergillus niger* and *Aspergillus oryzae*) produce and secrete a variety of industrial enzymes including cellulases, pectinases, xylanases and other hemicelluloses etc. *Aspergillus* species are the most mannolytic group among fungi [14]. (Siti Norita *et al*. [15] also reported that the easily grown fungus, *A. niger* was capable of producing a cocktail of enzymes (beta-mannanase, beta-mannosidase, endoglucanase, and alpha-galactosidase) required for the degradation of mannan-based polysaccharides. The main products obtained during the hydrolysis of mannan by beta-mannanases are mannobiose and mannotriose. Beta-mannanases from *Aspergillus tamarii* [11] and *Aspergillus niger* [16] all produced mainly mannnobiose and mannotriose and traces of higher oligosaccharides [11].

Many mannan-based carbon sources have been used to cultivate filamentous fungi. These include locust bean gum, guar gum, konjac flour and copra meal [17, 18, 19 and 16]. Although locust bean gum represents the most common carbon source; however, no data have demonstrated the best carbon source to cultivate microorganisms [3].

Palm press cakes are the leftovers after kernel oil is pressed out from the nut in the palm fruit. Palm press cake is commonly used as animal feed for dairy cattle because of its high protein content. Otherwise, it is usually treated as biomass to fuel up boilers to generate electricity for use at palm oil mills and surrounding villages. Palm pressed cake is an abundant waste produced by the palm oil industry and the potential use of this waste as animal feed have been reported [20].

Cocoyam (*Colocesia esculenta*) is an edible root crop belonging to the family Aracea. Two types of cocoyams are grown in South-eastern Nigeria and are both herbaceous plants. The most popular type available in almost all South-eastern Nigerian market is *ede-uli* in *Igbo* (*Colocasia esculenta*). It grows in marshy areas and its corms are used as soup thickeners in most South-eastern Nigerian communities. The second type which is less popular is called *ede-oku* in *Igbo* (*Xanthosoma sagittifolium*) [21]. Cocoyam (*Colocasia esculenta*) makes significant contribution both as root crops and vegetables in the diet of people, particularly Nigerians and Africans at large. Cocoyam (*Colocasia esculenta*) is composed of 72% of starch. [22].

This research is aimed at utilizing cocoyam as the sole carbon source in the Dox-medium for the production of mannanase enzyme by *Aspergillus* species isolated from decaying palm pressed cake. Thus, establishing the use of cheaper raw material for the production of mannanase enzyme, which is of very high biotechnological importance.

## MATERIALS AND METHODS

### Sample Collection

Decaying palm (*Elaesis guineensis*) press cake was obtained from various locations within the University of Nigeria, Nsukka campus, Enugu State, Nigeria. These locations included restaurants and household /domestic wastes. The samples were sun-dried, pulverised and then taken to the laboratory for microbiological analyses.

### Fungal Isolation and Sub culturing

Fungi were isolated from decaying palm press cake. Ten fold serial dilution was done and $10^6$ dilution was plated out using the pour plate technique onto SDA medium. The plates were incubated at $30^0$C for five days and the developing cultures purified by repeated subculture technique.

### Fungal Identification

This was done based on the description of the gross morphological appearance of fungal colonies on SDA medium and the slide culture technique for microscopic evaluation with reference to the manual of fungal Atlases [23, 24, 25 and 26].

**Screening of Aspergillus Isolates for Mannanase Production**

Dox-medium was used for screening the isolates. 500mls of the medium containing: locust bean gum =5g, $NaNO_3$ =1g, $K_2HPO_4$ =0.5g, $MgSO_4.7H_2O$ = 0.25g, KCL =0.25g, $FeSO_4.7H_2O$ =trace, agar-agar =10g. The final pH was adjusted to 5. The mannanase producing fungal isolates were inoculated onto the Dox medium and incubated at room temperature for 7days.

**Idenification of Zones**

Zones of inhibition were spotted using Congo red as indicator.

**Inoculum Preparation**

The inoculum was prepared by adding 5ml of distilled water to the agar slant. 1ml of this suspension served as the inoculum.

**Mannanase Production under Submerged Fermentation**

5g of cocoyam powder which served as the carbon source was used to substitute the locust bean gum in the Dox-medium. 100ml of the medium was dispensed into 500ml Erlenmeyer flasks each. The pH was adjusted to 5 and then sterilized by autoclaving. Each flask was inoculated with 1ml of fungal spore suspension obtained from a four-day old slant culture. Flasks were incubated statically at room temperature for 7days. Each treatment was carried out in triplicates and the results obtained throughout the work were the arithmetic mean of at least 3 experiments.

**Fig-1: Erlenmeyer flasks containing the culture medium for *Aspergillus* isolate C.**

**Enzyme Extraction**

The fermentation medium was filtered using Whatman no.1 filter paper. The filtrate served as the crude enzyme while the trapped particles on the filter paper were analysed for biomass content.

**Determination of final pH**

The final pH value of the crude enzyme solution was determined using pH meter (Hanna instruments, Italy).

**Extraction of Protein Content**

The protein content of the filtrate was determined by Standard methods described by Lowry *et al*. [27]. The developed purple-blue colour was measured at 750 nm (Pharmacia Biotech/Nova spec® spectrophotometer) and the standard curve constructed using crystalline Bovine Serum Albumin (Hopkin & William LTD, Chadwell Health Essex. England).

**Preparation of Standard Mannanase Assay Curve**

This was prepared by dissolving 0.1g of mannose in 100ml of distilled water (1mg/ml). Dilutions were made between 0-1mg/ml. 3ml of DNS was poured into each dilution in the test tubes and boiled for 10minutes. A control was prepared using 1ml of distilled water in place of the mannose solution. The test tube containing the mannose solution was made up to 4mls using distilled water and the absorbance of each solution was read at 550nm against the blank (control) and the standard curve obtained by plotting the absorbance against the concentration.

**Mannanase Assay**

The reaction mixture containing 1ml of 1% of locust bean gum dissolved in 0.05M acetate buffer at pH of 5 was introduced into a test tube containing 1ml of crude enzyme solution. The reaction mixture was incubated in a water bath at $40^0$C for 5minutes. 1ml of this mixture was removed, and 1ml of Dinitrosalycilic acid (DNS) was added to the reaction mixture. The mixture was left to boil for 10minutes and then a colour change was observed. The absorbance was read at 550nm. The released mannose due to mannanase activity was determined by DNS method [10] using mannose as standard. One Unit of activity is defined as

the amount of enzyme required to release one micromole of mannose reducing sugar equivalents per minute under the defined assay conditions.

**Determination of Dry Weight**

The cells collected by Whatman No. 1 filter paper were oven dried at 70$^{o}$C and weighed with a weighing balance until a constant weight was obtained.

**RESULTS**

Six different fungal strains were isolated from the palm pressed cake. Three out of the six, belonged to the genus *Aspergillus*

**Result of *Aspergillus* isolates Screening on Solid Media**

All the three *Aspergillus* isolates were able to utilize the locust bean gum present in the medium; this was characterized by their ability to grow in the Dox medium. This growth suggested a positive result for mannanase production.

**Result of Mannanase Production under Submerged Fermentation:**

**Final pH of the Crude Enzyme Solution**

The final pH of the *Aspergillus* species was within the acidic range as shown in figure 2. *Aspergillus tamari* and *Aspergillus flavus* had final pH values of 6.1, while *Aspergillus niger* had a pH of 6.2.

**Dry weight content of the fermentation culture**

After drying at 70$^{o}$C in the hot air oven (Gallen Hamp), the following values were obtained, *Aspergillus tamari* had a growth yield of 0.17g/ml, *Aspergillus flavus* had a growth yield of 0.21g/ml and *Aspergillus niger* had 0.25g/ml growth rate.

**Protein content of the fermentation filterate**

The method used was described by Lowry *et al*. [27], using bovine serum albumin as a standard. Figure 4 shows the protein content of the crude enzyme solution. *Aspergillus tamari* had a protein content of 0.73mg/ml; *Aspergillus flavus* had 0.75mg/ml while *Aspergillus niger* contained a higher quantity of protein (0.83mg/ml).

***Mannanase activity:***

Mannanase unit is defined as the amount of mannanase that releases 1mg of mannan in one minute under the defined assay conditions. *Aspergillus tamari* produced 0.1546 U/ml, *Aspergillus flavus* produced 0.1830 U/ml, and *Aspergillus niger* had a mannanase content of 0.3489 U/ml. The above result was displayed in figure 5.

**Table1: Morphological and Microscopic Characteristics of Fungal Isolates**

| Isolates | Cultural characteristics | Microscopic characteristics | Identity |
|---|---|---|---|
| A | On SDA, colonies were colourless at first, and then gradually turned orange-yellow to brown/light brownish-olive with age. | Conidial heads varied greatly in size in the same fruiting area, from more or less columnar to nearly, but not completely, globose and up to 300m in diameter, with radiating chains and columns of conidia. | *Aspergillus tamari* |
| B | On SDA, colonies were flat and compact with yellow basal felt covered by a dense layer of black conidial heads with powdery texture. | Conidiophores were hyaline or pale-brown, erect, simple, with foot cells basally, inflated at the apex forming globose vesicles, bearing conidial heads (up to 3 mm by 15 to 20 µm in diameter). | *Aspergillus niger* |
| C | On SDA, colonies were powdery, flat with radial grooves, yellow at first, but later turned to bright to dark yellow-green with age. | Conidial heads were radiate, splitting to form loose columns (300- 400 µm in diameter). Thus, conidiophores stipes was hyaline and coarsely roughened, noticeable near the vesicle and non-septate. Conidia were globose to subglose (3-6 µm in diameter), pale-green and conspicuously echinulate. | *Aspergillus flavus* |

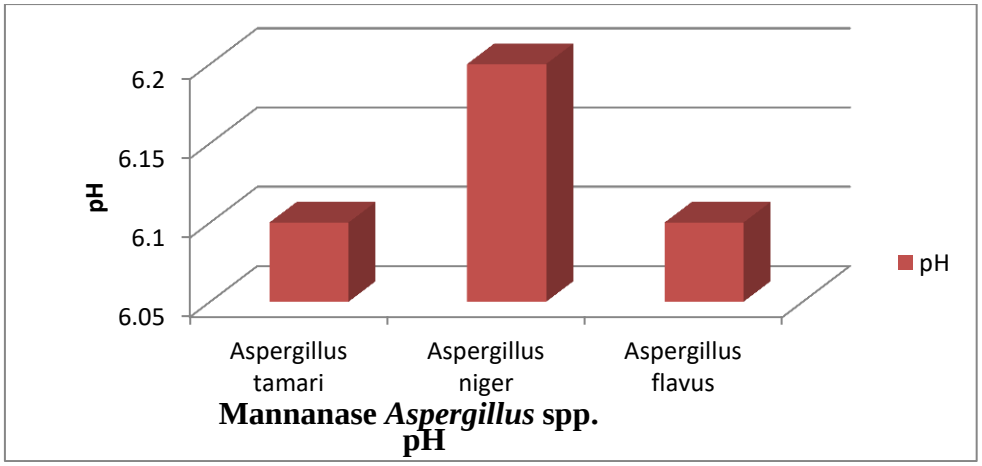


**Fig-2: Final pH ranges of the *Aspergillus* species in the Crude Enzyme Solution**

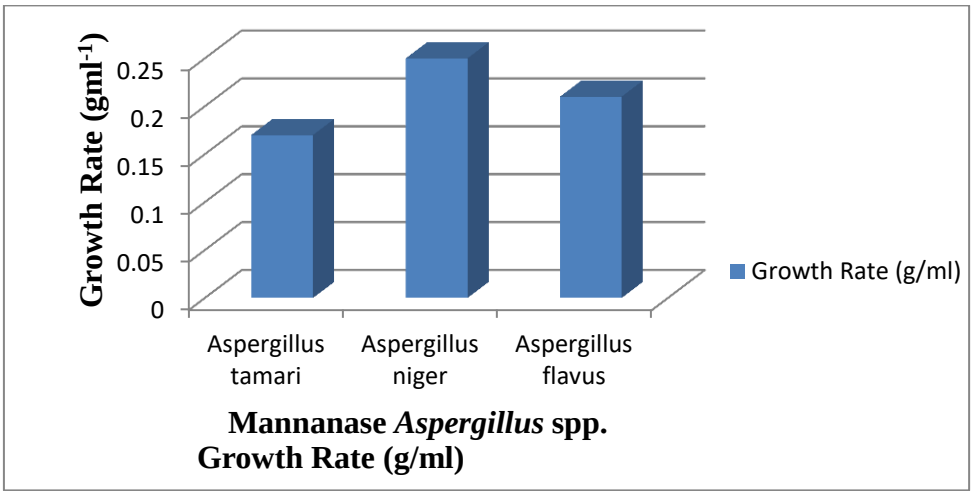


**Fig-3: Biomass content (g/ml) of the fermentation medium.**

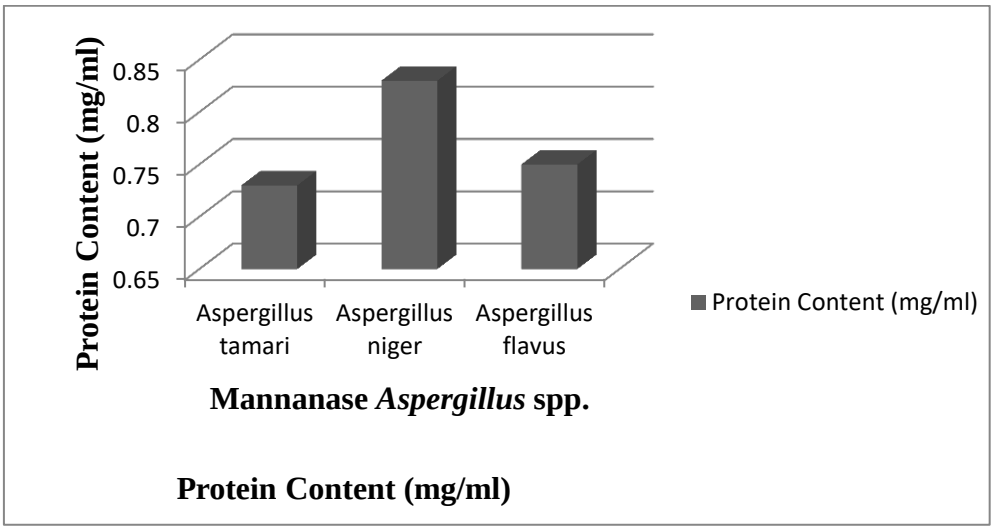


**Fig-4: Final protein content of crude enzyme in mg/ml.**

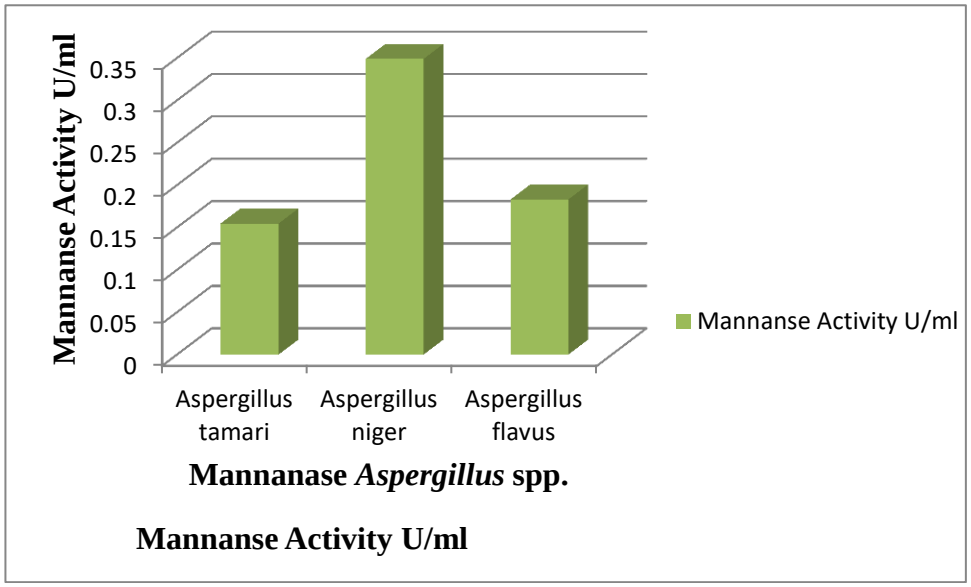


**Fig-5: Mannanase activity of the crude enzyme solution in U/ml.**

## DISCUSSION

β-mannanase enzyme is very important for the digestion of hemicelluloses, one of the most abundant groups of polymers in nature. This enzyme hydrolyzes mannan yielding mannotriose and mannobiose [28]. Fungi were isolated from decaying palm press cake. Howard *et al*. [29] and Blibech *et al*. [30] reported that large amounts of agricultural waste are generated through forestry and agricultural practices, paper pulp industries, timber industries and many agro-industries and they pose an environmental pollution problem. These singular activities tend to expose the agro-wastes to microbial contamination. The reports of Howard *et al.* [29] also revealed structural component of agro-wastes to contain lignin, cellulose, hemicellulose, and presence of some components (activators or inhibitors). The chemical properties of the components of lignocelluloses make them a substrate of enormous biotechnological value [31]. The chemical composition of the wastes might be linked to its ease of colonization, hence may account for the high fungal counts. Apart from this, fungal isolates may probably have originated from soil, water and material used during harvesting of agricultural produces, while the variations of the isolates may be due to the handling process and the prevailing environmental conditions [32].

The isolated fungi were identified macroscopically and microscopically, only three isolates were found to be *Aspergillus* species (Table 1). This is in line with the report of Abid-Aziz *et al*. [33] who reported the ability of *Aspergillus niger* to grow on palm press cake. Civas *et al*, [11] and Wong *et al*. [34] also had similar reports for *Aspergillus tamarii and Aspergillus Flavus*, respectively.

The fungal isolates were screened primarily on Dox medium containing 5g locust bean gum and the quantitative mannanase activity was determined on the basis of clear zones formed around the colonies. All the three *Aspergillus* species isolates showed clear zones of mannanase activity on solid medium at $30^0$C. The formation of clear zone by these isolates on agar medium supplemented with Locust Bean Gum could be attributed to the ability of their genetic make up to secrete active mannanase with high diffusion rate as reported [20].

Mannanase production was carried out using the three *Aspergillus* isolates under submerged fermentation. The final pH of the *Aspergillus* species was within the acidic range as shown in figure 2. *Aspergillus tamari* and *Aspergillus flavus* had final pH values of 6.1, while *Aspergillus niger* had a pH of 6.2. The acidic pH obtained for the static culture might be due to organic acid accumulation [20].

*Aspergillus niger* had a growth yield of 0.25g/ml, protein content of 0.83mg/ml and the amount of mannanase produced was 0.3489U/ml; while *Aspergillus tamari* had a growth yield of 0.17g/ml, protein content of 0.73g/ml and the amount of mannanase produced was 0.1546U/ml. The *Aspergillus niger* had a higher growth yield, protein content and mannanase production, when compared to *Aspergillus tamari* and *Aspergillus flavus.*

Although, many investigators used galactomannan (mannan) as a sole carbon source for the cultivation of some fungi [35]. The substitution of locust bean gum in the culture medium by cocoyam resulted in a maximum β-mannanase activity. This indicates that cocoyam can serve as a good carbon source for the isolates. It also showed that there was a direct relationship between the growth rate, protein content and amount of enzyme produced; as the organism, *Aspergillus niger* which had a growth yield of 0.25g/ml and protein content of 0.83g/ml was able to

produce a higher amount of mannanase (0.3489U/ml). This is in line with the results of Youssef *et al*. [10], who reported that there was a direct relationship between the biomass content and mannanase yield.

## CONCLUSION

This work collectively suggests that the locally isolated *Aspergillus* species and its extracellular enzyme have a significant role towards the enhancement of the industrial solubilisation of lignocellulose (mannan-based polysaccharides) under optimized conditions. Cocoyam (*Colocesia esculenta*) contains considerable amounts of carbohydrates which stimulates the cells to express the hydrolytic enzyme. In addition, it contains appreciable amounts of easily utilizable sugars, which encourage growth initiation. Cocoyam is readily available and its use reduces production cost. The results of the present investigation summarily indicate the possibility of cocoyam to serve as carbon source for *Aspergillus* in mannanase production, as was the case in this study. Nonetheless, other carbon sources could be used to substitute to locust bean gum.

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