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Full Length Research Paper

Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil

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Eight *Aspergillus niger* strains which produced strong starch degrading amylase were isolated from the soil using a medium containing Remazol Brilliant Blue (RBB) starch as substrate. Amylase production was detected by the disappearance of the blue colour around the colony. Among the isolates, *A. niger* AM07 produced the largest clear zone (7.0mm) on Remazol Brilliant Blue (RBB) agar plate and also gave the highest amylase yield (806 U/ml) in solid-state fermentation process, hence it was selected for further studies. The crude amylase preparation of *A. niger* AM07 had temperature and pH optima activities at 60°C and 4.0 respectively. The optimum substrate concentration was 3 %. The action of the crude amylase of *A. niger* on raw tuber starches of yam, cassava, sweet potato and cocoyam were studied in comparison with the well known maize starch which is a cereal starch. The crude amylase was able to hydrolyze all the raw starches tested. Hydrolysis was significantly ($p<0.05$) dependent on starch source and length of incubation. At 72-h incubation time, raw cassava starch gave the highest yield of 200.1 mg/g with a conversion efficiency of 198.91% while raw maize starch gave a yield of 109.6 mg/g with 108.95 % conversion efficiency. Raw cocoyam starch was more resistant to hydrolysis and incubation of cocoyam starch beyond 24 h, resulted in decreased yield of reducing sugars. Thin layer chromatography showed glucose as the main sugar produced with low level of maltose.

Key words: Amylase, Remazol Brilliant Blue, tuber starches, and hydrolysis.

INTRODUCTION

In recent years the new potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms (Bilinski and Stewart, 1990; Akpan et al., 1999b ; Buzzini and Martini, 2002). Amylases are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents (Mitchell and Lonsane, 1990; Akpan et al., 1999b). Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. Evidences of amylase in yeast, bacteria and moulds have been reported and their properties documented (Akpan et al., 1999b, Adebiyi and Akinyanju, 1998; Buzzini and Martini, 2002). Among the microorganisms, many fungi had been found to be good sources of amylolytic enzymes. Studies on fungal amylase especially in the developing countries have concentrated mainly on *Rhizopus sp* and *A. niger* probably because of the ubiquitous nature and non fastidious nutritional requirements of these organisms (Abe et al., 1988). The *A. niger* group is wide spread with many strains capable of producing amylases. The

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amylase producing strains of *A. niger* have spore bearing heads which are large, tightly packed, globular and may be black, or brownish black. They are considered to be mesophillic with optimal temperature for growth between 25°C and 35°C. They are aerobic in nature and can grow over a wide range of hydrogen ion concentration. These organisms can utilize different kinds of foods from simple to complex ones, which make them easy to cultivate and maintain in the laboratory (Manno and Pekka, 1989).

Solid-state fermentation has been used in the production of industrial enzymes like amylase and it has great potentials in the developing countries due to its simplicity of operations, low capital cost and high volume productivity. (Mitchell and Lonsane 1990, Akpan *et al* 1999b)

In most developing countries of the tropics, carbohydrate based agricultural products like starchy tubers and cereal occur abundantly. (Okolo et al., 1995; Anthony, 1996). Starchy tubers such as cassava, yam, sweet potato and cocoyam are important staple foods in the diet of people in most developing countries of the tropics. In these countries, they are widely distributed and more cultivated than cereal. However, despite their importance, a large proportion of these tubers are lost yearly due to inadequate and ineffective storage facilities (Jean- Claude et al*.,* 1993; Okolo et al., 1995; Anthony, 1996). These starches can be converted to reducing sugars by acid or enzymatic saccharification (Shambe et al., 1989). In terms of energy utilization and process simplicity, amylase conversion of raw starches is believed to be superior to the conventional method that make use of pregelatinized starch as substrate (Okolo, 1995, Adebiyi and Akinyanju, 1998).

Several microorganisms are known to produce raw starch digesting amylase, however most of these microorganisms were effective for cereal starches, but root or tuber starches were more resistant to the enzyme reaction (Bergmann et al., 1988, Okolo et al., 1995). Although the use of microbial amylase for the hydrolysis of raw starches has long been advocated and practiced to a limited extent, yet, there is not much information on the hydrolysis of raw starches of locally available tubers in the tropics. Most screening procedures used for the detection of amylase producing microorganisms involve growing the organism on solid media containing soluble starch and testing for starch hydrolysis by flooding the plates with either iodine solution or 95% ethanol.

These methods are time consuming and inconvenient for direct the isolation of intact cells (Akpan et al., 1999a). The present study is therefore aimed at: (1) Using a new screening technique involving the use of Remazol brilliant blue (RBB) agar for screening of starch digesting amylase microorganisms from the soil.
(2) Hydrolyzing several raw native tubers starches with crude amylase of A. *niger* AM07 isolated and selected from the soil through the screening procedure described.

MATERIALS AND METHODS

Raw materials and cultures

Cassava (*Mannihot utilissima*), Maize (*Zea mays*), Yam (*Dioscera rotundata*), Sweet potato (*Ipomea batatas*) and Cocoyam (*Xanthomonas sagittifolium*) were purchased at Kuto Market, Ogun State, Nigeria. These were prepared into starches in the laboratory according to standard procedures as described by Corbishley and Miller (1984). All other chemicals used were obtained commercially and of analytical grade.
Known amylase producing strains of *A. niger* (K-6) which had earlier been identified (Akpan et al., 1999b) was obtained from the culture collection of the Microbiology Laboratory of the University of Agriculture, Abeokuta. The culture was maintained on Potato Dextrose Agar at 30°C

Preparation of RBB starch

Remazol Brilliant blue (RBB) R salt (Sigma R-8001, Sigma Chemical Co. St. Louis, USA), corn-starch (Sigma S-4126) and commercial soluble starch (Sigma S-9765) were used for the media preparation. Remazol Brilliant blue (RBB)-starch agar medium used for amylolytic fungi isolation and selection was prepared by the method of Akpan et al. (1999b). Five grams (5 g) of corn-starch was suspended in 50 ml water (60°C) in a 250ml Erlenmayer flask with vigorous stirring. To this, 100 ml of 1% (w/v) RBB dye was added. After 30 mins, 8 g of sodium sulphate (Na_2SO_4, Sigma S-6547) was added and stirred at 60°C until all had dissolved. Sodium phosphate ($Na_3PO_412H_2O$, Sigma S-1001) (1 g) was used to fix the dye to the corn-starch. The mixture was then maintained at 60° C for 2 h with continuous stirring. Stained insoluble RBB-starch was collected by centrifugation and washed with warm water (55°C) until the supernatant was clear. This was then washed twice with methanol. The powder was dried and stored in a desiccator at room temperature before use.

Isolation of amylase producing *A. niger* from the soil

The basal medium for the isolation and selection of amylolytic *A. niger* from the soil consisted of 0.1% RBB-starch added to nutrient agar. The medium was autoclaved at 121°C for 15 min. Soil samples from compost and heaps (1 g) were suspended in 10 ml of sterile water and centrifuged at 2,500 x *g* using a table centrifuge (Hettich EBA 8S, Tuttlingen, Germany). The supernatant (0.1 ml) obtained after centrifugation was plated on the RBB-starch agar medium using pour plate method. Incubation was at 30°C for 48 h. Amylase production was detected by the disappearance of the blue colour of the medium around microbial colonies. Evaluation of the clear zones of each colony was estimated as radius (mm) of the clear zone minus the radius of the colony.

A. niger colonies producing large clear zones were picked up and purified by streaking on malt extract agar. Identification was based on cell and colonial morphological characteristics with references to the method of Rasper and Fennel (1965). Colonies of *A. niger* consisted of compact white or yellow basal felt covered by a dense layer of dark-brown to black conidial heads. Conidial heads are biseriate, large, globose and dark brown. Conidiophores are smooth-walled, hyaline or turning dark towards the vesicle. Conidial heads are biseriate with the phialides borne on brown, septate metulae. Conidia are globose to subglobose, dark brown to black and rough-walled.

Pure cultures were maintained on Potato Dextrose Agar slants, stored in a refrigerator and sub-cultured at 3-month interval.

Production of crude amylase

The selected *A. niger* isolates were cultured on rice bran and soya bean basal medium as described by Akpan et al. (1999b). The medium consisted of rice bran (10 g) and soya bean flour (3 g) in a 250 ml Erlenmeyer flask. It was moistened to 55% moisture content with aqueous mineral salts solution [$MgSO_47H_2O$, 0.1%; KH_2PO4, 0.1 %; $CaCl_2$, 0.1 %; $FeSO_4$, 0.05 % and $(NH_4)_2 SO_4$, 0.1 %]. This was sterilized at 12^{0}C for 15 min. Prepared media plates were inoculated with *Aspergillus niger* AM07 (2 x 10^6 spores / 100 ml) and were incubated at 30^{o}C for 72 h.

Extraction of enzyme

The moist mouldy bran obtained after incubation was mixed with citrate phosphate buffer (pH 4.5) in the ratio 1:10(w/v) in a 250ml Erlenmeyer flask. The mixture was shaken on an orbital shaker (LAB-LINE, U.K) at 150 rpm and 28^{o}C for 1 h. The supernatant obtained after filtration was used as crude enzyme source.

Enzyme assay

Amylase activity was assayed as described by Ramakirshna et al. (1982) using a reaction mixture comprising of 1 ml of crude enzyme, 1 ml of 1% (w/v) corn starch solution and 0.1 ml of citrate buffer solution (pH 4.5). Incubation was at 60^o C for 1 h and the reaction was terminated by immersing the reaction tube in boiling water (100^{o}C) for 2 min. The reducing sugars liberated were estimated by the DNS methods (Miller, 1959). One unit of amylase activity (U) was defined as the amount of enzyme that liberated 1.0 µmole of D-glucose from starch in 1.0 µl reaction mixture under the assay conditions.

Characterization of the crude enzyme

The effect of temperature on the crude amylase activity was assayed at temperature values ranging from 20^{o}C to 90^{o}C. The reaction mixture contained 1 ml of the crude enzyme, 1 ml of corn-starch (1% w/v) buffered with 0.1 M citrate phosphate buffer (pH 4.5). This was incubated for 60 mins at each chosen temperature. Effect of pH on the crude amylase activity was determined using 0.1 % corn-starch suspended in buffers consisting of citrate (pH 4-6), phosphate (pH 6 - 7.5) and Tris (pH 7.5 - 9) at 0.1 M.

The effect of various starch concentrations on the enzyme activity was also studied using starch solutions (1%, 2%, 3%, 4% and 5%) suspended in 0.05 M NaCl in 0.1 M-phosphate buffer (pH 6.9). Incubations were at 60^{o}C for 1 h except otherwise stated. Amylase activities at the different conditions studied were determined as described earlier.

Hydrolysis of raw starch

The ability of the crude enzyme to hydrolyze raw starch was studied using maize, cassava, sweet potato, yam and cocoyam starches. Commercial soluble starch was used as the standard. Enzyme solution, (1 ml); acetate phosphate buffer (pH 4.5, 1 ml) and 3 ml each of the raw starch solutions were incubated at 60^{o}C for 1 h. Susceptibility of the raw starches to hydrolysis was determined in terms of the quantity of reducing sugars (mg/ml) produced. The effect of time of incubation on raw starch hydrolysis was studied by incubating each raw starch at different time intervals between 0 h and 72 h.

Analysis of sugars by thin layer chromatography (TLC)

Sugars produced by the hydrolysis of the raw native starches with crude amylase of *A. niger* AM07 were identified by Thin Layer Chromatography (TLC). Commercially prepared TLC plates (Polygram, UK) were used as the stationary phase. Aliquot of each starch hydrolysate was spotted on the TLC plates along with standard mixture of known sugars. The reference sugar solution contained 0.1 g each of maltose, sucrose, glucose and raffinose, dissolved in 100 ml of 10% isopropanol. A one dimensional ascending chromatography was done at room temperature using a solvent system of n-butanol: acetic acid: diethyl ether: water (9:6:3:1, (v/v/v/vl). After 2 h, all the chromatograms were treated by dipping in locating reagents made up of 4-amino-benzoic acid in methanol. The plates were air-dried and placed in the oven at 100^{o}C for 15 min. The sugar spots appeared as dark brown spots. Identification of the sugars was done by comparing the relative fraction (Rf) values of the samples with that of the standards.

Table 1. Amylase producing ability of *A. niger* strains.

Isolates code	means of clear zones (mm)*	amylase activity (U/ml)*
**K-6	5.2	658[a]
AM07	7.0	806[b]
AM27	6.0	753[c]
AM38	5.5	705[d]
AM67	4.3	589[e]
AM50	3.5	435[f]
AM43	3.2	421[g]
AM72	2.0	325[h]
AM75	1.5	298[i]

*Means of triplicate readings
**Mean growth pattern of known amylase producing strain of *A. niger*
Means not followed by the same superscripts are significantly different ($p < 0.05$).

RESULTS

Using the method described, eight strains of *A. niger* which produced large clear zones on the RBB-agar medium were randomly selected from the soil. Of the eight *A. niger* AM07 was considered to be the best amylase producing strain with zone of clearing of 7.0 mm (Table 1). Table 1 also showed the quantitative assessment of the amylase productivity by the isolates using solid state fermentation process. *A. niger* AM07 also produced the highest enzyme activity (806 U/ml), hence it was selected for subsequent investigations.

Effect of temperature on amylase activity

The influence of temperature on amylase activity of the crude enzyme showed that enzyme activity increased

progressively with increase in temperature from 20°C reaching a maximum at 60°C (Figure 1). Above 60°C, there was a reduction in the amylase activity.

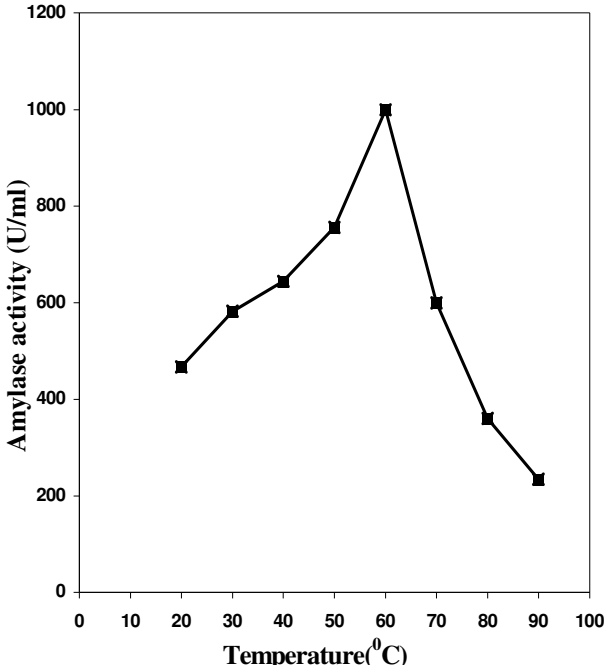

Figure 1. The effect of temperature on *A. niger* AM07 amylase activity.

Effect of pH on amylase activity

The optimum pH was observed at pH 4.0 as indicated in Figure 2. After pH 4, a continuous decrease in enzyme activity was observed.

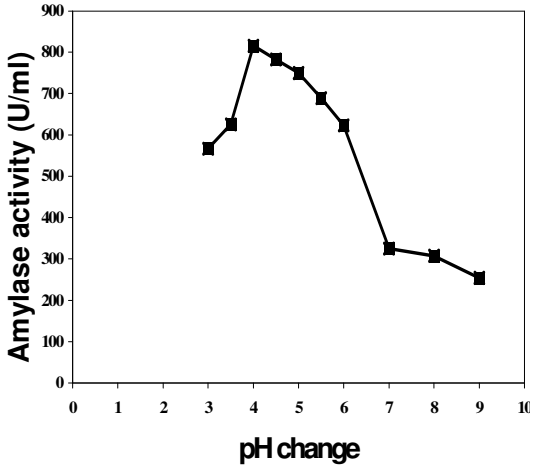

Figure 2. The effect of pH on A.iger AM07 amylase activity.

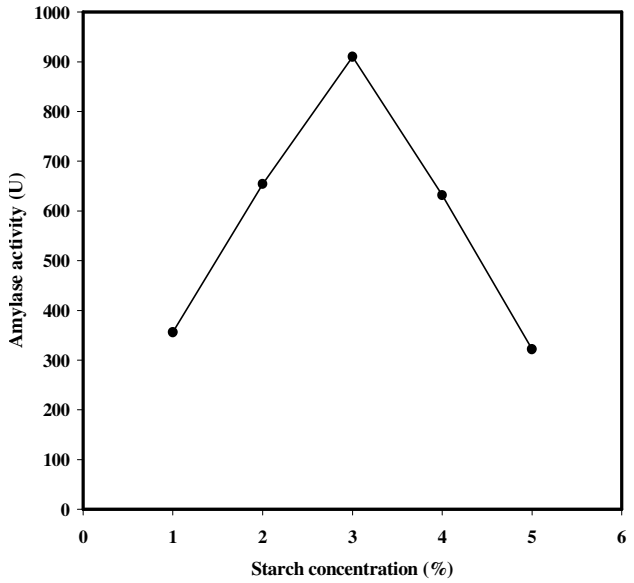

Figure 3. The effect of starch concentration amylase activity.

Effect of starch concentration on amylase activity

Amylase activity increased with increase in the starch concentration from 1% to 3%.
Beyond 3%, there was a decline in amylase activity (Figure 3).

Hydrolysis of raw starch

The ability of the crude amylase of *A niger* AM07 to digest different raw starches was studied using different tuber starches (yam, cassava, cocoyam, sweet potato) and a cereal starch (maize). Corn-derived soluble starch was used as the standard. The results are presented in Table 2 in terms of the amount of reducing sugars (mg/g) produced. The susceptibility of each starch to amylase hydrolysis was described as the conversion efficiency and it was calculated relative to the corn derived soluble starch used as the standard.

Results indicated that at 1 h incubation the crude amylase was able to hydrolyze some of the raw starches tested. Maize starch (a cereal) and the corn derived soluble starch used as the standard were rapidly hydrolyzed to give 10.2 mg/g and 9.2 mg/g reducing sugars respectively. These were followed by yam starch (7.8 mg/g) and potato starch (5.5mg/g). Cassava starch was least hydrolysed to give 2.5 mg/g of reducing sugars. Cocoyam was not hydrolysed at 1-h incubation. The conversion efficiency (Table 2) revealed that relative to the soluble starch used as the standard (100%), maize starch had conversion efficiency of 110.87% while yam,

Table 2. Raw starches hydrolysis by amylase of *A. niger* AM07.

Starches	Reducing sugars (mg/ml)*	**Relative conversion efficiency (%)
Soluble starch	9.2 [a]	100.00
Maize	10.2 [b]	110.87
Yam	7.8 [c]	84.78
Cassava	2.5 [d]	27.17
Sweet potato	5.5 [e]	59.78
cocoyam	0 [f]	00.0

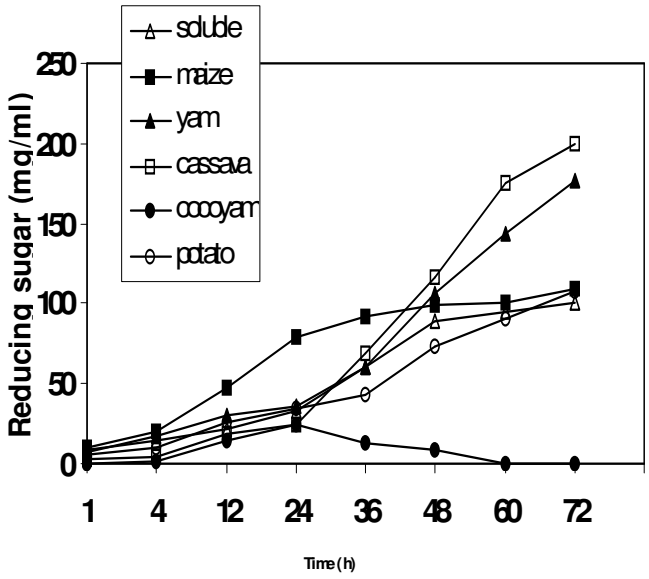

Figure 4. The effect of time of incubation on the hydrolysis of different raw starches.

potato and cassava starches had 84.78%, 59.78% and 27.17% respectively.

The effect of time of incubation on the hydrolysis of the different starches tested is summarized in Figure 4. At 4 h of incubation, maize starch had the highest reducing sugar yield (20.4 mg/g). Of all the tuber starches tested, yam starch was more susceptible to hydrolysis at 4 h incubation to give reducing sugars yield of 17.2 mg/g. This was followed by 10.6 mg/g and 4.2 mg/g for potato and cassava starches respectively. Cocoyam starch had the lowest yield of 2.0 mg/g. The relative conversion efficiency (Table 3) showed that at 4 h incubation maize starch gave conversion efficiency of 141.66 % which was significantly (p=0.05) higher than 119.44%, 73.61%, 29.17% and 13.89% obtained for yam, potato, cassava and cocoyam starches, respectively. Results (Figure 4) also showed that the ability of the crude amylase of *A. niger* AM07 to digest the raw starches increased significantly (p=0.05) with time for all the raw starches tested. The only exception was cocoyam starch in which the highest yield of reducing sugars (24.8 mg/g) was observed at 24-h incubation time. Further incubation of the cocoyam starch beyond 24 h resulted in decreased susceptibility to hydrolysis. On the other hand, the susceptibility of the other tuber starches to amylase hydrolysis increased highly after 36 h, particularly for cassava starch. At 36 h incubation, cassava starch had reducing sugar yield of 68.9 mg/g (113.70 %), however at the end of incubation (72 h), the yield for cassava has increased to 200.1 mg/g (198.91 %) which is almost twice the yield obtained for maize starch (109.6 mg/g (108.95 %). With the exception of cocoyam starch, all the tuber starches examined were more susceptible to hydrolysis by the amylase of *A niger* AM07 at 72 h when compared to the maize starch (cereal). Based on the quantity of reducing sugars, the raw cassava and yam starches are more susceptible to hydrolysis than the raw maize starch at 72 h. Comparison of means showed that there was no significant difference between the yield of the raw potato starch (107.8 mg/g) and maize starch (109.6 mg/g) at 72h incubation. No detectable amount of reducing sugars was observed after 36 h of incubation for cocoyam starch.

Calculation of the relative conversion efficiency at 72 h (Table 3) showed that 198.91% and 175.75% obtained for raw cassava and yam starches are significantly higher than 108.95 % obtained for the raw maize starch. In the same vein, no significant difference was observed between 108.95% and 107.16% obtained for the raw maize and potato starches, respectively.

The thin layer chromatography (TLC) analysis of the starch digest, showed glucose as the predominant product of hydrolysis with small amount of maltose for all the starches tested (Data not shown)

Table 3. Relative conversion efficiencies of different raw starches at 4 h and 72 h of incubation.

Starches	Relative conversion efficiencies (%)*	
	4 h	72 h
Commercial starch	100 [a]	100 [a]
Maize	141.66 [b]	108.95 [g]
Yam	119.44 [c]	175.75 [h]
zCassava	29.17 [d]	198.91 [i]
Potato	73.61 [e]	107.18 [g]
Cocoyam	13.89 [f]	0 [j]

* Amount of reducing sugars produced by the commercial soluble starch was taken as 100%

** Percentages not followed by the same superscripts are significantly different.

DISCUSSION

The occurrence of amylolytic organisms from the soil agrees with earlier reports of Rehana (1989) and Adebiyi and Akinyanju (1998) that the soil is known to be a repository of amylase producers. All the isolates selected from the RBB plates produced detectable quantities of amylase in the solid-state fermentation process and as such the use of RBB-starch agar present a simple and rapid way to screen for amylolytic microbes from the soil. It gives a direct visual indication of starch hydrolysis. It requires no flooding, no prior replication of colonies on slants; the zones are very sharp and contrast sharply with the blue-black background (Akpan et al., 1999a).

This study showed that the susceptibility of the raw starches to the crude enzyme of *A. niger* AM07 was significantly dependent on the starch source and time of incubation. This agrees with earlier reports of Okolo et al., 1995 that the susceptibility of starch granules to digestion by amylase is dependent on starch source and length of amylase treatment. Contrary to previous reports (Taniguich et al., 1982, Okolo et al., 1995) that potato starch is not easily hydrolysed, the amylase of A *niger* AM07 was able to hydrolyse the raw potato starch used for this study and at 72 h incubation, the reducing sugars obtained from the hydrolysis of the potato starch was higher as compared to that of maize (a cereal starch). This study also revealed that cocoyam starch is less hydrolyzed than potato starch, which was considered as one of the less susceptible to enzyme hydrolysis.

Conversely cassava starch, which is a tuber starch, is as susceptible to hydrolysis as the maize cereal starch if incubated for longer period. It is significant to note that at 72 h incubation, cassava starch, which is a tuber starch, produced the highest reducing sugar (200.1mg/g) with the maximum conversion efficiency of 198.91%. The high digestibility of raw cassava starch observed in this study is similar to the findings of Okolo et al., 1995.

The ability of the crude amylase of *A. niger* AM07 to hydrolyze the root starches especially cassava starch presents a remarkable property since these root starches are abundantly available in the tropics. According to Anthony et al., 1996 and Oluwole et al., 1999, over 30 million tonnes of cassava are lost yearly since cassava is perishable after harvesting. Conversion of raw cassava by this enzyme means that some of the cassava could be used as raw materials by the starch industry for value added products. This will reduce wastage and improve economic gain. However there is a need to carry out further research on cocoyam starch because it is considered as one of the most important root crop in West Africa after yam and cassava (Oluwole et al., 1999).

The appearance of glucose and low level of maltose in the starch digest indicates that the amylase of *A. niger* AM07 consists principally of glucoamylase and low level of alpha-amylase. The low level of maltose is a major of alpha-amylase. The low level of maltose is a major advantage in the industrial production of glucose syrup.

In conclusion, the crude amylase of *A. niger* AM07 selected for this study is capable of hydrolyzing both cereal and tuber starches into glucose which can then be used directly in the production of ethanol and fructose.

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