Production and Partial Purification of Amylase By *Aspergillus niger* Isolated from Cassava Peel

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Abstract: *Aspergillus niger* strains 1, 2 and 3 isolated from cassava dumpsites were used for the production of amylase enzyme. The *Aspergillus niger* strains 1, 2 and 3 had diameter (mm) zone of clearance of 17.0, 23.0 and 8.0 respectively using Potato dextrose agar plates fortified with starch. Studies on the amylase enzyme activity (mg/ml) of *Aspergillus niger* strains 1 and 2 showed 19,340 and 16,510 respectively. These values were higher than the commercially available amylase enzyme that had an activity of 5,722.2. The protein (mg/ml) and specific activity (units/mg) for amylase from *Aspergillus niger* strain 1 was 28.39 and 681.23 while 21.76 and 758.73 from *Aspergillus niger* 2 respectively. Purification using ammonium sulphate (% w/v) at 60, 80 and 100 on amylase enzyme from *Aspergillus niger* strain 1 for enzyme activity, protein and specific activity was 44405.49, 17.01 and 2610.55, 28949.76, 23.62 and 1225.65, 36220.25, 16.67, and 2172.787 respectively. The microbial production of Amylase enzyme in Nigeria from Cassava peel will reduce cost of production, convert cassava peel from waste condition to wealth, and will boost economy through indigenous industrialization.

Keywords: *Aspergillus niger*, Amylase enzyme, Cassava peel, Partial purification.

INTRODUCTION

Amylases are enzymes that catalyses the breakdown of starch into sugars. Amylase was the first enzyme to be discovered and isolated in 1983 [1]. All amylases are glycoside hydrolases and act on α - 1,4-glycosidic bonds. The α-amylases (EC 3.2.1.1), 1, 4- α-D-glucan glucanohydrolase are calcium metalloenzymes, completely unable to function in the absence of calcium. By acting at random locations along the starch chain, α-amylase breaks down long-chain carbohydrates ultimately yielding maltotriose and maltose from amylase , or maltose, glucose and “limit dextrin” from amylopectin by an endoacting hydrolytic mechanism, thereby making it amenable to the action of amyloglucosidase for saccharification to reducing sugar [2, 3]. Β-amylases (EC 3.2.1.2), 1,4-α-D-glucan maltohydrolase,working from the non-reducing end, β-amylase catalyzes the hydrolysis of the second α-1,4glycosidic bond, cleaving off two glucose units (maltose) at a time. Amylases, starch degrading enzymes, have numerous biotechnological applications, e.g in the production of syrups containing oligosaccharides, maltose and glucose. The precise composition of the end product can be controlled so that products with desired physical properties may be obtained [4]. Enzymatic degradation of starch on an industrial scale has been practised for many years and has replaced to a considerable extent, the traditional acid- catalysed processes. The properties and mechanisms of amylases depend on the source of the enzyme. They are all endo - acting enzymes and therefore affect a rapid decrease in iodine- staining power and a simultaneous decrease in the viscosity of starch solutions. Amylases produced from agricultural raw materials could be used in the production of glucose syrup. Patenting of microbial production of enzymes is generally beneficial to local Industries [5]. The use of agro waste residues and microorganisms in enzyme production will increase industrial advantages in the area of research and development [6]. This will reduce foreign exchange in importation towards and furthermore control agro waste industrial resources. The objectives of this study were to isolate, screen for Amylase hyper producing strains of *Aspergillus* species, produce Amylase from hyper producing strains, and establish partial purification of produced Amylase using Ammonium Sulphate precipitation.

MATERIALS AND METHOD

Collection of Soil Sample

Soil samples from cassava processing areas were obtained from the following locations; Cassava processing site of the pilot plant section, FIIRO Cassava processing site at Tigbo’lu, Abeokuta, Ogun State, Nigeria. The soil samples were collected on 17th November, 2010 using sterile McCartney bottles and taken to the laboratory within 3hours of collection for immediate laboratory analyses.

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Isolation of *Aspergillus niger* Strain from Soil Samples

Using serial dilution method of isolation, 0.1ml was plated on Potato Dextrose Agar (fortified with 0.1% streptomycin). The moulds were purified to obtain pure culture of *Aspergillus niger.* These relevant *Aspergillus niger* mould strain were then transferred on PDA slants kept in the refrigerator at 4^0C. The mould isolate were identified following microbiological method of identifications through staining reagents of lactophenol cotton blue and observing under the microscope, and comparing with standard Mycological Atlas.

Screening for the Best Strain of *Aspergillus niger*

Spore suspension of each mould strain of *Aspergillus niger* was carefully introduced within 0.3mm diameter of PDA plates fortified with 1% starch. The plates were then incubated separately at 30^0C for 24-28 hr after which the plates were flooded with grams iodine reagent. Zones of clearance around the smeared portion was measured and recorded against the blue black colouration background of PDA-Starch - Agar plates. The zone of clearance produced is the measure of the ability of the strains with the highest ability of producing yield of amylase.

Enzyme Production Medium

The culture medium used in this work for amylase production contained $MgSO_4.7H_2O$ -0.417g, $CaCl_2$-0.417g, $FeS0_4.7H_2O$- 0.208g, $(NH_4)_2SO_4$- 0.417g, KH_2PO_4- 0.417g, Distilled H_2O- 333.3ml, the pH was adjusted to 7.2; followed by the addition of Rice bran-333.3g, Soya bean flour-100g. The above mixture was autoclaved at 121^0C, 15PSI for 1hour. It was allowed to cool overnight in the autoclave. A 5days culture of microorganism (*Aspergillus niger*) in petri-dishes were washed using Sterile Tween 80 (5mls) and Tween 80 containing the fungal spores was carefully dispensed into the cooled substrate, mixed thoroughly in a safety cabinet and incubated at room temperature for 96h.

Extraction of Amylase

The enzyme was harvested on the 6th day using citrate phosphate buffer (pH 4.5). After which it was allowed to stand for 30mins, it was then filtered using sieved packed muslin cloth. The pH of the extracted enzyme was taken and the enzyme was centrifuged. The supernatant was decanted which serves as the crude enzyme. The crude enzyme was preserved in the refrigerator after incorporating appropriate concentration of Sodium benzoate as preservative.

Amylase Assay

Amylase activity was determined using the method of Ramakrishna using the curve of known concentration for maltose [7]. Amylase activity was measured as follows, the reaction mixture consisted of 1ml of the enzyme extract, 1ml of 1% (w/v) soluble starch solution and 0.1ml citrate buffer (pH 4.5), incubation was at 60^0C for 1hr. The reaction mixture was stopped by immersing the tubes in boiling water bath (100^0C). The reducing sugars liberated were estimated by 3, 5 Dinitrosalicylic (DNS) acid method [8].The reaction mixture consisted 1ml DNS reagent and 3ml of starch hydrolysate in a test tube. The test tubes were covered and placed in boiling water (100^0C) for colour development for 5mins, after which the tube was allowed to cool at ambient temperature. The absorbance was read at 540nm against a blank using the spectrophotometer (Unispec 23D model). The blank was made up of 1ml of the reagent with 3ml distilled water. One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1.0µmole of maltose from starch in 1.0µL reaction mixture under the assay conditions.

Protein Determination

Protein content of the enzyme extracts were determined by following the method of Lowry and co [8] with Bovine Serum Albumin as standard. 0.2ml of protein extract was measured into tubes and 0.8ml distilled water was added to it. Distilled water was used as blank while BSA standard curve was equally set up, (10mg/ml), 1-10mg/ml, 5.0ml of alkaline solution was added to all the tubes, mixed thoroughly and allowed to stand for 10 mins, 0.5ml of Folin- C solution was added to all the test tubes and left for 30mins after which the optical density was read at 600nm wavelength in a spectrophotometer (T70 PG Instrument UV model). The protein concentration was estimated using values extrapolated from the standard graph of protein.

$$\text{Protein concentration (mg / ml)} = \frac{\text{Absorbance value}}{\text{Gradient}}$$

Determination of Specific Activity

The Specific activity of an enzyme gives the measurement of the activity of the enzyme.

This is the activity of an enzyme per milligram of total protein (expressed in units/mg).

It is the amount of product formed by an enzyme in a given amount of time under given condition per milligram of protein. Specific activity of the amylase was determined using the formula below.

$$\text{Specific activity} = \frac{\text{Enzyme activity (units / ml)}}{\text{Protein concentration (mg / ml)}}$$

Ammonium Sulfate Fractionation of Amylase

200ml of the amylase samples were first brought to 20% (w/v) saturation with solid ammonium sulphate (enzyme grade). The precipitated proteins were regimented by centrifugation for 15mins at 500min^{-1}. The resulted pellet was dissolved again with ammonium sulphate to achieve 60, 80 and 100% (w/v) saturation; the precipitated proteins were centrifuged for 15mins at 500min^{-1}. Both enzyme activity and protein content were determined for each separate fraction.

RESULTS AND DISCUSSION

Screening for the Best Strain of *Aspergillus niger*

All the mould strains were identified as *Aspergillus niger* but, three (3) were the most relevant strains, which were then preserved on PDA slants and kept in the refrigerator at 4^{0}C for further use. The result is shown in the Table **1**.

Table 1: Diameter of Zones of Clearance (mm) of *Aspergillus niger* Strains

Aspergillus niger strains	Diameter of zones of clearance (mm)
1	17.0
2	23.0
3	8.0

From the screening result, all the strains of *Aspergillus niger* could produce hydrolytic enzymes, however based on the results as obtained, *Aspergillus niger* strain 2 had the highest zone of clearance of 23mm and was used for further production studies inoculated into the enzyme production medium for the production of Amylase (Table **1**).

Ohimain and co studied the amylase producing microorganisms from palm oil mill effluent, and reported amylase producing ability of *Pseudomonas, Bacillus, Micrococcus, Candida, Aspergillus fumigatus, Penicillium, Mucur and Fusarium* [9]. The production of α-amylase from *Fusarium* species isolated from Eastern Nigeria Soil on fermentation of mineral salt medium supplemented with 25% corn starch has also been reported [10]. The amylase producing potentials of bacterial isolates associated with Cassava Peels obtained in five different locations within Delta State, Nigeria has also been reported [11].

Production of Amylase

Among the several growth factors, particularly substrate particle size is one of the most critical parameter affecting the productivity and growth of microorganisms [12]. It has been reported in literature that a low cost substrates like wheat flour, soya bean flour, wheat bran, rice straws (husk), rice bran and molasses are suitably effective for growth and enzyme production [13].

Enzyme Activity of Crude Amylase

Akpomie *and colleagues* reported the activity of amylase from bacteria from 100unit/ml to 700unit/ml [11]. The activity of produced amylases from rice bran and the commercial sample were determined and from the table below, the concentration of amylases produced were 6.62mg/ml, 19,340.0 μmol/L and 5.65 mg/ml, 16,510.0 μmol/L whereas the commercial amylase was 1.03mg/ml, 5,722.2μmol/L. The result gives a clear indication that amylase enzymes produced from our strain of *Aspergillus niger* in our laboratory had a higher activity than the commercial enzyme sample (Tables **2a** and **2b**). There are also existing reports on Amylase activity within 07.85 to 28.81 Unit/ml from fungal isolates [14]. The optimum pH for the Amylase reported by that study was 6.9 [14]. The study also documented synergistic α-amylase activity on involvement of mixed microbial culture [14].

Extraction and Purification of Amylase

The enzyme was harvested on the 6th day using 500ml of 0.01M citrate phosphate buffer (pH 4.5), and allowed to stand for 30 minutes and thereafter filtered. The pH of the enzyme was 9.01. 10 ml of the crude enzyme was centrifuged at 8,500rpm for 20 mins to get clear supernatant. The supernatant with amylase activity of 19340U/ml and specific activity of 681.23U/mg was used as crude enzyme solution and subjected to partial purification by ammonium sulphate precipitation in three fractions; 60, 80 and 100%. The crude enzyme was precipitated at 60, 80 and 100% saturation with specific activity of 2610.55, 1225.65 and 2172.78U/mg respectively (Table **3**).

Table 2a: Activity of Amylase Samples Using Dinitrosalicylic Acid Reagent @ 540nm

Sample	Volume of Standard (ml)	Volume of Water (ml)	Optical Density (O.D)	Conc. (mg/ml)	Conc. (μ/mol/L)
Blank	-	3.0	0.000	-	
STD. 1	0.6	2.4	0.360	0.6	
2	1.2	1.8	0.856	1.2	
3	1.8	1.2	1.376	1.8	
4	2.4	0.6	1.861	2.4	
5	3.0	-	2.282	3.0	
Amylase 1	-	-	1.771	6.62	19,340.0
Amylase 2	-	-	1.512	5.65	16,510.0
Commercial Enzyme	-	-	0.808	1.03	5,722.2

Table 2b: Enzyme Activity of Crude Amylase

Sample	Enzyme Activity (unit/ml)	Protein Content (mg/ml)	Specific activity (unit/mg)
AMYLASE 1	19340	28.39	681.23
AMYLASE 2	16510	21.76	758.73

Aspergillus niger strains 1 and 2 are the sources of Amylase 1 and 2 respectively.

Table 3: Purification Summary of Amylase Produced from *Aspergillus niger* after Ammonium Sulphate Precipitation

% (w/v) Ammonium Sulphate Saturation	Enzyme Activity (unit/ml)	Protein Content (mg/ml)	Specific activity (unit/mg)
60	44405.49	17.01	2610.55
80	28949.76	23.62	1225.65
100	36220.25	16.67	2172.78

CONCLUSION

Studies carried out so far have shown that *Aspergillus niger* are good potential producer of amylase using rice bran which is an agricultural raw material found greatly in our local environment. Research and development into local production of amylase for industrial use using local resources has proven cost effective and requires improvement into standardization as required by international organizations working on industrial enzymes. Industries also have the potential of adopting the enzymes produced for optimal production and this saves cost due reduction or even eradication of enzyme importation. The results showed that cassava peels is an adequate substrate for Amylase enzyme production. In Nigeria, there is a presidential mandate on Federal Ministry of Science and Technology through its research agencies to develop through research waste to wealth programmes. Several attempts have been made with different success stories. Similarly, the Federal Institute of Industrial Research, Oshodi, Nigeria has developed this innovative enzyme technology of utilizing the readily abundant and available cassava peel for Amylase enzyme production. This technology will definitely become sustainable as Nigeria still remains among the major producers of Cassava all over the world. In addition, the Cassava Industries in Nigeria produce the peel as waste and disposal is still a source of Environmental pollution. Globally, there are conventions and non-governmental agencies such as Gratitude that have sponsored and advocated for reduction or possible elimination of losses in Cassava and other root crops all over the world. Utilization of Cassava peels waste as substrate for enzyme / Amylase production is an

innovation requiring much fund for upgrading to pilot plant in Nigeria.

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