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Full Length Research Paper

Production and partial purification of glucoamylase from *Aspergillus niger* isolated from cassava peel soil in Nigeria

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Glucoamylase is an enzyme that hydrolyses 1,4α and 1,6β-glucosidic linkages in polysaccharides yielding glucose. *Aspergillus niger* strains 1, 2 and 3 were locally isolated from cassava peel dumpsite for the production of glucoamylase enzyme. *A. niger* strains 1, 2 and 3 were screened for their hyper producing ability on potato dextrose agar using plate assay method fortified with starch agar, and showed zone of clearance of 17.0, 23.0 and 8.0 mm, respectively. The glucoamylase activity for *A. niger* strains 1 and 2 were 13 000.0 and 11 740.0, respectively. These values were however higher than the activity as obtained from the commercial enzyme with 2 500.0. Investigations on the protein (mg/ml), and specific activity (units/mg) on glucoamylase produced by *A. niger* strains 1 and 2 was 24.20, 537.19, 23.13 and 507.57, respectively. Fractionation of the enzyme ammonium sulphate (% w/v) using 60, 80 and 100% showed that the enzyme activities were 33 179.86, 47 985.86 and 19 167.65 units/ml, respectively. Protein concentrations were 16.29, 16.29 and 21.55 units/mg, respectively, while specific activities were 2 036.82, 2 945.725 and 889.45 units/mg, respectively. The production, packaging, and commercialization of glucoamylase in Nigeria will save a lot of foreign exchange earnings, and boost the economy of Nigeria.

Key words: Glucoamylase, specific activity, *Aspergillus niger*, fractionation, cassava peel.

INTRODUCTION

Glucoamylase is one of the oldest and widely used enzymes in food industry. They are microbial enzymes, present in bacteria, archaea, and fungi but never in plants and animals. Fungal glucoamylases are biotechnologically very important as they are used industrially in large amounts, and have been extensively studied during the

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past 30 years (James and Lee, 1997). Glucoamylase (EC 3.2.1.3) hydrolyzes polysaccharides from the non-reducing bonds consecutively. They hydrolyze both 1, 4α and 1,6β-glucosidic linkages in polysaccharides yielding glucose only (Kumar and Satyanarayana, 2009). Glucoamylases are used mainly in the production of glucose syrup, high fructose corn syrup and in whole grain and starch hydrolysis for alcohol production. The major characteristic of glucoamylase is the saccharification of partially processed starch/dextrin to glucose, which is an essential substrate for numerous fermentation processes in a range of food and beverage industries (Lowry et al., 1951). Glucoamylase for commercial purposes has traditionally been produced employing filamentous fungi; although a diverse group of microorganisms is reported to produce glucoamylase, since they secrete large quantities of the enzyme extracellularly.

The commercially used fungal glucoamylases have certain limitations such as moderate thermostability, acidic pH requirement and slow catalytic activity that increase the process cost, consequently, the search for newer glucoamylase and protein engineering to improve pH and temperature optima leading to amelioration in catalytic efficiency of existing enzymes have been the major areas of research over the years (Marin-Navano and Polaina, 2011). The high cost of importation had over time being as a result of patent rights (Okafor, 1989) but improvement in optimal enzymatic activities has lead to this investigation. The use of locally available agro waste resources and imploring microorganisms in production is expected to reduce cost of importation and eventually improve quality of products in relevant industries. The objectives of this study were to isolate, screen for glucoamylase hyper producing strains of *A. species* and establish partial purification of produced glucoamylase using ammonium sulphate precipitation.

MATERIALS AND METHODS

Collection of soil sample

Soil samples from cassava processing areas were obtained from the cassava processing pilot plant site of The Federal Institute of Industrial Research, Oshodi extention at Tigbo'lu, Abeokuta, Ogun State. The soil samples were obtained aseptically and transferred to the laboratory for further analysis. The decision to isolate from this soil receiving cassava effluent is that the soil has high concentration of polysaccharide, and surviving microorganisms in the soil must have acquired genes for utilizing the polysaccharides. These genes include glucoamylase expression genes.

Isolation and Identification of *Aspergillus* spp. from soil samples

Using serial dilution method of isolation, 0.1 ml was plated on potato dextrose agar (fortified with 0.1% streptomycin). The molds were purified to obtain pure culture of *A. niger.* These relevant *A. niger* mould strain were then transferred on PDA slants kept in the refrigerator at 4°C. The mould isolates were identified following microbiological method of identifications through staining reagents of Lacto phenol cotton blue and observed under the microscope. Morphological and microscopic appearances of the moulds were compared with standard Atlas.

Screening for hyper-producing strain of *Aspergillus niger*

Spore suspension of each mould strain of *A. niger* was carefully introduced within 0.3 mm diameter of PDA plates fortified with 1% starch. The plates were then incubated separately at 30°C for 24 to 28 h, and flooded with Grams iodine reagent. Zones of clearance around the smeared portion was measured with the aid of meter rule 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 glucoamylase.

Enzyme production medium

The culture medium used in this work for glucoamylase production contained $MgSO_4.7H_2O$ (0.417 g), $CaCl_2$ (0.417 g), $FeSO_4.7H_2O$ (0.208 g), $(NH4)_2SO_4$ (0.417 g), KH_2PO_4 (0.417 g), distilled H_2O (333.3 ml), rice bran (333.3 g), soya bean flour (100 g). The mineral water was prepared as stated above and the pH was adjusted to 4.5 using 2 N NaOH.

The above mixture was autoclaved at 121°C for 1 h. It was allowed to cool overnight in the autoclave. The culture microorganism (sporulating *A. niger*) in petridishes were washed using Tween 80. The inoculum was mixed thoroughly in a safety cabinet previously sterilized using ultraviolet (UV)-light with the substrate and covered with foil paper.

Glucoamylase assay

The reaction mixture consists of 1 ml of the enzyme extract; 1 ml of 1% (w/v) soluble starch solution and 0.1 ml citrate buffer (pH 4.5), incubation was at 60°C for 1 h. The reaction mixture was stopped by immersing the tubes in boiling water bath (100°C). The reducing sugars liberated were estimated by 3, 5 dinitrosalicylic (DNS) acid method (Mehta et al., 2006). The reaction mixture consisted 1 ml DNS reagent and 3 ml of starch hydrolysate in a test tube. The test tubes were covered and placed in boiling water (100°C) for colour development for 5 min, after which the tube was allowed to cool at ambient temperature. The absorbance was read at 540 nm against a blank using the spectrophotometer (Unispec 23D model). The blank was made up of 1 ml of the reagent with 3 ml distilled water. One unit of enzyme activity (U) was defined as the amount of the enzyme that liberated 1.0 µmole of maltose from starch in 1.0 µL reaction mixture under the assay conditions.

Protein determination

Protein content of the enzyme extracts were determined by following the method of Miller (1959) with bovine serum albumin as standard. 0.2ml protein extract, was measured into tubes and 0.8 ml distilled water was added to it. Distilled water was used as blank

while BSA standard curve was equally set up 10 mg/ml, 1 to 10 mg/ml, 5.0 ml of alkaline solution was added to all the tubes, mixed thoroughly and allowed to stand for 10 min, 0.5 ml of Folin- C solution was added to all the test tubes and left for 30 min after which the optical density was read at 600 nm wavelength in a spectrophotometer (T70 PG Instrument UV model). The protein concentration was estimated using values extrapolated from the standard graph of protein.

Ammonium sulfate fractionation of glucoamylase

Glucoamylase samples (200 ms^{-1}) were first brought to 20% (w/v) saturation with solid ammonium sulphate (enzyme grade) as mention by (Dixon and Webb (1964). The precipitated proteins were regimented by centrifugation for 15 min at 500 min^{-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 15 min at 500 min^{-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 *A. niger* but screening was carried to identify three (Lowry et al., 1951) most relevant strains with hyper production properties, which were then preserved on PDA slants and kept in the refrigerator at 4°C for further use (Table 1)

In addition, all the strains of *A. niger* could produce hydrolytic enzymes, however based on the results as obtained, *A. niger* strain 2 had the highest zone of clearance with 23 mm and was inoculated into the enzyme production medium for the production of glucoamylase (Table 1). Among the several growth factors particularly substrate particle size is one of the most critical parameter affecting the productivity and growth of microorganisms (Sen et al., 2009). 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 (Zadrazil and Punia, 1995).

Table 1. Diameter of zones of clearance (mm) of *Aspergillus niger* strains.

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

Enzyme activity of crude glucoamylase

The activity of produced glucoamylases from rice bran and the commercial sample were determined, and from Table 2, the concentration of amylase produced was 4.45 mg/ml, 13000.0 µmol/L and 4.02 mg/ml, 11740.0 µmol/L, respectively; whereas the commercial amylase was 0.45 mg/ml, 2,500.0 µmol/L. The result thus shows a higher activity of produced glucoamylase enzymes when compared with the commercial enzyme sample (Table 2a and b). Ominyi et al. (2013) reported activity within 0.068 to 1.327 (Unit/ml) for glucoamylase enzyme from different fungal strains (Ominyi et al., 2013). Ominiyi et al. (2013) also reported the increased glucoamylase activity when mixed cultures of fungal strains were involved in the fermentation process (Ominyi et al., 2013). This synergistic increase in the activity of glucoamylase on utilization of mixed microbial cultures for fermentation has been reported also by Ueda (Oyewole and Agboola, 2011). There have been several attempts by other Indigenous Nigerian Scientists to extract glucoamylase from non-microbial sources including kilned and unkilned malted sorghum and corn. Oyewole and Agboola (2011) reported a glucoamylase activity of 2.78, 0.94, 1.22, and 0.75 (Mg/Maltose/ml/min) in malted unkilned sorghum, kilned sorghum, unkilned corn and kilned corn, respectively (Oyewole and Agboola, 2011). The general problems with plant enzymes are that their production is not sustainable for commercialization because continuous use of the grains will lead to loss or threat to the plant species (negative impact on biodiversity), again the malting process of the grains is not only labour intensive but also time-consuming.

Extraction and purification of glucoamylase

The enzyme was harvested on the 6th day using 2.5 L of 0.01 M citrate phosphate buffer (pH 4.5); 5 g of sodium benzoate was added to prevent enzyme deterioration by microbial actions. After adding the buffer and sodium benzoate to the medium, it was allowed to stand for 30 min and thereafter filtered using sieve packed with muslin cloth. The pH of the enzyme was 4.5. Ten (10) ml of the crude enzyme was centrifuged at 8 500 rpm for 20 min to get clear supernatant. The supernatant with glucoamylase activity of 13 000 U/ml and specific activity of 537.19 U/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 2 036.82, 2 297.04 and 889.45 U/mg, respectively (Table 3).

Studies carried out so far have shown that *A. niger* strains

Table 2a. Activity of glucoamylase samples using dinitrosalicylic acid reagent at 540 nm.

Sample	Volume of standard (ml)	Volume of water (ml)	Optical density (O.D)	Concentration (mg/ml)	Concentration (µ/mol/L)
Blank	-	3.0	0.000	-	
STD. 1	0.6	2.4	0.360	0.6	
STD. 2	1.2	1.8	0.856	1.2	
STD. 3	1.8	1.2	1.376	1.8	
STD. 4	2.4	0.6	1.861	2.4	
STD. 5	3.0	-	2.282	3.0	
Glucoamylase 1	-	-	1.192	4.45	1 3000.0
Glucoamylase 2	-	-	1.075	4.02	1 1740.0
Commercial Enzyme	-	-	0.808	0.45	2 200.0

Aspergillus niger strains 1 and 2 were the sources of Glucoamylase 1 and 2 respectively.

Table 2b. Enzyme activity of crude glucoamylase.

Sample	Enzyme activity (unit/ml)	Protein content (mg/ml)	Specific activity (unit/mglml)
Glucoamylase 1	13000	24.20	537.19
Glucoamylase 2	11740	23.13	507.57

Aspergillus niger strains 1 and 2 were the sources of glucoamylase 1 and 2, respectively.

Table 3. Glucoamylase activity after Ammonium Sulphate precipitation.

Percentage (%) (w/v) Ammonium sulphate saturation	Enzyme activity (unit/ml)	Protein content (mg/ml)	Specific activity (unit/mg/ml)
60	33179.86	16.29	2036.82
80	47985.86	16.29	2945.725
100	19167.65	21.55	889.45

are good potential producers of glucoamylase using rice bran and other agro waste residues. This in turn shows the relevance and importance of agricultural raw materials found greatly in our local environment. This investigation also shows the control of regulatory patenting rights through proper research and development. Industries also have the potential of adopting the enzymes produced for optimal production and by saving cost in enzyme importation. The report also shows the need for improved enzyme production through analysis of better substrates and also adopting improved technology in production medium.

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