PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR *Aspergillus flavus* GLUCOAMYLASE ISOLATED FROM DECOMPOSING CASSAVA *(MANIHOT SPP)* FLOUR

Supported by tetfund

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Abstract: In this present study, extracellular glucoamylase was secreted from *Aspergillus flavus* an organism isolated from cassava flour and identified with the feature of the strain. The crude enzyme was precipitated at 80 % ammonium-sulphate, followed by ion-exchange chromatography on carboxyl-methylSephadex C-25, affinity chromatography on sepharose 4B and gel-filtration on sephacryl S-200. The peak with the highest activity was pooled from latter chromatographic step and characterized afterwards. The specific activity of the purified enzyme was approximately 0.59 to 7U/mg of protein with a yield of 11.58 % and 22.1 purification fold. The optimal pH and temperature of the enzyme were 7.0 and 50 ^{0}C respectively. The enzyme was observed to be thermostable at the 50 ^{0}C for 15 to 30 mins. The kinetic parameters Km and Vmax for the enzyme were 0.934 mg/ml and 6.452U/min respectively. The effect of selected cations and chemical compounds at concentrations of 1 mM, 5 mM and10 mM revealed the activating effect of Ca^{2+} while Al^{3+}, $Cu^{2+,}$ Mg^{2+}, K^+, Na^+, EDTA, mercaptoethanol and urea inhibit the enzyme's activity. The apparent molecular weight of the enzyme as determined by gel filtration on sephacryl S-200 was 28 KDa while the subunit molecular weight as determined on SDS PAGE was approximately 25 KDa.

Keywords: Glucoamylase, *Aspergillus flavus*, starch hydrolases, chromatography, Electrophoresis.

Introduction

Starch is the plant storage polysaccharide made up of amylose and amylopectin. The amylose component is a polymer of glucose linked by α-1, 4-glucosidic bonds while the amylopectin is a polymer of glucose linked by α-1, 4-glucosidic bonds in the straight chain and α-1, 6 at the branch point (Okwuenu *et al*., 2017). It occurs mainly in the seeds, roots and tubers of higher plants (El-Fallal *et al*., 2012). Starch degrading enzymes like amylases have received great deal of attention because of their perceived technological significance and economic benefits (Negussie *et al.*, 2019). Glucose as the reaction product can be further used as a substrate for ethanol production during fermentation, as raw material for chemical synthesis (sorbitol), or for the production of fructose by isomerization (Crabb and Shetty, 1999). Glucoamylase is used in many fermented food products as well as in textile, leather, and detergent industries. With the enhancement in industrial prerequisite, the utility of glucoamylase for the production of various food grade metabolites has increased. Among hydrolases, glucoamylase is an inevitable primary requirement in food industries (Imran *et al*., 2017).

Glucoamylase (EC. 3.2.1.3) is also referred to as amyloglucosidase industrially, it an exoamylase catalyzing the hydrolysis of α-(1, 4) glycosidic bonds, and α-(1,6) glucosidic bonds of polysaccharides (Lam, Kwan, and Lin, 2015). The amylolytic enzyme attacks the non-reducing ends of polysaccharides like starch giving glucose as the end product (Norouzian *et al*. 2006; Sauer *et al*. 2000). During saccharification, partially hydrolysed amylose and amylopectin molecules are depolymerised by the action of glucoamylase which removes glucose units in a stepwise manner from the nonreducing chain ends (Jensen and Norman, 1984; Hebeda, Nagodawithana and Reed, 1993). The enzyme cleaves α-(1,6) glucosidic bonds at a much slower rate than the hydrolysis of α-1-4-bonds (Norouzian *et al*. 2006; Robertson *et al*. 2006).

Enzymatic starch saccharification has several advantages including improved yield of glucose, fructose, maltose, and oligosaccharides as compared to the chemical hydrolysis methods. Enzymatic hydrolysis is a cost effective, reaction-specific method with better control over amylolysis and usually generates more stable products with limited by-product formation. Starch hydrolyzing enzymes are classically used in starch liquefaction, saccharification, and in isomerization.

Many researchers have purified and characterized glucoamylase from various fungi used commercially from strains of either *Aspergillus sp.*, (Deshmukh *et al*., 2011) *Aspergillus awamori* (Negi and Banerjee 2009), *Aspergillus niger* (Slivinski *et al*. 2011), or *Rhizopus spp* (Morita and Fujio, 2000). Other sources also reported include those from two ectomycorrhizal fungi, *Tricholoma matsutake* and *Lyophyllum shimeji* (Adeoyo *et al*., 2018). Kusuda *et al*., in 2004 characterized an extracellular glucoamylase from *L. shimeji*, stating that the enzyme was most active around 40°C. More recently, this enzyme has been reported to be produced from some microorganisms, viz, *Aspergillus flavus* (Karim *et al*., 2017) and *Tetracladium* sp. (Carrasco *et al*., 2017).

The enzymes can be produced from various substrates by different methods, including submerged, semi-solid and solid-state fermentation processes (Ramesh and Murty, 2019). Solid-state fermentation is a potent protocol for producing enzymes particularly glucoamylase on large scale (El-Gendy and Alzahrani, 2020). Beside several

fermentation strategies, mutational approaches could also be employed for the hyper-production of glucoamylase to meet the industrial requirements and thus improve the upstream processing (Ghani *et al*., 2019*).*

Due to the increasing demand for the enzyme, there is immense interest in developing enzyme with raw starch degrading capacity which could be useful industrially and possibly cost effective. We therefore isolated and identified a good glucoamylase producing fungus from which glucoamylase was extracted, purified and partially characterized.

Materials and Methods

The organism used in this study was isolated from decomposing cassava flour purchased from Falawo market, Sagamu Ogun State, Nigeria and taxonomically characterized at the Medical Microbiology Laboratory of Olabisi University of Olabisi Onabanjo University. D-(+)-glucose (CAS No: 50-99-7), 3, 5-dinitrosalicylic acid and sodium hydroxide were purchased from Sigma Aldrich Chemie GmbH (Germany). Rochelle salt (potassium sodium tartrate) was obtained from Fluka (Switzerland). Glass distilled water was used for all preparations. All media and other chemicals used were of analytical grade while equipment used were located in the department of Biochemistry, Olabisi Onabanjo University.

Methods

The various media used for the isolation, cultivation and identification of the microorganism were sterilized by autoclaving at 121ºC for 15 min under pressure while all glass wares used were sterilized in hot air oven at 180ºC for 3 hours. Inoculating loop was sterilized by flaming.

Isolation of the Fungi

Cassava flour (1g) was homogenized in 9 ml of sterile distilled water. 1ml of the homogenate was serially diluted to 10^{-5}**,** 0.1ml of the selected dilutions was plated out on Sabouraud Dextrose Agar (SDA) using sterilized disposable syringe. The plates were incubated at 28°C±2°C for 168h. Discrete colonies on the plates were counted and the isolates were purified by repeated sub culturing on SDA. All the mould cultures were identified and confirmed by studying the morphology of colonies and microscopic examination was compared with the standard Atlas. All the mould isolates were stored on SDA slants at 4°C until required.

Primary Screening of Amylase Producing Fungi

The fungal isolates were screened for their starch hydrolyzing ability. The fungal isolates were inoculated on 1 % starch containing SDA Plate. After 3 days of fungal growth, the plates were flooded with iodine solution. Starch reacted with iodine to form a dark blue starch-iodine complex that covered the entire agar. When starch was broken down into sugars, there were clear zones surrounding streaked lines which indicated starch hydrolysis (Alfred, 2007). The zones of hydrolysis formed by each isolate were measured in mm of diameter and the fungal isolates which showed maximum zone of starch hydrolysis were selected for enzyme production.

Identification of the amylase producing isolate

The selected fungus with highest zone of inhibition was identified growth characteristics of the fungi isolate which are texture of the colony, pigment and the shape of the colony, staining technique using lacto phenol in cotton blue. The lacto phenol act to kill the organism and also to fix the cells on the glass slide. The prepared glass was examined under micro scope at x40 objective. Drawings of the various structures (i.e. sporagium, sporangiophores, collumellae, mycelium and spores) of the isolate were made and compared with the drawings of described commonly encountered mould.

Enzyme Secretion and Extraction

The enzyme secretion was carried out using solid state fermentation with a medium for the cultivation of the fungus that was formulated by the modified method of Puri *et al.*, (2013) and Zambare, (2010). Mineral medium was prepared by weighing 0.50 g $MgSO_4.7H_2O$, 0.50 g KCl, 0.15 g $FeSO_4.7H_2O$, 1.00 g NH_4NO_3, 2.00 g KH_2PO_4 in 1L of distilled water followed by sterilization. The solid state medium was prepared by adding to an Erlenmeyer flask, 10g of wheat bran, 3g soya bean meal and 1g cassava starch; moistened with the previously prepared mineral medium at 4:6 (w:v), stoppered and sterilized by autoclaving at 121ºC for 15 mins.

The sterile medium prepared was then inoculated with the serially diluted 2x106ml of the spore of the pure isolate of the fungus on the slant after reactivation and incubated at 35^0C for 168 hrs without shaking. At the end of the incubation, the mycelium was separated from the culture broth by filtering through a sterile filter paper (Whatman No 1), the filtrate was centrifuge at 6,000 x g f0r 15 min at 100C to remove the suspended particles, the supernatant was carefully collected and stored under refrigeration as crude enzyme for further analysis after adding 1 % Phenyl Methyl Flouride (PMSF) solution.

Confirmation of amylase type by sugar analysis on thin layer chromatography (TLC)

To confirm the type of amylase from the fungal isolates based on the starch hydrolysates TLC system (Feinberg 1965 and Oyefuga *et al*., 2011) followed. First 0.9 ml of 2% soluble starch was incubated with 0.3ml of the crude enzyme for 30 min at 50° C. Then the hydrolysate obtained was spotted on TLC plate along with standard known sugars (glucose, maltose and mannose) solution. A one dimensional ascend was done by using a solvent system (v/v) of butanol: ethanol: water (5:3:2). After a total of five ascends, the plate was air-dried; sprayed with 50% (v/v) methanol-H_2SO_4 and heated for 10 min at about 100° C. The sugar spots that appeared were identified by comparing with the standard sugars.

Enzyme and Protein Assays

Glucoamylase activity was determined by measuring the rate at which glucose is released from starch due to its ability to reduce 3, 5- Dinitrosalicylic acid (DNSA) using the modified Bernfeld (1955) method. One unit of glucoamylase activity was described as the amount of enzyme that releases 1 µg of glucose per minute at 50ºC The reaction mixture consisted of 0.10 ml of enzyme solution and 0.50 ml of the substrate (1% cassava starch in 0.10 M Citrate-Phosphate buffer at pH 5.5) incubated for 5 minutes at 50ºC and the reaction stopped by the addition 1.0 ml DNSA colour reagent. The mixture was heated in a water bath at 50ºC for 5 minutes so as to develop the reddish-brown coloration, cooled and made up to 10 mL using distilled water. The reaction mixture was allowed to stand for 15 min at room temperature and the optical density read at 540 nm. One unit of enzyme activity was defined as the amount that released one microgram of reducing sugar as glucose per minute under the same

conditions as described for the assay and calculated as shown below. The protein concentration was determined with Bradford reagent assay kit using bovine serum albumin as standard (Bradford, 1976).

$$\text{Enzyme Activity (U/min)} = \frac{\mu g \text{ of glucose released X dilution factor}}{\mu g \text{ of protein in reaction mixture x incubation time}}$$

Enzyme Purification

Ammonium Sulphate Fractionation

The crude enzyme was brought to 70 percent ammonium sulphate fractionation; the salt was added slowly by gently stirring of the solid ammonium sulphate in an ice-cold environment. The stirring continued occasionally for 30 minutes and the mixture was then left overnight at 4 ^{o}C. The precipitated protein was recovered by centrifugation at 4,000 rpm at 4 ^{o}C for 10 minutes. The precipitate was reconstituted with 50 mM Sodium Phosphate buffer, pH 7.5 containing 10 percent ammonium sulphate and 1 % PMSF, a protease inhibitor. The supernatant resulting from the fractionation was discarded as no protein was detected in it and the precipitate was saved at 4 ^{o}C for further analysis.

Dialysis

The dialysis tubing was pre-treated by boiling in water bath until it softens. The tube was secured at one end while the precipitated sample was poured in through the other end. The bag was tied securely at the other end and suspended in 50 mM Sodium Phosphate buffer, pH 7.5 for about 12 hours while changing the buffer every 4 hours. The dialysate was saved and at 4 ^{o}C with the addition 1 % PMSF.

Ion–Exchange Chromatography on CarboxyMethyl Sephadex

About 20g of CM sephadex powder was pretreated according to Whatmann Product Instruction Manual. 100 ml of 0.05 M HCl was added to the resin and allowed to stand for 30 minutes while stirring intermittently on settling. The washing continued until the pH of the slurry was 7.0. The 100 ml of 0.05 M NaOH was added afterwards and the washing continued by adding water until the pH of the slurry was at 7.5. The pre-treated resin was then loaded in the column (2.5×40cm) and equilibrated with 50 mM Sodium-Phosphate buffer, pH 7.5. The dialysate was loaded onto the pre-packed column and eluted into 5 ml fractions at a flow rate of 20 ml/hr with a 250 mL linear salt gradient (0- 0.10 M NaCl) in the same buffer (50 mM Sodium Phosphate buffer, pH 7.5). Enzyme activity and protein concentration were routinely determined in all the fractions collected. The active fractions were pooled quantitatively and brought to 70 percent ammonium sulphate.

Gel Filtration on Sephacryl-S-200

Sephacryl-S-200 was swollen in distilled water; the slurry loaded into a column (1.5×40 cm) and equilibrated with 50 mM sodium phosphate buffer, pH 7.5. The precipitated sample from the ion-exchange chromatography step was dialyzed and loaded onto the Sephacryl-S-200 in the column. The column was then eluted with the same buffer and 2.5 mL fractions were collected at a flow rate of 20 ml/hr. Enzyme activity and protein concentration were routinely determined on each of the fractions collected. The active fractions were pooled, precipitated, dialyzed against same buffer and saved at 4^{o}C for characterization studies.

Homogeneity test

The homogeneity test was performed by polyacrylamide gel electrophoresis (PAGE) in the absence of sodium dodecyl Sulphate (SDS) on a 7% gel according to Pharmacia handbook (Polyacrylamide gel electrophoresis, Laboratory Techniques, Revised Edition, Feb. 1983). The electrophoresis gel buffer was a0.2 M phosphate buffer, pH 7.2. An aliquot of the purified enzyme in 0.01 M phosphate buffer was mixed with glycerol and tracking dye (0.05% bromophenol blue in 0.01 M phosphate buffer), after which 0.01 ml of this was layered on a rod gel and ran at room temperature at 8 mA per gel. The gels were stained with Coomasie brilliant blue R250 solution (1.25 g of Coomasie brilliant blue in 400 ml of methanol, 70 ml glacial acetic acid, and distilled water to 1 L) followed by distaining in a solution containing 5% methanol and 7.5% glacial acetic.

Enzyme kinetics

The Michaelis–Menten substrate saturation curve was used to determine the *Km* and *Vmax* value of the glucoamylase by measuring the rate of soluble hydrolysis under standard assay conditions. The reaction mixture was 20 mM acetate buffer (pH 5.0), with the soluble starch substrate at concentrations ranging from 0.2 to 10 mg/mL. The values for *Km* and *Vmax* were then determined using Lineweaver-Burk's plot.

Effect of pH on activity of the glucoamylase

The optimum pH for the enzyme was determined by incubating enzyme with the substrate (1%, w/v) prepared at various pH values using 0.10 M of the following buffers having pH values of 1.0, 2.0 (hydrochloric acid–potassium chloride); 3.0 (citrate); 4.0-5.0 (acetate); 6.0-7.0 (phosphate); 8.0 – 10.0 (Tris–HCl) at a temperature of 45 °C for 1 h, after which the enzyme activity was measured before determining the residual activity by the standard procedure.

Effect of temperature on activity and stability of the glucoamylase

The optimum pH for the enzyme was determined by incubating enzyme with the substrate (1% w/v) prepared in 0.10 M Phosphate buffer (pH 7.5). The enzyme was incubated at different temperatures of 30, 40, 50, 60, 70, 80, 90 and 100 °C for 1 h, after which, the enzyme activity was measured. The temperature stability was investigated by pre-incubating the enzyme at different temperatures (30, 40, 50, and 60 °C) for 15, 30, 45 and 60 min before determination of the residual activity by the standard procedure.

Effect of metal salts and inhibition study on glucoamylase enzyme activity

The method of Lee et al. ( ) was used to study the effect of various salts on the activity of the from sprouted maize grains. The salts tested were $MgCl_2$,$AlCl_3$, KCl, $CuSO_4$,Na_2SO_4,$CaCl_2$ at 1.0 mM and 5mM concentrations and reagents (EDTA, 2-mercaptoethanol and urea) at 1.0 mM and 5mM concentrations in a typical glucoamylase assay mixture. The reaction mixture without the salts was taken as control with 100% activity.

Substrate Specificity

The substrate specificity of the enzyme activity was carried out. The enzyme was assayed using different substrates; banana peel, cassava starch, corn starch, carboxyl methyl cellulose (CMC), Pectin, Apple pome and soluble starch as control. The glucosidase activity was assayed routinely using each of the listed substrates.

Determination of Native Molecular Weight

The native molecular weight of the enzyme was determined using gel filtration on Sephacryl-S-200 column. The protein markers used were, Bovine Serum Albumin (66 kDa), ovalbumin (45 kDa), trypsin (24 kDa) and Lysozyme (14 kDa). The void volume was determined using blue dextran.

Molecular weight determination by SDS-PAGE electrophoresis

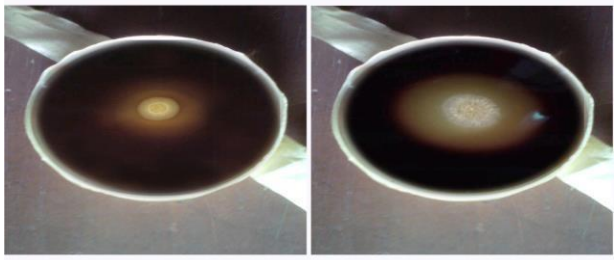
SDS-PAGE was performed using 8×10×0.75 cm gels in a Mini-Protean II (Bio-Rad) gel apparatus. Samples were treated with reducing (containing 2-mercaptoethanol) sample buffer and boiled for 5 min before loading the gel. After electrophoresis, proteins in the gel were visualized by staining with Coomassie Blue R-250 (Laemmli, 1970).

Results

The photographs of the amylase-producing fungal strains isolated were shown on Plate 1 and Plate 2. The morphological characteristics of the organism with higher starch hydrolysis revealed rapid growth of greenish yellow with a white boarder having a moldy texture and the transverse has a yellow colour. The fungal isolate was identified as *Aspergillus flavus*.

A: *Aspergillus flavus* B:*Aspergillus fumigatus*

Plate 1: (A) Pure culture of *Aspergillus flavus* and (B)*Aspergillus fumigatus*



A: *Aspergillus fumigatus* B:*Aspergillus flavus*

Plate 2: (A) zone starch hydrolysis shown by *Aspergillus fumigatus* and (B) *Aspergillus flavus*

Enzyme Purification

The results of the purification procedures were summarized in Table 1, producing glucoamylase with specific activity of 6.83 Umg^{-1} of protein with a yield of 22.1%. The elution profile of the dialysate on the CM Sephadex column (Figure 1) showed more than one peak of glucoamylase activity but the one with highest activity was pooled and purified further while the elution profile of the post CM Sephadex on Sepharose-4B column (Figure 2) showed a major and minor peak of glucoamylase activity but the one with highest activity was pooled and purified further. The elution profile of the post Sepharose-4B on Sephacryl S-200 gel filtration column is presented in Figures 3. Two major peaks were noted in the latter figure and the one with the highest peak was pooled (Fraction number 25 to 35).

Table 1: Summary of the Purification of *Aspergillus flavus* glucoamylase

Fractions	**Volume (ml)**	**Total Protein (mg)**	**Total Activity (U)**	**Specific Activity U/mg**	**Purification fold**	**Yield (%)**
Crude Enzyme	400	218.6	128.70	0.59	1.0	100
Ammonium Sulphate Precipitation (80%)	35	121.38	93.46	0.81	1.37	72.60
CM Sephadex c25 Ion Exchange Chromatography	10	42.13	71.90	1.70	2.88	55.90
Sepharose-4B Affinity Chromatography	16	8.40	38.69	4.61	7.80	30.10
Sephacryl-200 gel filtration	18	6.66	28.43	6.83	11.58	22.10

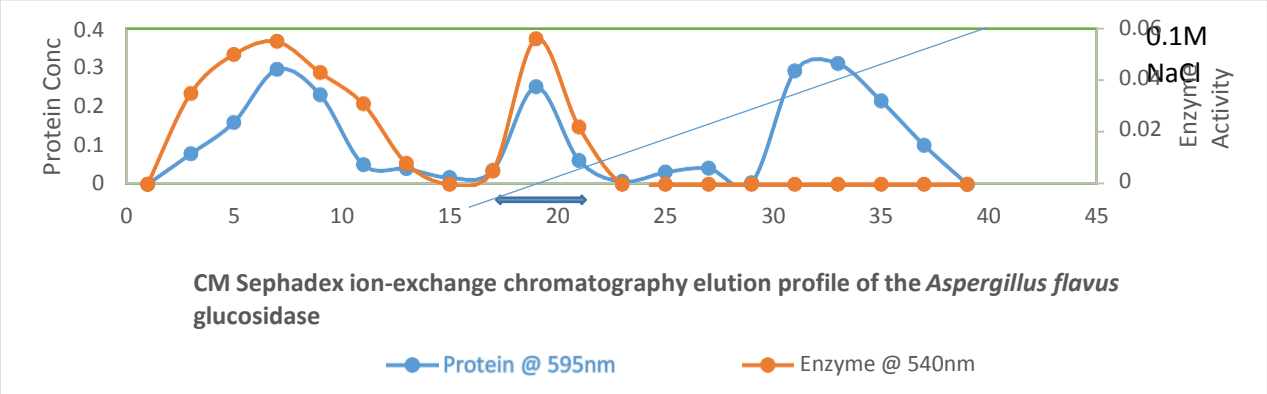


Fig. 1: CM Sephadex ion-exchange chromatography elution profile of the *Aspergillus flavus* glucosidase obtained from cassava flour

Legends: Protein concentration at 595 nm, Enzyme Activity at 540 nm, pooled fractions of the enzyme for further analysis.

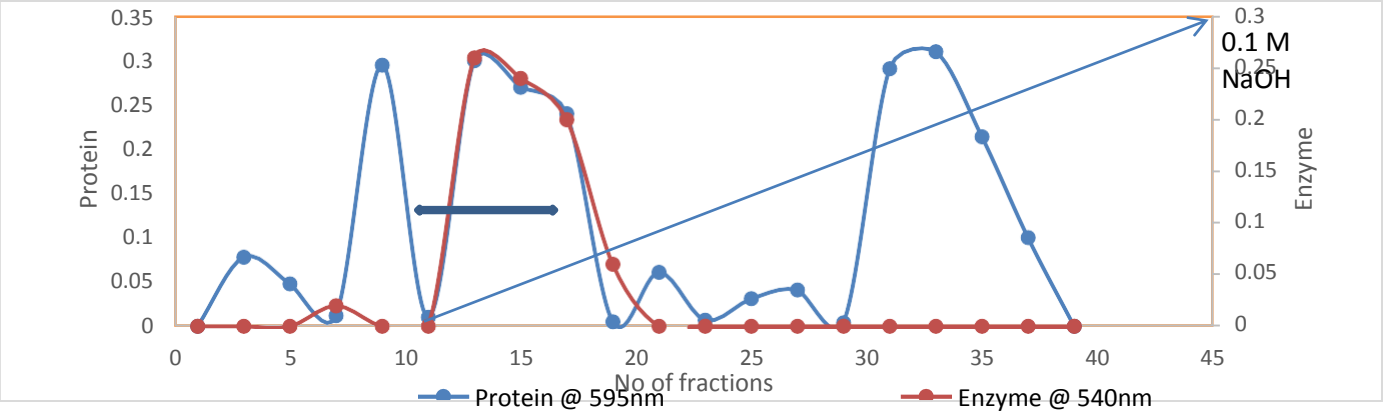


Fig. 2: Sepharose 4B Affinity chromatography elution profile of the *Aspergillus flavus* glucoamylase obtained from cassava flour.

2ml fractions were collected from the column; Enzyme activity profile OD was measured at 540 nm) Protein profile OD was measured at 595 nm Salt concentration gradient pooled fractions of the enzyme for further analysis

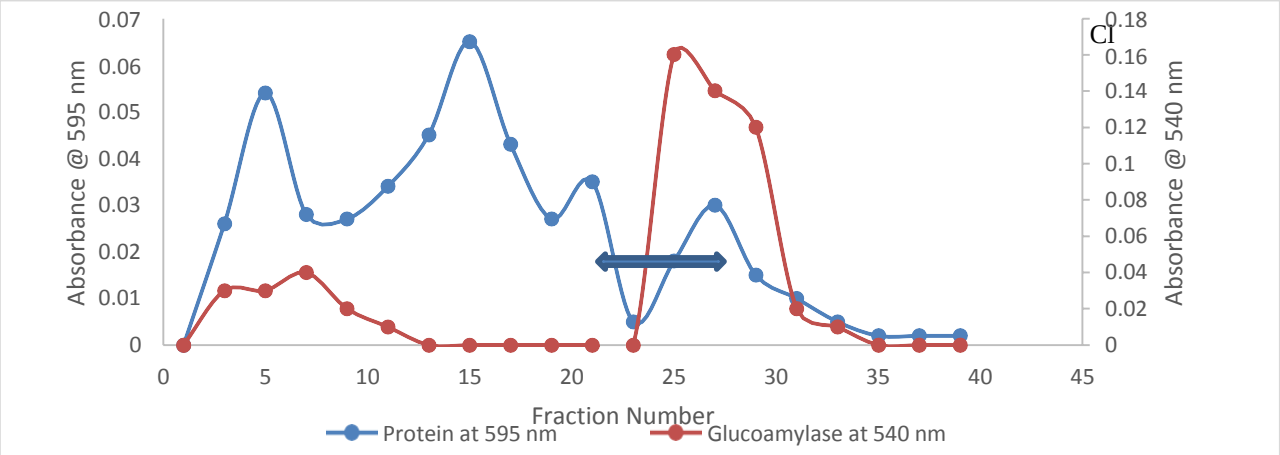


Fig. 3: Sephacryl S-200 Gel filtration chromatography elution profile of the *Aspergillus flavus* amyloglucosidase from cassava flour. Fractions of 2ml were collected from the column Activity profile (OD 540 nm) Protein profile (OD 595 nm) Pooled fractions of the enzyme for further analysis**.**

Properties of the Purified glucoamylase Isolated from Decomposing Cassava flour

The maximum glucoamylase activity was obtained at 50ºC. A reduction in enzyme activity was observed at values above 50°C (Figure 4) beyond this temperature, the activity was reduced drastically and enzyme was completely inactivated. The stability of glucoamylase to temperature is shown in Figure 5, glucoamylase was observed to be stable at 50ºC for 15 to 45 min. A sharp decrease in stability was observed as the incubation time increased beyond 45 min. The optimum pH was 7.0 (Figure 6). The graph of velocity versus substrate concentration (Figure 7) and gave a Vmax value of 14.60±0.04 U/min and kinetic constant (Km) of 0.2818 ± 0.04 g/ml. Effect of metals salts and other compounds on the activity of the enzyme is shown in Figure 8 & 9 revealing the activating effect of Ca^{2+} while Al^{3+}, $Cu^{2+,}$ Mg^{2+}, K^{+}, Na^{+}, EDTA, mercapto-ethanol and urea inhibit the enzyme's activity. Substrate specificity of purified *Aspergillus flavus* glucoamylase isolated from cassava flour on different substrates showed that the enzyme has high specificity for cassava starch (Figure 10). The native molecular and the sub unit molecular weight of the purified enzyme gave an estimated value of 28 kDa and 25 kDa from the plot of partition coefficient against Logarithm of molecular weight

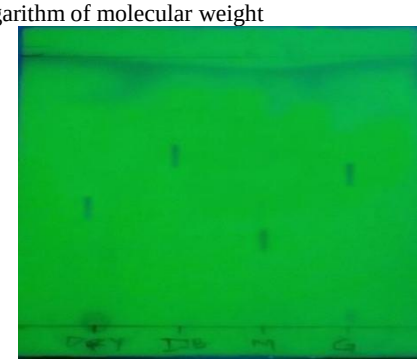


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Plate 3: Thin layer chromatogram of *Aspergillus flavus* glucoamylase starch hydrolysates

Properties of the Purified glucoamylase Isolated from Decomposing Cassava flour.

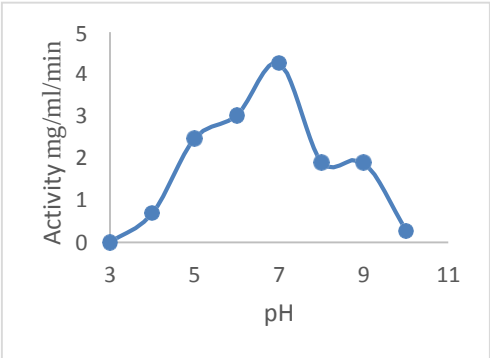

Fig. 4: The pH profile of purified Aspergillus flavus glucoamylase isolated from decomposing cassava flour

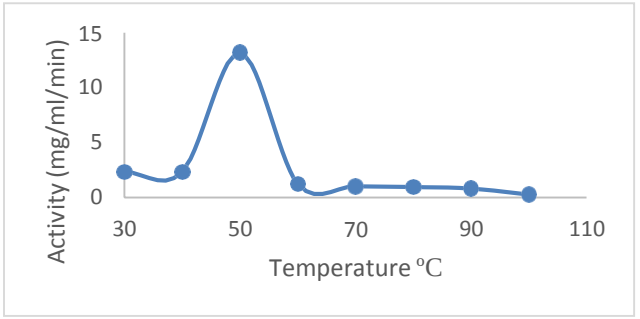

Fig. 5: The Temperature profile of purified *Aspergillus flavus* glucoamylase isolated from fermenting cassava starch

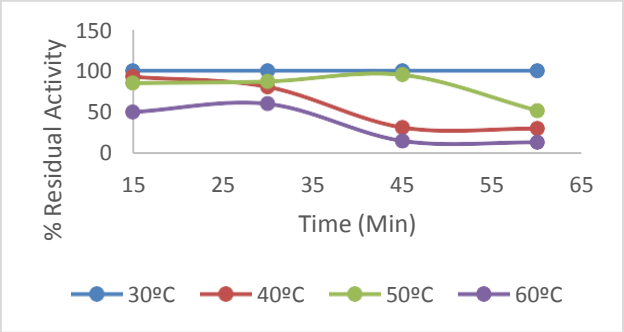

Fig. 6: The thermal stability curves of purified *Aspergillus flavus* glucoamylase isolated from fermenting cassava flour

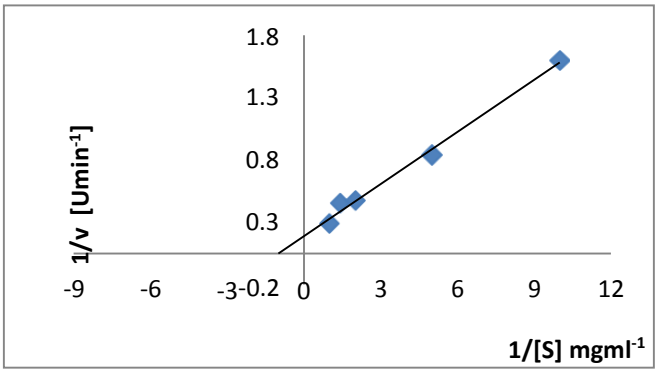

Fig.7: Lineweaver-Burk plot for the determination of kinetic parameters. The concentration of glucoamylase used varied between 0.1-1.0mg/ml. The lines of best fit were through the points using regression analysis.

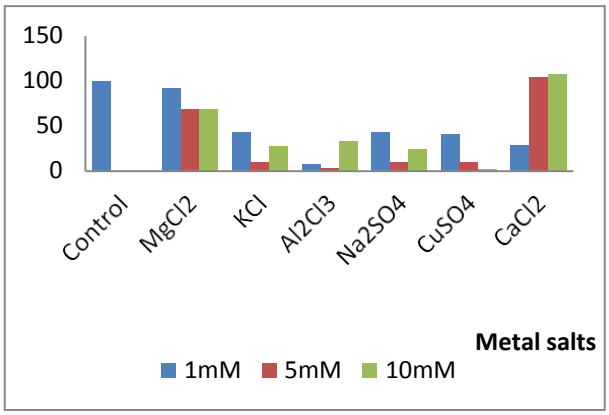

Fig. 8: Effect of metals on the purified *Aspergillus flavus* glucoamylase isolated from decomposing cassava flour

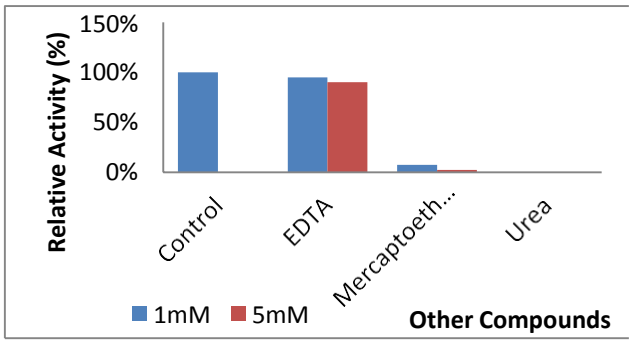

Fig. 9: Effect of other compounds of the purified *Aspergillus flavus* glucoamylase isolated from fermenting cassava flour.

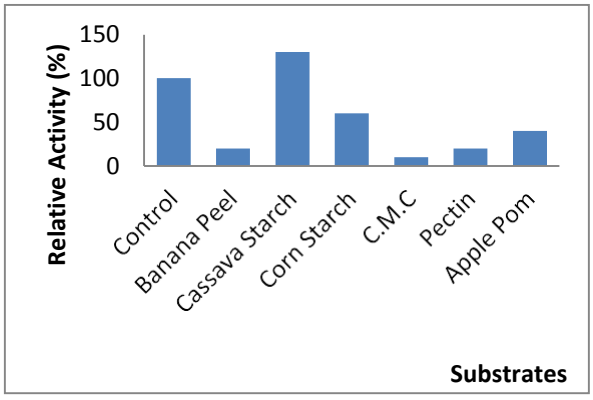

Fig. 10: Substrate specificity of purified *Aspergillus flavus* glucoamylase isolated from decomposing cassava flour on different substrates

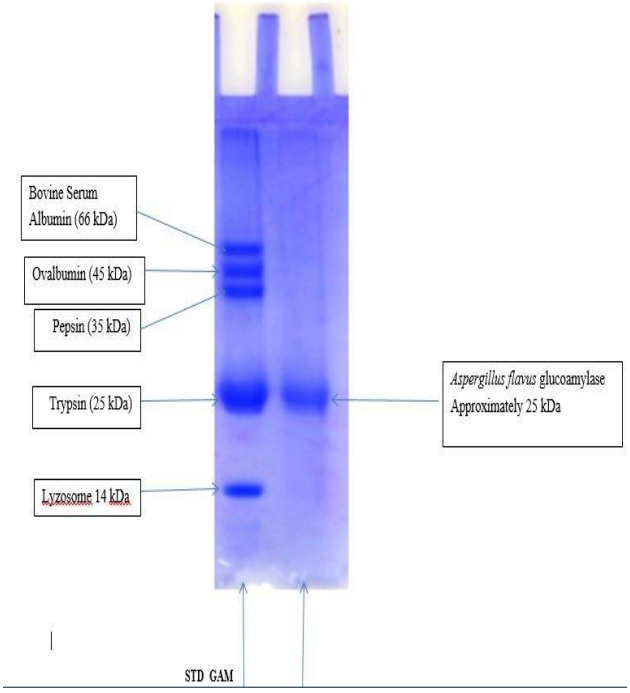

Plate 4: Polyacrylamide gel electrophoresis of *Aspergillus flavus* glucoamylase in the presence of SDS.

Legends:
STD Standard protein
GAM *Aspergillus flavus* Glucoamylase.

Discussion

Generally, microorganisms survive on plant waste and soil because they shield starch hydrolyzing enzymes which are used in extracellular digestion of substrate (the source of energy for these organisms) and they secrete the enzymes extracellularly into the surrounding medium. Also, the native substrate is water-insoluble and cannot penetrate into cells; the biodegradation of starch therefore occurs extracellularly (El-Fallal *et al*., 2012). The fungal amylase mainly developed spot that was parallel with the standard glucose indicated that the test enzyme was glucoamylase. This was direct evidence for the presence of glucoamylase in the fungal extract that resulted in the hydrolysis of starch to glucose. This agrees with similar analysis the reports that most fungi produced glucose as the product of glucoamylase hydrolysis (Adeoyo *et al*., 2018; Gudi *et al*., 2013; Negussie *et al*., 2019). Amylases have been purified from various microorganisms by similar purification processes as followed in this work wherein a three step purification process involving ion-exchange and gel-filtration including ammonium Sulphate precipitation at 80 %. The purification steps in this study were similar to the works of Negi and Banerjee, 2009 and Arpana *et al*., 2013. The specific activity of the enzyme in each step increased, with the specific activity after ammonium precipitation being 0.81 U/mg and after gel filtration the specific activity was 6.83 U/mg, a value lower than the one reported for *Aspergillus awamori* from Tapioca powder by Arpana *et al*., (2013). The purification fold also increased with the different steps of purification which showed that the

enzyme was being freed from impurities with each step of purification. Currently, with expanding application areas, research is primarily focused on the development of new amylases with high pH and temperature stability to achieve enhanced rate of catalysis, improved gelatinization of starch, decreased media viscosity and diminished possibility of microbial contamination (Li *et al*, 2011). It was noted in the enzyme secreted by this organism with maximum activity at a temperature of 50ºC. This value is exactly same with the one obtained by El-Gendy (2012) who reported optimum temperature of 50°C from *Aspergillus sp* JAN 25. A higher activity at a temperature of 60ºC was obtained by *Adefisoye and Sakariyau* (2018) for *Aspergillus niger* while 30ºC was obtained from study *by Zambare* (2010) for *Aspergillus oryzae*. Its thermal-stability at the optimum temperature of 50°C for 15 to 45 min. also conformed with the work of Koç and Metin., 2010 where *Aspergillus flavus* HBF34 was stable at temperatures ranging from 30-50°C though for 1 h and but the isolated enzyme was found to be more active as it retained 100% activity at 50°C. Thermostable glucoamylase are desired as they minimize contamination risk and reduce reaction time, thus saving considerable amount of energy. Also when hydrolysis is carried out at higher temperatures, the polymerization of D-glucose toiso-maltose is minimized (Konsoula and Liakopoulou-Kyriakides, 2007). pH optimum for *Aspergillus flavus* glucoamylase has been reported to be in the acidic to neutral range (Bozic *et al.,* 2011). The pH optimum of 7.0 obtained for *Aspergillus flavus* glucoamylase is in agreement with Koc and Metin, 2010 and Ezugwu *et al*., 2015 who stated in their report that most fungal enzymes function between a pH range of 6 and 8; the exact value of 7.0 was reported by Oyewale, 2012 for *Aspergillus flavus glucoa*mylase strain. It is noteworthy to find out that the *Aspergillus flavus* glucoamylase obeyed Lineweaver-Buck plots. So, the lower concentration of substrate indicated that glucoamylase had a strong affinity for the substrate (Imran *et al*., 2012). The results of the kinetic studies (Fig 8) showed that V_{max} and K_m values obtained for glucoamylase from *Aspergillus flavus* for starch were similar to those obtained from other sources: Kelkar and Desphande. (1993) reported apparent V_{max} and K_m of 8.33U/min and 0.6 mg/ml, for starch for glucoamylase produced by *Sclerotium rolfsii* while 283U/min and 0.25 mg/ml, respectively were reported for glucoamylase from *A. niger* reported by Raiz *et al.,* (2012). The result of the K_m value from this study shows that the *Aspergillus flavus* glucoamylase has an affinity for starch. The study on the effects of some cations in the present study is similar to the ones reported from *Aspergillus awamori* glucoamylase by Arpana *et al.,* (2013). On the contrary, Selvakumar *et al., (*1996) reported that the enzyme was unaffected by Na^+, K^+, Ca^{+2}, $Mg^{2+}$and Glycine, Kareem *et al*., (201) also reported that Mn^{+2}, Ca^{+2}, and Fe^{2+}increased the activity of glucoamylase obtained from *Rhizopus oligosporus* SK5 mutant. On the other hand, mercaptoethanol and urea were found to result in a 90 per cent loss of enzyme activity (Fig 10). In the present study, glucoamylase seems to be a calcium metalloenzyme as Ca^{2+}exerted a strong activating effect on glucoamylase activity. The substrate specificity study involving the use of other starch substrates (Fig 11) showed that the glucoamylase from *Aspergillus flavus* has a preference for cassava starch while it can still use other starchy substrates for its catalytic activity. Pavezzi *et al*. (2014) reported the use of different starchy substrates by glucoamylase. Other researchers have also shown the specificity of glucoamylase for starch from different sources (James and Lee, 1995). Okwuemu *et al*. (2017) also reported the importance of glucoamylase in starch hydrolysis. The high affinity of *Aspergillus flavus* glucoamylase for starch could explain the role of the enzyme in starch hydrolysis.The result obtained from Sephacryl-200 gel filtration was approximately 28 kDa and SDS-PAGE had a single protein band with a molecular weight of approximately 25 kDa, this revealed that the enzyme is a monomeric enzyme. Several works on glucoamylase have revealed that the enzyme could be monomeric or dimeric. The value obtained is lower than the one reported for *Aspergillus niveus* with native and subunit molecular weights of 77 kDa and 76 kDa respectively; it is also lower than the one reported for *Aspergillus niger* glucoamylase with subunit molecular weight of 36 kDa by Gudi *et al*., (2013). But the result obtained from SDS-PAGE of this work is different from the one reported by Jebor *et al.,* (2014) for *Aspergillus niger* glucoamylase as a dimeric enzyme, glucoamylase A was reported to have 66 kDa and glucoamylase B as 52 kDa. In previous studies, molecular weights of glucoamylase from fungi were reported to be in a range of 25 to 112 kDa (Koç and Metin, 2010).

Conclusion

This organism produces a thermostable glucoamylase which could be used in heat stable reaction and can be optimized for use in the industry in production of-sweeteners, glucose syrup among others from starch.

References

Adefisoye, S.A., Sakariyau, A.O.,(2018). Production of glucoamylase by Aspergillus niger in Solid nstate fermentation, *Advances in Biological Researches* 12(1):07-11

Adeoyo O.R., Pletschke B. I. and Dames J. F.(2018) Purification and characterization of anamyloglucosidase from an ericoid mycorrhizal fungus (*Leohumicola incrustata*) *AMB Expr (2018) 8:154* https://doi.org/10.1186/s13568-018-0685-1

Arpana Mittal, Neeraj K Aggarwal, Varun Gupta3 Gulab Singh, Anita Yadav (2013) Purification and Characterization of Amyloglucosidase Produced by *Aspergillus awamori* NA21 under Solid State Fermentation using Tapioca Powder \International peer-reviewed *journal Octa. J. Biosci.* Vol. 1(2): 122-131 ISSN 2321 – 3663

Bernfeld P. (1955).Amylases alpha and beta. *Meth Enzymol.* 1: 149

Bradford, M. M. (1976) A rapid and sensitive method for the quantification of microgramqualities of proteins utilizing the princnple of protein-dye binding. *Anal Biochem.* 72:248-254 ,

Bozic N, Ruiz J, Lopez-santin J, Vujcic Z (2011).Production and properties of thehighlyefficient raw starch digesting α-amylase from a *Bacillus licheniformis* ATCC 9945a. *Biochem. Eng. J.* 53:203-209.

Carrasco M, Alcaíno J, Cifuentes V, Baeza M (2017) Purification and characterization of a novelcold-adapted fungal glucoamylase. *Microb Cell Fact* 16:75.https://doi.org/10.1186/s1293 4-017-0693-x

Crabb WD, Shetty JK (1999) Commodity scale production ofsugars from starches. Curr Opin *Microbiol* 2:252–256

Deshmukh KB, Taur SA, Cherekar MN, Kothari MN, Pathak AP. (2011). Process Optimization,purification and characterization of glucoamylase from different Sorghium varieties. *J. Chem Pharmacol. Res.* 3(2) 732-737.

El-Fallal A., Dobara M.A., El-Sayed A. and Omar N. (2012). Starch and Microbial α- A m y l a se s : F r o m C o n c e p t s t o Biotechnological Applications. Chapter 21 in "Carbohydrates–Comprehensive Studies on Glycobiology and Glycotechnology,http://dx.doi.org/10.5772/51571

El-Gendy MMAA, Alzahrani NH (2020) Solid State Fermentation of Agro-industrial Residuesfor Glucoamylase Production from Endophytic Fungi *Penicillium javanicum* of *Solanumtuberosum* L. *J Microb Biochem Technol.* 12:426. doi: 10.35248/1948-5948.20.12.426

El-Gendy NMA (2012) Production of glucoamylase by marine endophytic Aspergillus sp.JAN 25 under optimized solid state fermentation conditions on Agro-residue .*Australian journal of Basic and Applied science* 6:41-54

Ezugwu, A. L., Eze, S. O. O. and Chilaka, F. C. (2015). A study of the optimal conditions forglucoamylases obtained from Aspergillus niger using amylopectin from cassavastarch as carbon source. *African Journal ofBiotechnology* Vol. 14(36), pp. 2693-2702,

Ghani Maria, Ansari Asma, Haider Muhammad Samee, Asif Tayyaba, Ibrahim Fariba, Qader Shah Ali UI, and Aman Afsheem,(2019). Purification and characterization of a thermostable starch-saccharifying alpha-1,4-Glucanhydrolase produced *by Bacillus licheniformis Starch-Strike* Vol 71 issue 11-12.

Hebeda, R. E. Nagodawithana, T. and Reed G. (1993). "Starches, sugars, and syrups," in Enzymes in Food Processing, pp. 321– 343, 3rd edition, 1993.

Imran, M., Asad, M.J., Gulfraz, M., Mehboob, N., Jabeen, N., Hadri, S.H., *et al*., 2017. Hyperproduction of glucoamylase by Aspergillus niger through the process of chemical mutagensis. *Int. J. Phys. Sci.* 6, 6179–6190.

Ivor M., FeinBerg K. (1965). Paper and thin Layer Chromatography and Electrophoresis ,Ateaching level manual 2nd Edn.,Schadon ScientificCo Ltd., pp 62-65

Jensen B. D. and Norman B. E. (1984). "Bacillus acidopullyticus pullulanase: applications andregulatory aspects for use in food industry," *Process Biochemistry*, vol. 1, pp. 397–400,1984

Karim KMR, Husaini A, Tasnim T (2017) Production and characterization of crude glucoamylase from newly isolated *Aspergillus flavus* NSH9 in liquid culture. *Am J Biochem Mol Biol* 7(3):118–126

Koc, Q. and Metin, K. 2010. Purification and Characterization of a thermostableglucoamylaseproduced by *Aspergillus flavus* HBF34. *Afr. J. Biotechnol. 9(23), 3414-3424*

Kelkar hS & Desphande MV(19930. purification and characterization of pullulan hydrolyzingenzyme from Slerotium rolfsii, *starch/Starke*,45, 361-368

Konsoula and Liakopoulou-Kyriakides (2006). Thermostable α-amylase production by Bacillussubstilis entrapped in calcium alginate gel capsules, *Enzyme and Microbial Technology*39:690-696

Kusuda M, Ueda M, Konishi Y, Matsuzawa K, Shirasaka N, Nakazawa M, Miyalake K, Terashita T (2004) Characterization of extracellular glucoamylase from the ectomycorrhizal mushroom *Lycophyllum shimeji*, *Mycosci*, 45:383-389

Li, H., YunyunGao, W.S., Huang Y.W.L., Huang, E.Z, 2011. Cloning recombinant expression and characterization of a new glucoamylase Aureobasidium pullulans NRRL 12974 andits potential application in raw potato starch degradation. *Afr, J. Biotechnol.* 10 (45)91229131.

Morita H, Fujio Y. (2000) Effect of organic nitrogen sources on raw starch-digestingglucoamylase production of *Rhizopus sp*. MKU 40 *starch*, 52:18-21

Negi S, Banerjee R (2010) Optimization of culture parameters to enhance production of amylaseand protease from Aspergillus awamori in a single fermentation. *African Journal ofBiochemistry Research* 4(3): 73-80.

Negussie F Bussa, Mesfin Moges, Manikandan Muthuswamy, Melese Abdisa (2019). Isolationand Characterization of Amylase Enzyme from Selected Fungal Strains of Wof Washa Forest of North Shoa, *Ethiopia. Sci J Biol & Life Sci.* 1(2): 2019. SJBLS.MS.ID.000506.

Okwuenu P. C., Ezugwu A. L. and Chilaka F. C. (2017) Production and optimization ofAspergillus niger glucoamylase using amylopectin from guinea corn starch as the sole carbon source *Bangladesh J. Sci. Ind. Res.* 52(4), 263-272.

Oyefuga O. H., Adeyanju M. M., Adebawo. O. O. and Agboola F. K.(2011) Purification and some properties of β-amylase from the nodes of sugar cane, Saccharium offinacium *International Journal of Plant Physiology and Biochemistry*Vol. 3(7), pp. 117-124

Oyewale, M. O (2012). Partial Purification of Amylase from the Culture Filtrates of Aspergillus flavus Grown on Cassava Peels,*Fountain Journal of Natural and Applied Sciences*:1(1): 19 - 24

Puri S, Arora M, Sarao L (2013) .Puri S, Arora M, Sarao L. Production and optimization of amylase and glucoamylase using *Aspergillus oryzae* under solid state fermentation.*Int J Res Pur Appl Microbiol*. 3:83-88

Riaz M, Rashid MH, Sawyer L, Akhtar S, Javed MR, Nadeem H (2012) Physiochemical properties and kinetics of glucoamylase produced from deoxyglucose resistant mutant of Aspergillus niger for soluble starch hydrolysis. *Food Chem* 130:24–30

Ramesh V, Murty VR. Optimization of glucoamylase production by *Humicola grisea* MTCC 352 in solid state fermentation *CMU. J Nat Sci*. 2019;18(3):251- 266.

Sarojini R, Deepika K and Rangabhashiyam S (2012), Production, characterization, kinetic studies of glucoamylase through solid-state fermentation by *Aspergillus niger* using agricultural residues as substrate, *International Journal of Current Research* **4:** 198-201.