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Partial purification and some physicochemical properties of *Aspergillus flavus* α-amylase isolated from decomposing cassava peels

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α-Amylase is one of the most important enzymes of great significance due to its wide area of potential application in food, fermentation, textile, paper, detergent and pharmaceutical industries. This study aimed at production of α-amylase from an indigenous fungal source and also ascertaining the properties of the enzyme for maximal activity. The enzyme was isolated from decomposing cassava peels, fractionated at 70% ammonium sulphate and characterized. The fungal isolate was characterized as *Aspergillus flavus.* The crude enzyme extract had a specific activity of 2.40 Umg^{-1} which increased to 7.88 Umg^{-1} on fractionation with ammonium sulphate with a yield of 11.10% and purification fold of 3.28. The K_m and V_{max} values of 0.52±0.009 g/dL and 62.57±0.23 U/min were obtained, respectively, at 2% cassava starch substrate. The enzyme also demonstrated maximum activity at 70°C and pH 5.0. It thus produces α-amylase which is thermostable, a property which could be exploited for industrial purposes where hydrolysis of starch and other complex carbohydrates are required.

Key words: Cassava, α-amylase, starch hydrolysis, *Aspergillus species*, industrial application.

INTRODUCTION

Amylases constitute a class of industrial enzymes representing approximately 30% of the world enzyme production (Calik and Ozdamar, 2001; Hmidet et al., 2008). α-Amylases (endo-1,4-α-D-glucan-4-glucanohydrolase: EC.3.2.1.1) constitute the family of endo-amylases which randomly cleave the 1, 4- α-D-glycosidic bonds between adjacent glucose units in the linear amylose chain retaining α-anomeric configuration in the products (Ramachandran et al., 2004). Although, amylases can be obtained from several sources, such as plants and animals, the enzymes from microbial sources generally meet industrial demand (Pandey et al., 2000). Amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from

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fungal and bacterial sources have dominated applications in industrial sectors (Pandey et al., 2006; Prabakaran et al., 2009; Alariya et al., 2013). It has been reported that fungi have high secretion capacity and are effective hosts for the production of foreign proteins (Tsukagoshi et al., 2001) with many species of *Aspergillus* and *Rhizopus* used as a source of fungal α-amylase (Gupta et al., 2008; Kim et al., 2011; Irfan et al., 2012). This probably could be due to the ubiquitous nature and non-fastidious nutritional requirements of these organisms (Abe et al., 1988). Specifically, starch degrading enzymes like amylases have received a great deal of attention because of their technological significance and economic advantages which include less time and space required for production, ease of process modification and optimization, cost effectiveness and consistency (Burhan et al., 2003).

α-Amylases are ubiquitous hydrolytic enzymes which play significant roles in the utilization of polysaccharides (Reddy et al., 2003). The ubiquitous nature, ease of production and broad spectrum of applications make α-amylase an industrially important enzyme. The production of α-amylase is essential for conversion of starches (an important constituent of the human diet and a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and cassava) into oligosaccharides. This polysaccharide hydrolysis yields products such as glucose, maltose, maltotriose, and α-dextrins (Stryer, 1995; Sajitha et al., 2011) which are of immense industrial use. The enzyme has numerous applications in brewing and fermentation industries for the liquefaction of starch to fermentable sugars; textile industry for resizing of fabrics; food industry for preparation of sweet syrups, to increase diastase content of flour and improve digestibility of some of the animal feed ingredients; paper industry for sizing (Rao and Satyanarayana, 2003; Aiyer, 2005; Sajitha et al., 2011). Others include detergent, pharmaceutical and fine chemical industries (Ashwini et al., 2011). Amylase is also used in the bread-baking process, to increase its volume and keeps its softness longer (Ammar et al., 2002). In addition, they are used in sewage treatment for reducing the disposable solid content of sludge (Parmar et al., 2001).

Meanwhile fungi belonging to the genus *Aspergillus* have been commonly employed for the production of α-amylase as posited that soils around mills and cassava farms after harvesting and treatment of tubers represent media where natural amylolytic activities occur, therefore, amylolytic microorganisms are expected to be present in these areas. Hence, this study was aimed at production and characterization of indigenous α-amylase with highest amylolytic activity from the fungi isolated from decomposing cassava peels.

MATERIALS AND METHODS

Phenylmethane-sulfonyl fluoride (PMSF) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich Co., USA while Dinitrosalicylic Acid (DNSA) was obtained from BDH Chemicals Ltd., Poole England. Potato dextrose agar (PDA) was purchased from Oxoid Ltd. (Basingstoke, England). Other chemicals used were of analytical grade.

Isolation of organism and culture conditions

The fungi were isolated from decomposing cassava peels in a local cassava milling industry in Ikenne, Ogun State and cultured on Potato Dextrose Agar (PDA) incubated at 37°C for 7 days.

Screening of fungal isolates for amylolytic activity

The fungal isolates were sub-cultured on 1% starch-agar, pH 6.5 and incubated at 37°C for 7 days. These isolates were screened for amylolytic activity detected as clear zones after flooding with iodine solution. The fungus with maximum zone of hydrolysis was isolated and saved on a slant to be reactivated prior to use.

Identification of the isolate

The isolated organism was identified based on its morphological characteristics. The pure isolate of the fungus was obtained by repeated sub-culturing and used as inoculum in enzyme secretion.

Enzyme secretion and extraction

Prior to inoculation, the isolated organism on the slant was reactivated by sub-culturing on 1% starch-PDA agar plates and incubated at 37°C for 72 h. Thereafter, the growing culture was used as inoculum in sterile starch broth containing cassava starch (2.0 g/L), KH_2PO_4 (1.4 g/L), NH_4NO_3 (10 g/L), KCl (0.5 g/L), $MgSO_4.7H_2O$ (0.1 g/L), $FeSO_4.7H_2O$ (0.01 g/L) in replicates, pH 6.5 and incubated on a shaker incubator at 150 rpm for 72 h at 37°C. Culture was harvested and cells separated by centrifugation at 8,000 X g for 20 min at 4°C using a high speed cold centrifuge according to Ramachandran et al. (2004). The cell-free supernatant (crude enzyme) was assayed for the α-amylase activity and protein concentration.

Enzyme assay

The amount of reducing sugar liberated by the enzyme was measured using the method of Miller (1959); a modification of Bernfeld method (1955). The reaction mixture consisted of 1.25 ml pre-gelatinised 1% (w/v) cassava starch, 0.25 ml, 0.1 M sodium acetate buffer (pH 5.0), 0.25 ml of distilled water and 0.25 ml of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by adding 1.0 ml of 3, 5-dinitrosalicylic acid reagent followed by heating in a boiling water bath for 10 min and cooling at room temperature. The concentration of reducing sugar released was measured at 540 nm using glucose as the standard. One unit of α-amylase was defined as the amount of enzyme that releases one milligram of glucose under the assay conditions and expressed as:

$$\text{Alpha-amylase activity (U)} = \frac{\text{mg of glucose released} \times \text{dilution fold}}{\text{Time of incubation} \times \text{mg of enzyme in reaction mixture}}$$

Protein concentration determination

The protein concentration of the enzyme was determined using

Table 1. Purification table of *Aspergillus flavus* α-amylase from decomposing cassava peels.

Step	Fraction	Volume (ml)	Total Protein (mg)	Total activity (U)	Specific activity (U/mg)	Yield (%)	Purification fold
1	Crude extract	760.00	3120.00	7491.20	2.40	100.00	1.00
2	70% Ammonium sulphate fractionation	16.00	105.60	832.40	7.88	11.11	3.28
3	Dialysate	25.00	67.50	2447.55	36.26	32.67	15.64

Biuret method (Gornall et al, 1949) using bovine serum albumin (BSA) as standard. The protein concentration of each purification step was then extrapolated from the standard protein calibration curve.

Ammonium sulphate fractionation

The crude enzyme was brought to 70% ammonium sulphate saturation by adding slowly, followed by gentle stirring of the solid ammonium sulphate (472 g/L) in an ice cold environment. The stirring continued occasionally until all the salt had been dissolved. The mixture was then allowed to stand overnight at 4°C. The precipitated protein was recovered by centrifugation at 8,000 X *g* at 4°C for 20 min using a high speed cold centrifuge. The supernatant resulting from the 70% ammonium sulphate fractionation was discarded while the precipitate was kept and re-suspended in a minimal volume of 10% ammonium sulphate in 0.01 M Sodium acetate buffer. The enzyme activity and protein concentration were also determined.

Effects of pH

The optimum pH of the enzyme was determined by the measurement of activity of the partially purified enzyme at different pH values ranging from 4.0 to 9.0 using three buffer system comprising: 0.1 M sodium acetate buffer at pH 4.0 to 5.0, sodium phosphate buffer (pH 6.0 to 8.0), and Tris-HCl buffer (pH 9.0) while assay was carried out routinely.

Effect of temperature

Optimum temperature of the enzyme was determined by routine measurement of the activity of the enzyme by incubating at temperatures between 40 to 100°C for 10 min.

Determination of kinetic parameters

The kinetic constants K_m and V_{max} were determined using aqueous 2% cassava (pre-gelatinised) starch solution as substrate. The reaction mixture contained 1.25 ml of the substrate, 0.25 ml of appropriately diluted enzyme solution, 0.10 ml of 80 mM $CaCl_2$ and 0.25 ml of 0.10 M sodium acetate buffer. 0.40 ml of distilled water was added to the reaction mixture and incubated at 50°C for 10 min after which 1.0 ml of dinitrosalicylic acid (DNSA) was added and heated at 100°C for 10 min. On cooling, the absorbance was read at 540 nm.

RESULTS

Morphological identification of the Isolate

Four fungi were observed after culturing the sample and the one with the highest amylolytic activity, that is, the fungus having largest zone of hydrolysis on flooding with iodine was isolated and morphologically characterized. The colonies of the pure strain of the fungus showed pale-yellowish colouration; centrally rising with close textured velvety and regular margins. This fungus was identified as *A. flavus* and used for further studies.

Enzyme purification

The purification table of the *A. flavus* α-amylase is shown in Table 1. The total protein concentration and activity of the crude extract were 3120.0 and 7491.20 mg while on fractionation at 70% ammonium sulphate it gave 105.60 mg of total protein concentration and activity of 832.40 U. The result of varying substrate concentration with reaction velocity resulted in hyperbolic curve and the kinetic constants (K_m and V_{max}) were determined from the Line weaver-Burk's plot (Figure 1). The maximum velocity, V_{max} obtained was 62.57±0.23 U/min while the Michaelis-Menten constant, K_m was 0.52±0.009 g/dL. The optimal temperature of 70°C and pH 5.0 were obtained for the *A. flavus* α-amylase (Figures 2 and 3), respectively.

DISCUSSION

Fungi are known to utilize polysaccharide substrates through extracellular digestion catalyzed by secreted enzymes. This high capacity secretory system has driven the exploitation of filamentous fungi as cell factories for provision of enzymes used in a wide variety of applications (Archer, 2000). *A. flavus*, a filamentous fungus isolated from decomposing cassava peels was identified in this study, an indication that the organism was able to utilize starch, a naturally occurring glucose polymer in plants, as food. This genus has been reported to produce a large variety of extracellular enzymes with amylases being the most significant and of industrial importance (Hernández et al., 2006) which are known to produce considerable quantities of enzymes that are used extensively in the industry. Specifically, *A. flavus* had been reported to secrete α-amylase (Ali and Abdel-Moneim, 1989). Abou-Zeid (1997), isolated filamentous fungi from cereals and screened to test the alpha-amylase producing potential. The strain which showed

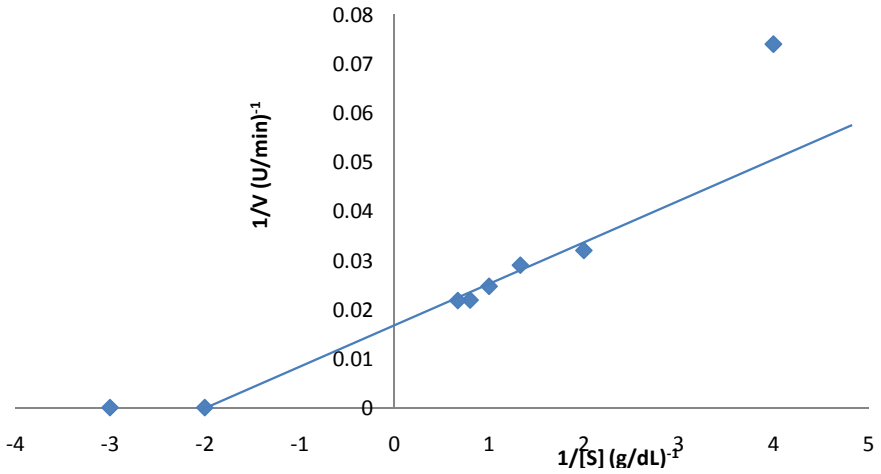

Figure 1. Lineweaver-Burk's plot of the kinetic parameters of the *Aspergillus flavus* α-amylase.

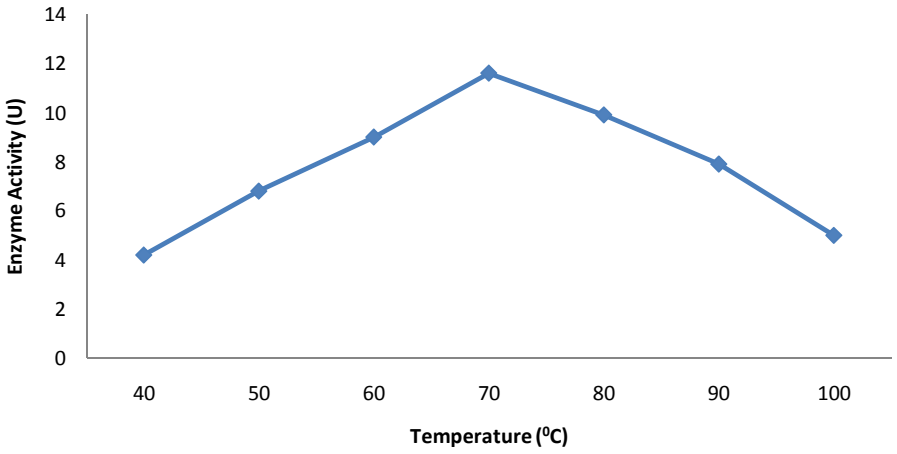

Figure 2. Temperature profile of the *Aspergillus flavus* α-amylase.

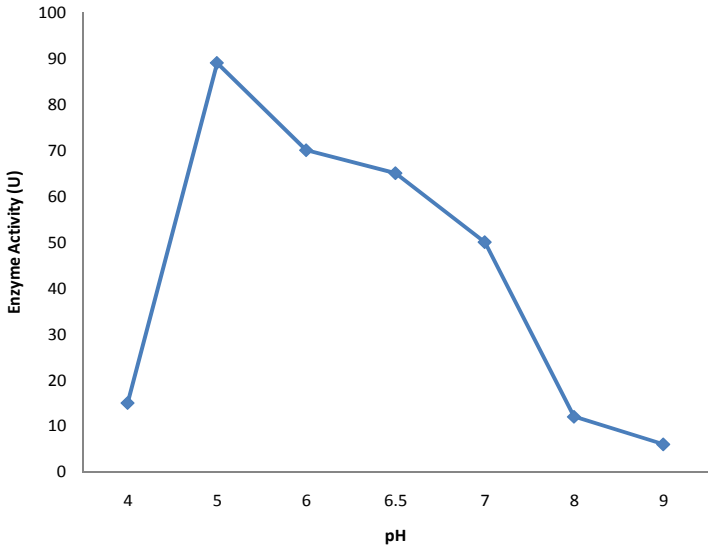

Figure 3. pH profile of the *Aspergillus flavus* α-amylase.

highest ability for α-amylase production was identified as *A. flavus*. Likewise, a species of the genus *Aspergillus oryzae* has received increased attention as being a favourable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, for example α-amylase (Jin et al., 1998). Different investigators have reported differing specific activity values of the enzyme isolated from various sources: *A. oryzae* from mangrove associated fungi (2.29±0.03 µmol/min/mg; Joel and Bhimba, 2012) and *A. oryzae* from waste water (8.77 IU/mg; Shah et al., 2014). The specific activity of 7.88 U/mg obtained at 70% ammonium sulphate fractionation in this work is not all that different from value reported (Shah et al., 2014), though on dialysis it increased to 36.26 U/mg which reflected progress of the purification procedure. It is pertinent to mention that *A. flavus* α-amylase in this study obeyed Michaelis-Menten kinetics as evident in the Line weaver-Burk's plot (Figure 1), a pattern also corroborated by Shah et al. (2014) who also reported the K_m value of 1.4 mg/ml, a value lower than 0.52±0.009 g/dL obtained in study though substrate concentration also differs. The K_m value was known as the criterion for the affinity of enzymes to substrates, and lower value of K_m represents higher affinity between enzymes and substrates (Shuler and Kargi, 2002). The reverse was the case for V_{max} where it was 62.57±0.23 U/min in this study and 46.56 IU/mL by same researchers.

The optimum temperature of 70°C obtained in this study for the *A. flavus* α-amylase is higher than the optimum temperature of 30°C reported for the same enzyme sourced from cereal (Abou-Zeid, 1997); 40°C for *Rhizobium s*train (Oliveira et al., 2010) and temperature range between 50 to 55°C for the thermophilic fungal cultures such as *Talaromyces emersonii*, *Thermomonospora fusca* and *Thermomyces lanuginosus* (Jensen and Olsen, 1992; Bunni et al., 1989) and 50°C for *Aspergillus niger* α-amylase isolated from potato (Siddique et al., 2014). Thus, the strain of the fungus isolated in this study is one of very few with this thermophilic property producing the thermostable α-amylase. pH is another factor that determines the growth and morphology of the microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Earlier studies have revealed that fungi required slightly acidic pH for optimum growth (Sivaramakrishnan et al., 2006). pH is known to affect the synthesis and secretion of alpha-amylase just like its stability (Fogarty, 1983). Fungi of *Aspergillus sp*. such as *A. oryzae, Aspergillus ficuum* and *A. niger* were found to give significant yields of α-amylase at pH 5.0 to 6.0 in submerged fermentations (Moller et al., 2004); Knox et al., 2004). Therefore, the pH optimum 5.0 obtained in this work compares well with the works of Ali and Abdel-Moneim (1989); Sudo et al. (1994) and Odibo and Ulbrich-Hofmann (2001) who reported optimum pH of 5.0 for alpha-amylase from *A. flavus var. columnaris, Aspergillus kawachi* IFO 4308 and *T. lanuginosus* respectively which indicated that the organism prefer acidic condition for better enzyme production.

Conclusion

The organism therefore is regarded as a viable local strain since it secreted α-amylase with thermostability property at 70°C. This could therefore be exploited for use in liquefaction of starch and or other industrial purposes where hydrolysis of starch and other complex carbohydrates are required.

Conflict of Interests

The author(s) have not declared any conflict of interests.

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