Journal of Bioscience and Biotechnology Discovery
Volume 8(2), pages 57-65, April 2023
Article Number: 9CB3731F6
ISSN: 2536-7064
https://doi.org/10.31248/JBBD2023.183
https://integrityresjournals.org/journal/JBBD
Full Length Research

Biochemical properties of α-amylase isolated from *Aspergillus tamarii from cassava starch* using solid state fermentation

Opeyemi Olumuyiwa Otuewu[1]*, Sherifat Tolulope Akindele[1], Solomon Aderemi Akapo[1], Abdulrasak Alao Abdulkareem[1], Temitayo Adewale Adefuwa[2] and Moronke Muinat Adeyanju[2]

[1]Department of Science Laboratory Technology, School of Science, Abraham Adesanya Polytechnic, Ijebu-Igbo, Nigeria.
[2]Department of Biochemistry, Faculty of Basic Medical Sciences, Obafemi Awolowo College of Health Sciences, Olabisi Onabanjo University, Sagamu, Nigeria.

*Corresponding author. Email: opotuewu@gmail.com

Received 26th March 2023; Accepted 29th April 2023

ABSTRACT: Cassava starch can be completely hydrolyzed to produce a sweetener that can be used as a substitute for refined sugars and artificial sweeteners in the food and pharmaceutical industries. This work was designed to isolate and identify a good amylase producing fungus from cassava flour and to extract, purify, and partially characterize α-amylase produced. The enzyme was produced through solid-state fermentation followed by 70% ammonium sulphate precipitation and ion-exchange chromatography on Carboxyl-Methyl (CM) Sephadex C25. The physicochemical properties of the purified enzyme were determined. The peak with the highest activity was pooled from the latter chromatographic step and characterized afterward. The enzyme's specific activity rose from 0.11 to 2.1 U/mg having a yield of 15.8% and a purification fold of 19.1. The optimal pH and temperature of the enzyme were 6.0 and 50°C respectively. The enzyme was observed to be thermo-stable at 50°C for 15 to 30 minutes. The kinetics revealed that the V_{max} was 1.25 U/min while K_m was 0.2 mg/ml. The enzyme's native and sub-unit molecular weights were found to be 22 and 18.5KDa respectively. The results revealed conclusively that the isolated enzyme from *Aspergillus tamarii* exhibited the properties of glucoamylase.

Keywords: α-Amylase, *Aspergillus tamari*, cassava flour, ion-exchange chromatography, sweeteners.

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is one of the most popular and widely consumed food crops in Nigeria; serve as a cornerstone of food security in most developing countries in Africa. Nigeria is one of the world's leading cassava producers (Aisien and Igbinosa, 2019; Kayode, 2016). Cassava is the second most important starch source in the world (Hwang *et al*., 2014). Starch is a polymer of glucose linked by the glycosidic bond, amylose and amylopectin are the two types of glucose polymers present in starch. Amylose is a straight chain polymer containing up to 6000 glucose units with α-1,4 glycosidic linkage, while amylopectin contain little α-1,4 linked to straight chains of 10-60 glucose units and about 15 to 45 glucose units linked with α-1,6 linkage to side chains (de Souza and Magalhães, 2010). Amylase enzyme has received a great recognition due to their economic and technological importance therefore, isolating novel microbes capable of producing them could provide potential new sources of the enzyme (Asrat and Girma, 2018; Aullybux and Puchooa, 2013). Amylase is a class of industrial enzymes that accounts for about 30% of the world enzyme production (Adeyanju *et al*., 2014). The name amylase is the generic name given to enzymes hydrolyzing starch. Amylases act by hydrolyzing the linear α-1, 4 and branched α-1, 6 glycsidic bonds between adjacent glucose units of the polymer (Ominyi *et al*., 2013). The family of amylase consist of three major groups, which are α-amylase (EC 3.2.1.1), β-amylase (EC 3.2.1.2), and

glucoamylase (EC 3.2.1.3). Alpha amylase (α-1, 4-D-glucan glucanohydrolase EC 3.2.1.1) is an enzyme that digest starch randomly by cleaving α- 1, 4-D- glycosidic bond in starch molecules releasing short chain oligosaccharides and alpha limit dextrin, in addition to maltotriose and maltose. Neither terminal glucose residues nor α-1, 6 linkage can be cleaved by α-amylase (Akhter *et al*., 2017). Due to their significant relevance in today's biotechnology, they are among the most sought after enzymes. Amylases differs in molecular mass; ranging from about 10–210 kDa (Deepika and Satyanarayana, 2018). The molecular weights of some organisms' amylases are raised by presence of carbohydrate moieties in the amylase (Saranraj and Stella, 2013). Alpha-amylase have wide applications in starch processing, brewing, detergent, textile, pharmaceutical and among other industries (Metin *et al*., 2010; Siddique *et al*., 2014). Although, α-Amylases can be obtained from almost all living organisms, most modern biotechnological purposes are basically of microbial sources especially bacteria and fungi (Akhter *et al*., 2017). The major significant of using fungi for the production of amylase is the capacity to produce pure strain in large quantity economically (Asrat and Girma, 2018; Schallmey *et al*., 2004). Solid State Fermentation (SSF) is the growth of microorganisms in the absence of free water on solid material. It has been used for the production of enzymes, organic acids, antibiotics, alkaloids, aroma compounds etc. Solid materials, rich in protein nutrients that are cheaply available have been used in SSF for microbial production of α–amylase. Such materials are agro-industrial cassava, bagasse, coffee, husk, coconut oil cake, rice bran, sugarcane bagasse, sweet sorghum, tea waste, wheat bran and yam bean. Solid State Fermentation holds tremendous potentials for the production of crude enzymes (Akhter *et al*., 2017). This work is therefore aimed at isolating and identifying a good amylase producing fungus from cassava and extracting, purifying and partially characterizing the alpha amylase.

MATERIALS AND METHODS

Collection of materials

The organism (fungus) used was isolated from the cassava flour purchased from Atikori market in Ijebu-Igbo Ogun State, Nigeria and taxonomically characterized at the Microbiology Laboratory of Abraham Adesanya Polytechnic. Standard glucose was purchased from Merck (Germany). 3, 5-dinitrosalicylic acid and sodium hydroxide were bought from Sigma Aldrich Chemie GmbH (Germany). Rochelle salt (potassium sodium tartrate) was obtained from Fluka (Switzerland). All the preparations were done with the use of glass distilled water. All media and other chemicals used were of analytical grade.

Sample preparation and collection

Cassava flour was purchased from Atikori market in Ijebu-Igbo and taken to the laboratory in a sterile container. Thereafter, the moistening of the cassava four with water was done and stored at room temperature (28±2°C) until spoilage sets in. During the spoilage, the samples were observed for change in colour, firmness, texture, odour and appearance of moulds. Portions of samples showing signs of spoilage were taken and used as sources for the isolation of amylase producing moulds.

Isolation of fungi

The homogenization of 1g of cassava flour sample was done in 9 ml of sterile distilled water. Then 1 ml of the homogenate was serially diluted to 10^{-5}, 0.1 ml 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 120 h. 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 (Chay *et al*., 2017) and microscopic examination was compared with the standard Atlas. All the mould isolates was stored on SDA slants at 4°C until required.

Primary screening of amylase producing-fungi

The starch hydrolyzing ability of the fungal isolates was done by inoculating them on 1% starch containing SDA Plate. The plates were flooded with iodine solution after 3 days of fungal growth. 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 indicate starch hydrolysis (Aneja, 1996). 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 clear zone was identified by the characteristic growth of the fungi isolate, which are texture of the colony, pigment and the shape of the colony by 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 mold (Chay *et al*., 2017).

Enzyme production

Aspergillus tamari was used to carried out solid state fermentation using the isolate for α-amylase production. The enzyme production was done using mineral media as described by Bagheri et al. (2014), by adjusting the pH to 6.5 before sterilization. Mineral media (50 mL) were prepared in 250 mL Erlenmeyer flask. The fungal isolate spore disc was inoculated with sterilized 8 mm size cork borer into 250 ml Erlenmeyer flasks containing 50 ml production medium followed by incubation at 28°C for 168 h without shaker. The supernatant was collected by agitating the flask in shaker at 180 rpm for 1 h, the mixture was filtered through Whatman No. 1 filter paper and centrifuged at 10000 rpm at 4°C for 5 min and the supernatant was carefully collected and stored under refrigerated conditions less than 4°C as the crude enzyme for further analysis after adding Phenyl Methyl Fluoride (PMSF) solution.

Determination of α-amylase activity and protein concentration

Amylase activity was determined using method described by Bernfeld (1955). The absorbance was measured at 540 nm by spectrophotometer. The concentration of the enzyme produced and kinetics were evaluated against a standard amylase enzyme. One unit of enzyme activity was defined as the amount that released one microgram of reducing sugar as maltose per minute under the same conditions as for the assay described and calculated as shown below while the protein concentration was determined by the method of Bradford *et al*. (1976) using bovine serum albumin as standard.

$$\mathrm{EA} = \frac{\mu\text{g of glucose released x Dilution factor}}{\mu\text{g of enzyme in reaction x time of incubation}}$$

Where EA = Enzyme activities in U/min/

Enzyme purification

Ammonium sulphate fractionation

The crude enzyme was brought to 70 percent ammonium sulphate saturation; 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°C. The precipitated protein was recovered by centrifugation at 4,000 rpm at 4°C for 10 minutes. The precipitate was reconstituted with 50 mM Sodium Phosphate buffer, pH 7.5, containing 10 percent ammonium sulphate and 1% phenyl–methyl-sulfonyl-fluoride (PMSF), a protease inhibitor. The supernatant resulting from the fractionation was discarded as no protein was detected in it and the precipitate was saved and stored below 4°C for further analysis.

Dialysis

The dialysis tubing was pre-treated by boiling in water bath until it softened. 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 h. The dialysate was saved and stored at 4°C with the addition 1% PMSF.

Ion–exchange chromatography on CM Sephadex C25

About 30 g of CM Sephadex C25 powder was pretreated according to Whatmann Product Instruction Manual. Then 100 ml of 0.05 M HCl was added to the resin and allowed to stand for 30 min 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 × 40 cm) 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/h with a 250 mL linear salt gradient (0.00 - 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 saturation.

Characterization of enzyme

Effect of temperature on the activities of the enzyme and the thermostability studies

The effect of temperature on the activity of enzyme was investigated at different temperature values ranging from 40 to 90°C. The substrate was first incubated at the indicated temperature for 10 min before the reaction was initiated by the addition of 0.10 ml of the enzyme preparation that had also been equilibrated at the same

Table 1. Summary of the purification of *Aspergillus tamarii*α-amylase.

Purification step	Total protein (mg)	Total Activity (U)	Specific activity (U/mg)	Purification fold	% Yield
Crude	10660	1142.8	0.11	1	100
Dialysate	233	270	1.2	10.9	24
Ion Exchange	85	180	2.1	19.1	15.8

temperature. The thermal stability of the enzyme was also determined at 50°C by incubating for 45 min while aliquots were withdrawn at 5 min time intervals for enzyme assay.

Determination of optimum pH

Optimum pH of the enzyme was determined by measurement of the activity of the enzyme at different pH values ranging from 4.0 to 9.0 using four buffer systems comprising sodium acetate (3.5-4.5), citrate phosphate (5.5), phosphate (6.5-7.5) and Tris HCl (7.5-9.0) buffers. Each buffer solution was used to prepare the 1% soluble starch solution used as substrate in assaying the enzyme.

Effect of metal salts and other compounds on alpha-amylase enzyme activity

The effect of metal salts in the enzyme was determined by adding 1 ml of enzyme +1 ml of metal salt concentration (NaCl, KCl, $SnCl_2$, $ZnCl_2$, $MnCl_2$, $HgCl_2$.) 1 ml of phosphate buffer (pH 7.5) incubated it at 50°C. The assay for glucoamylase activity of each tube was performed and measured absorbance at 550 nm. The effect of some compounds on the activity of the glucoamylase from the fungi was determined. The compounds (urea and EDTA) at concentrations between 1.0 mM and 5 mM were used in a typical glucoamylase assay mixture. The reaction mixture without the salts was taken as control with 100% activity.

Determination of kinetic parameters

The kinetic constants Km and V_{max} of the enzyme was determined using 1.0 mL varying concentration of cassava starch (as substrate) from 2 to 20% in 50 mM sodium phosphate buffer (pH 7.5) following the method described by Metin *et al*. (2014).

Determination of native molecular weight

The native molecular weight of the enzyme was determined using gel filtration on Bio-Gel P-100 column. The protein markers used were; Bovine Serum Albumin (mw 66 kDa), Ovalbumin (mw 43 kDa), Carbonic Anhydrase (mw 29 kDa), Trypsin inhibitor (mw 20 kDa) and Lysozyme (mw 14.5 kDa). The void volume was determined using blue dextran.

Determination of subunit molecular weight

The molecular weight of the enzyme was determined using SDS-PAGE (10%) (Weber and Osborn, 1975). As a molecular weight marker, protein mixture SDS6H2 (SIGMA) containing bovine serum albumin (66,000 Da), ovalbumin (45,000 Da), Pepsin (35,000 Da), trypsin (24,000 Da) and lysozyme (14,000 Da), was used and the bands were detected by staining with Coomassie brilliant blue R-250.

RESULTS

Four fungal isolates were observed after culturing the sample, the fungus with the largest zone of hydrolysis on flooding with iodine was taking as the fungi with highest amylolytic activity 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. tamarii* and used for further studies.

Enzyme purification

The results of the purification procedures were summarized in Table 1 yielding α-amylase with specific activity of 2.1 Umg^{-1} of protein with a yield of 46.32%. The elution profile of the dialysate on CM Sephadex C25 column (Figure 1) showed one peak of amylase activity which was (pooled from Fraction number 8 to 14).

Properties of the purified α-amylase isolated from decomposing cassava flour

The maximum α-amylase activity was obtained at 50°C. A reduction in enzyme activity was observed at values above 50°C (Figure 2) after this temperature, the activity was decreased drastically and enzyme was completely

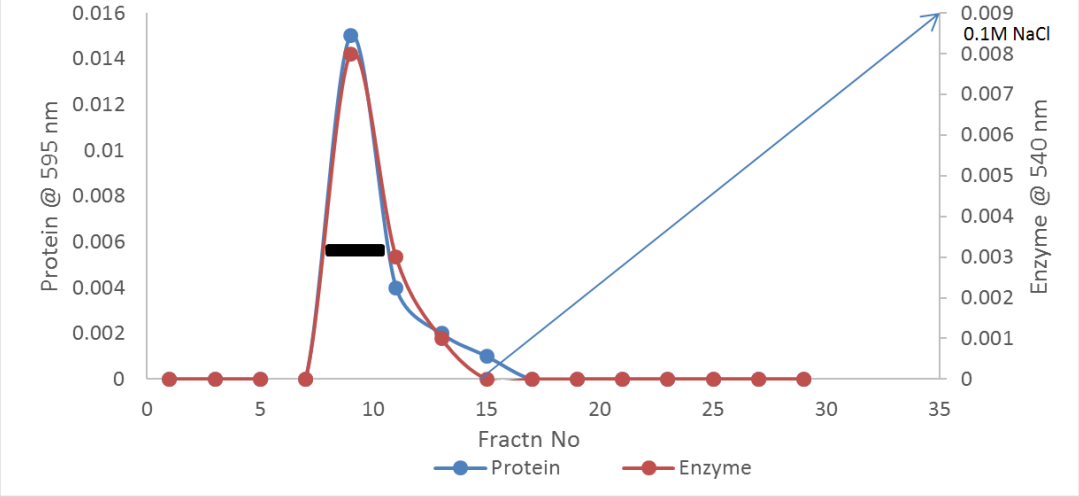

Figure 1. CM Sephadex ion-exchange chromatography elution profile of the *Aspergillus tamari* α-amylase obtained from cassava.

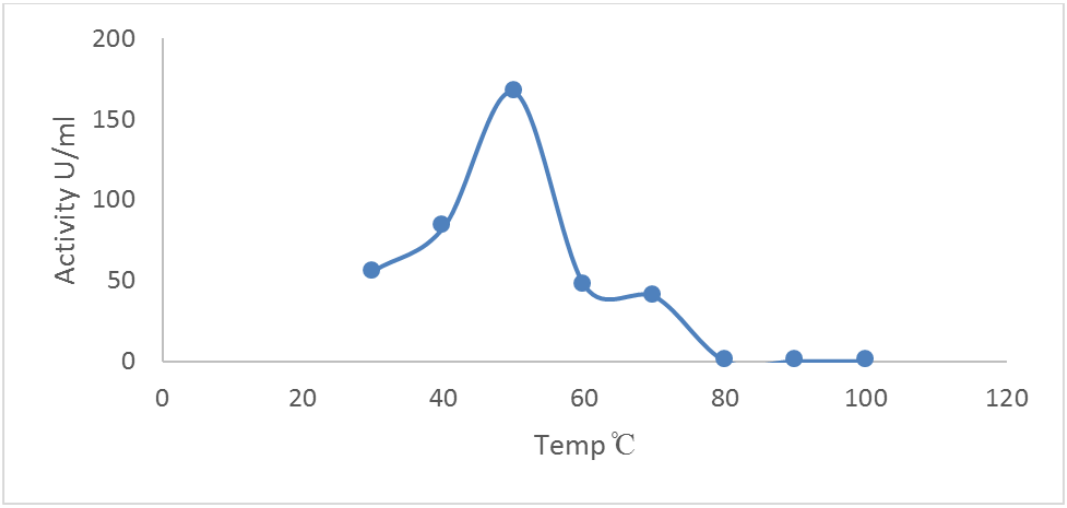

Figure 2. The temperature profile of the purified *Aspergillus tamarii* α-amylase isolated from fermenting cassava.

inactivated. The stability of α-amylase to temperature is shown in Figure 3. α-amylase was observed to be stable at 50°C for 25 to 30 min. A sharp decrease in stability was observed as the incubation time increased beyond 30 min. The optimum pH of α-amylase activity was 6.0 (Figure 4). The activity of the enzyme was greatly inhibited by metals at all concentration except for Potasium (K) that stimulated the enzyme at all concentration and Sodium at 1 mM as shown in Figure 5. The activity of the enzyme was greatly inhibited by urea at all concentration except for EDTA that stimulated the enzyme at all concentration as shown in Figure 6. Figure 7 shows the graph of velocity versus substrate concentration from which the K_m and V_{max} were extrapolated. The native molecular weight of the partially purified enzyme is shown in Figure 8.

DISCUSSION

The recent interest and needs of enzymes with special properties are enormous in many industries; this has led to discovery of different type of amylases with unique properties. The properties with respect to specificity of the amylases determine its application (Ashwini *et al*., 2011). Amylases from microbial sources, especially fungi (*Aspergillus spp.*), have gained much attention because of

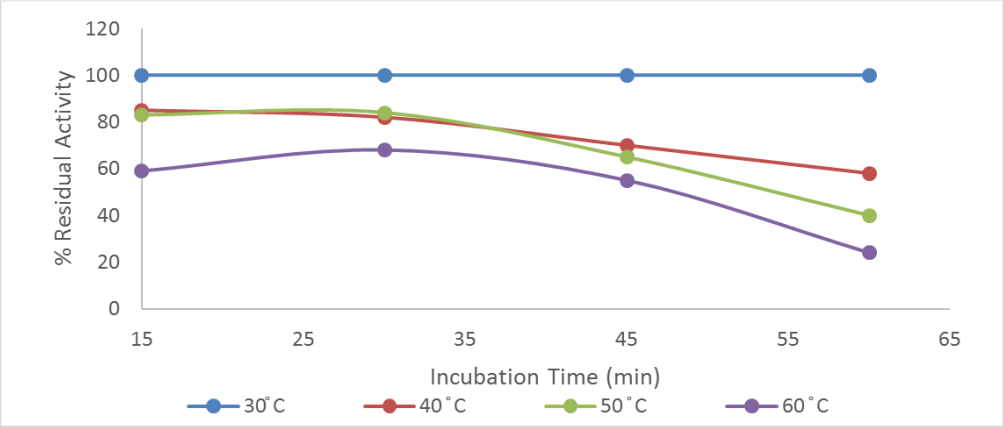

Figure 3. The thermal stability curves of the purified *Aspergillus tamarii* α-amylase isolated from fermenting cassava starch.

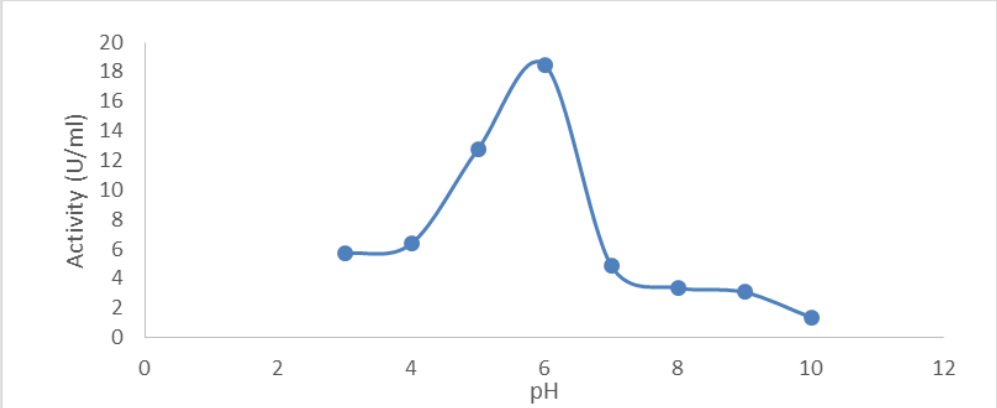

Figure 4. The ph profile of the purified *Aspergillus tamari* α-amylase isolated from fermenting cassava.

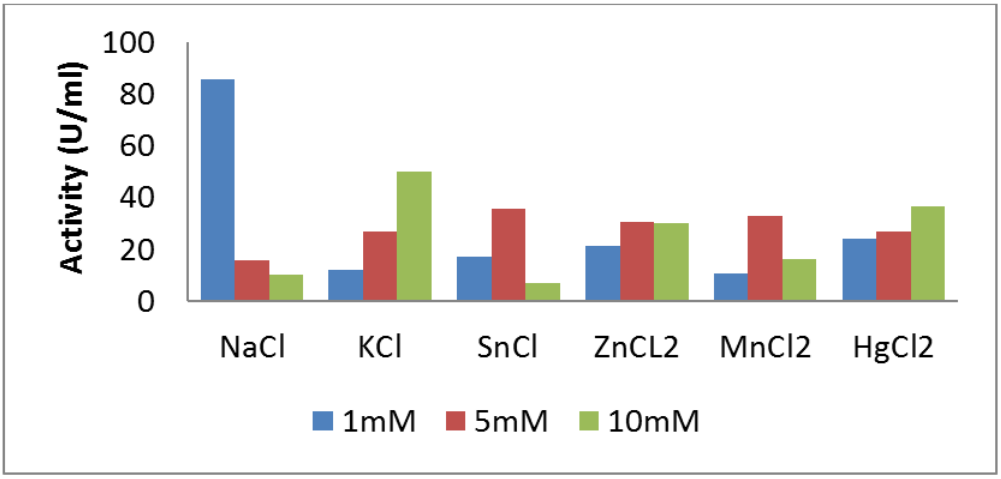

Figure 5. Effect of metals on the purified *Aspergillus tamari* α-amylase isolated from fermenting cassava.

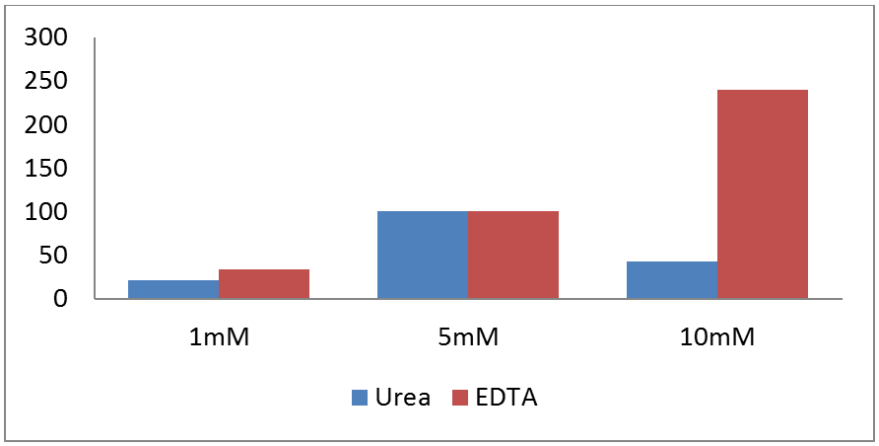

Figure 6. Effect of other compounds of the purified *Aspergillus tamari* α-amylase isolated from fermenting cassava.

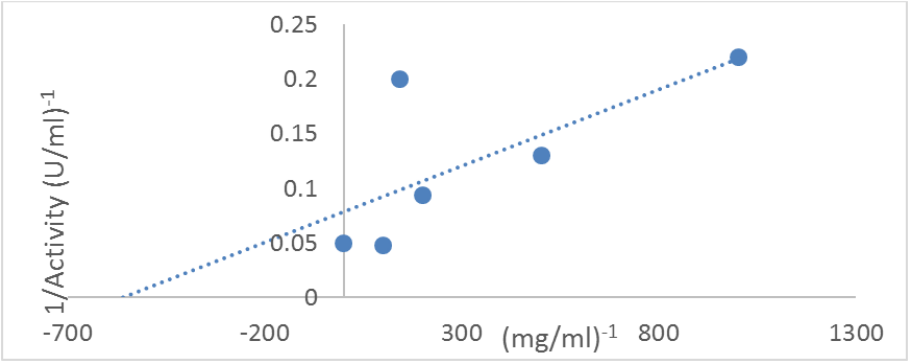

Figure 7. Lineweaver-burk plot for the determination of kinetic parameters. The concentration of α-amylase used varied between 0.1-1.0mg/ml.The lines of best fit were through the points using regression analysis.

the availability and high productivity of fungi, which are also amenable to genetic manipulation (Karlström *et al*., 2016; Moreira *et al*., 1999; Moreira *et al*., 2001). In this research, we were able to isolate *Aspergillus* species having the highest hydrolyzing activity thriving on the decomposing cassava flour, as fungi belonging to the genus *Aspergillus* represent the most important strains for the industrial production of secreted proteins (Schallmey *et al*., 2004). The back side of the colony was pale yellow in colour and microscopic feature showed septate hyphae and conidiophore was attached to the septate. According to these morphological features, this isolate was tentatively *Aspergillus tamarii*. The fungal amylase mainly produced spot similar to the standard maltose and glucose, this showed that the enzyme isolated was α-amylase. This indicated that the hydrolysis of starch by the fungal extract to glucose and maltose indicated that there is α-amylase in the extract. This agrees with similar analysis reporting that most fungi produced glucose and maltose as the product of α-amylase hydrolysis (Adeyanju *et al*., 2012; Mesfin *et al.,* 2019).

Aspergillus has been reported to survive harsh environmental condition and strive at temperature higher than the usual; a property also noted in the enzyme secreted by this organism with maximum activity at a temperature of 50°C. This value is exactly same with the one obtained by Siddique *et al*. (2014), who reported *A. niger* amylase isolated from potato to have 50°C optimum temperature. Lower temperatures of 30°C was obtained by Sidkey *et al*. (2011), while 70°C was obtained from a study for *A. flavus* isolated from cassava peels (Adeyanju *et al*., 2012). The thermostablity at 50°C for 25 to 30 min as the optimum temperature also conformed to the work of Hwang *et al*. (2013) where thermophilic *Streptomyces avermitilis* was stable at temperatures ranging from 20-50°C though for 1 h, however, the isolated enzyme was found to be more active as it retained 100% activity at 50°C. This is unlike the *A. oryzae* α-amylase from soil that retained almost 50% of

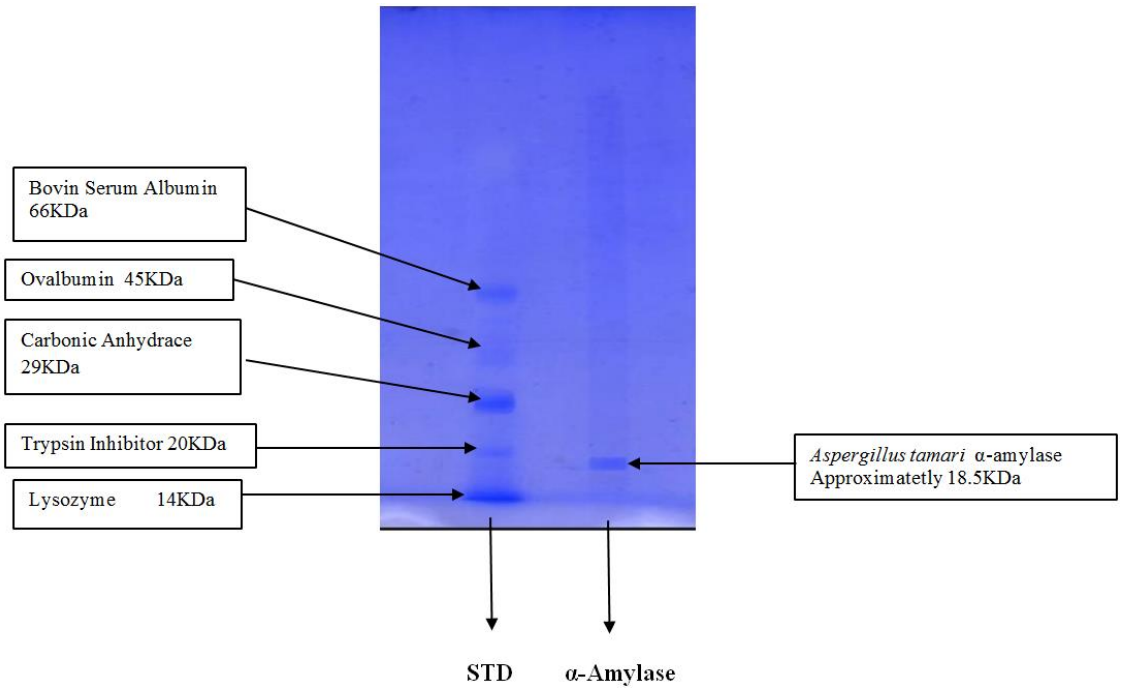

Figure 8. Polyacrylamide gel electrophoresis of *Aspergillus tamari* α-amylase in the presence of SDS.

its maximum activity at 50°C (Shah *et al*., 2014). The risk of contamination and reaction time are minimized by thermostable α-Amylases, thus they are desired to save energy and minimized polymerization of D-glucose to iso-maltose since polymerization is carried out at lower temperatures (Konsoula and Liakopoulou-Kyriakides, 2007). pH optimum for *A. tamarii* α-amylase was discovered to be 6.0. This is in agreement with the work reported by Oboh (2005). It is also similar to pH optimum of 6.4 obtained for *A. flavus* α-amylase reported by Sidkey *et al*., 2011. The results of the kinetic studies (Figure 3) showed the V_{max} and K_m values obtained for α-amylase. The V_{max} values was 1.25 U/min while K_m 0.2 mg/ml at 50°C and pH 6.0 with 0.2 M phosphate buffer. Higher V_{max} and lower K_m has confirmed the efficiency of this enzyme for diverse applications. This Km value is similar to Km value (0.5 mg/ml) of α-amylase from *Aspergillus flavus (*Adeyanju e*t al*., 2012). On the other hand, V_{max} of amylase for starch by *Aspergillus awamori:* nakazawa MTCC 6652 was calculated as 56.18 mg/ml/min and K_m as 9.79 mg/ml (Mesfin *et al*., 2019). The result of the K_m value from the current study shows that the *Aspergillus tammarii* alpha-amylase has an affinity for starch. In spite the differences of microbial alpha-amylases properties, there are usually similarities in their molecular weights which ranges from 10 - 120 KDa (Deepika and Satyanarayana, 2018; Koç and Metin, 2010). Therefore, the native and sub unit molecular weight of 22 and 18.5KDa respectively obtained falls within this range, this shows that the enzyme is a monomeric enzyme.

Conclusion

This organism produces a thermostable α-amylase which could be used in heat stable reaction and can be optimized for use for the industry in production of malt syrup, sweeteners, glucose syrup among others, from starch. The α-amylase in this study is a potential hydrolyzing enzyme for use in starch hydrolyzing industries. The α-amylase isolated may be genetically modified to a thermophilic variant because the α-amylase isolated is mesophilic and in the manufacturing process of starch the raw material is gelatinized at high temperature before the process of hydrolysis commence.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

REFERENCES

Adeyanju, M. M., Ojewunmi, O., Akande, T., Ezima, E. N., Akeredolu, A. A., & Fagbohunka, B. S. (2014). Partial purification and some physicochemical properties of Aspergillus flavus α-amylase isolated from decomposing cassava peels. *African Journal of Biotechnology*, *13*(52), 4657-4662.

Aisien, E. T., & Igbinosa, I. H. (2019). Production, purification, and characterization of α-amylase from Aspergillus niger, *Aspergillus flavus* and *Penicillium expansum* using cassava

peels as substrate. *Nigerian Journal of Biotechnology*, *36*(2), 114-126.

Akhter, P., Mishra, A., Mishra, V., & Raghav, A. (2017). Alpha amylase production from *Aspergillus terreus* UF39 using oil cakes, *European Journal of Biotechnology and Bioscience, 5(*6) 15-,21.

Aneja, K. R. (1996). *Experiments in microbiology, plant pathology, tissue culture & mush room cultivation* (2nd edition). Wishwa Prakashan New Delhi. p. 451.

Ashwini, K., Gaurav, K., Karthik, L., & Bhaskara Rao, K. V. (2011). Optimization, production and partial purification of extracellular α-amylase from *Bacillus sp. marini*. *Archives of Applied Science Research, 3*(1), 33-42.

Asrat, B., & Girma, A. (2018). Isolation, production and characterization of amylase enzyme using the isolate Aspergillus niger FAB-211. *International Journal of Biotechnology and Molecular Biology Research*, *9*(2), 7-14.

Aullybux, A. A., & Puchooa, D. (2013). α-Amylase production on low-cost substrates by Naxibacter sp. isolated from Mauritian soils. *British Microbiology Research Journal*, *3*(4), 478-491.

Bagheri, A., Khodarahmi, R., & Mostafaie, A. (2014). Purification and biochemical characterisation of glucoamylase from a newly isolated *Aspergillus niger*: Relation to starch processing. *Food Chemistry*, *161*, 270-278.

Bernfeld, P. (1955). Amylase, α and β. *Methods in Enzymology*, 1, 149-158.

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry*, *72*(1-2), 248-254.

Chay, C., Dizon, E. I., Elegado, F. B., Norng, C., Hurtada, W. A., & Raymundo, L. C. (2017). Isolation and identification of mold and yeast in medombae, a rice wine starter culture from Kompong Cham Province, Cambodia. *Food Research, 1*(6), 213-220.

de Souza, P. M., & Magalhães, P. D. O. (2010). Application of microbial α-amylase in industry-A review. *Brazilian Journal of Microbiology*, *41*, 850-861.

Deepika, M., & Satyanarayana, T. (2016). Bacterial and archaeal α-amylases: Diversity and amelioration of the desirable characteristics for industrial applications. *Frontiers in Microbiology*, *7*, Article number 1129.

Hwang, K., Shin, S. G., Kim, J., & Hwang, S. (2008). Methanogenic profiles by denaturing gradient gel electrophoresis using order-specific primers in anaerobic sludge digestion. *Applied Microbiology and Biotechnology*, *80*, 269-276.

Karlström, A., Calle, F., Salazar, S., Morante, N., Dufour, D., & Ceballos, H. (2016). Biological implications in cassava for the production of amylose-free starch: impact on root yield and related traits. *Frontiers in plant science*, *7*, Article number 604.

Kayode, O. (2016). Exploring the cassava industry's value chain opportunities for MSMEs. Retrieved from https://www.linkedin.com/pulse/exploring-cassava-industrys-value-chain-msmes-kayode-omosebi.

Koç, Ö., & Metin, K. (2010). Purification and characterization of a thermostable glucoamylase produced by Aspergillus flavus HBF34. *African Journal of Biotechnology*, *9*(23), 3414-3424.

Konsoula, Z., & Liakopoulou-Kyriakides, M. (2007). Co-production of α-amylase and β-galactosidase by *Bacillus subtilis* in complex organic substrates. *Bioresource Technology*, *98*(1), 150-157.

Mesfin, M., Negussie, F. B., Mesfin, M., Manikandan, M., Melese, A. (2019). Isolation and characterization of amylase enzyme from selected fungal strains of Wof Washa Forest of North Shoa, *Ethiopia. Science Journal of Biology & Life Science,* 1(2): 1-10.

Metin, K., Koc, O., Ateşlier, B. B., & Biyik, H. H. (2010). Purification and characterization of α-amylase produced by Penicillium citrinum HBF62. *African Journal of Biotechnology*, *9*(45), 7692-7701.

Moreira, F. G., Lenartovicz, V., de Souza, C. G., Ramos, E. P., & Peralta, R. M. (2001). The use of alpha-methyl-D-glucoside, a synthetic analogue of maltose, as inducer of amylase by Aspergillus sp in solid-state and submerged fermentations. *Brazilian Journal of Microbiology*, *32*, 15-19.

Moreira, F. G., Lima, F. A. D., Pedrinho, S. R. F., Lenartovicz, V., Souza, C. G. M. D., & Peralta, R. M. (1999). Production of amylases by Aspergillus tamarii. *Revista de Microbiologia*, *30*, 157-162.

Oboh, G. (2005). Isolation and characterization of amylase from fermented cassava (*Manihot esculenta* Crantz) wastewater. *African Journal of Biotechnology*, *4*(10), 1117-1123.

Ominyi, M. C., Ogbonna, J. C., Nwoba, E. G., Nwagu, K. E., & Ukachi, R. (2013). Isolation, and screening of α-amylase and Glucoamylase producing fungi and their Glucomaylase producing fungi and their application in Bioethanol, production. *International Journal Science Nature*, *4*(1), 44-50.

Saranraj, P., & Stella, D. (2013). Fungal amylase: A review. *International Journal of Microbiological Research, 4*(2), 203-211.

Schallmey, M., Singh, A., & Ward, O. P. (2004). Developments in the use of Bacillus species for industrial production. *Canadian Journal of Microbiology*, *50*(1), 1-17.

Shah, I. J., Gami, P. N., Shukla, R. M., & Acharya, D. K. (2014). Optimization for α-amylase production by Aspergillus oryzae using submerged fermentation technology. *Basic Research Journal of Microbiology*, *1*(4), 1-10.

Siddique, F., Hussain, I., Mahmood, M. S., Ahmed, S. I., & Iqbal, A. (2014). Isolation and characterization of a highly thermostable alpha-amylase enzyme produced by Bacillus licheniformis. *Pakistan Journal of Agricultural Sciences*, *51*(2), 309-314.

Sidkey, N. M., Abo-Shadi, M. A., Balahmar, R., Sabry, R., & Badrany, G. (2011). Purification and characterization of α-amylase from a newly isolated Aspergillus flavus F2Mbb. *International Research Journal of Microbiology*, *2*(3), 96-103.

Weber, K., & Osborn, M. (1975). Proteins and sodium dodecyl Sulphate: Molecular weight determination on polyacrylamide gels and related procedures. In: Hill, R. L.(ed.). *The proteins* (vol 1). New York, Academic Press. Pp. 179-223.