Vol. 24 (1)23-30 June 2012

Biokemistri

An International Journal of the Nigerian Society for Experimental Biology

Original Article

Characterization of a thermostable *Bacillus subtilis* β-amylase isolated from decomposing peels of Cassava (*Manihot esculenta*)

Muinat M. Adeyanju*, Adeshola O. Adetoro, Adeleye O. Adeshakin, Titilayo Kasumu, Oghenedaji Mowoe, Oluwaseyi A. Famakinwa, Oluwatumininu Lawal

Department of Biochemistry, Faculty of Basic Medical Sciences, Olabisi Onabanjo University, Remo Campus, Ikenne, Ogun State, Nigeria.

**Corresponding author: Muinat M. Adeyanju, E-mail: ronkalli2000@yahoo.com, Phone: +2348067191759*

Received: 23 November 2011

ABSTRACT: **β-Amylase (α-1, 4 glucan maltohydrolase; E.C:3.2.1.2) is used in the food processing, brewing and distilling industries due to its capacity to produce maltose syrup from starch. Here, we report the purification and characterization of β-amylase from *Bacillus subtilis* isolated from cassava peel waste obtained from a milling factory in Ikenne-Remo, Ogun State, Nigeria. The enzyme was extracted, fractionated at 90% $(NH_4)_2SO_4$ and further purified using DEAE-cellulose ion exchange chromatography. The molecular weight of the purified enzyme was estimated to be 34.67 Kd. The specific activity of the partially purified enzyme was approximately 1.35 units per mg of protein (Umg^{-1}), Kinetic analysis of its starch hydrolysis activity gave a K_m value of 2.496 ± 0.025% and a V_{max} of 1.136 ± 0.055 units min^{-1}. The optimum pH and temperature were determined to be 5.5 and 70 ^0C respectively, and the thermal stability curve gave a maximum activity of 9.75 U at 70°C for 60 min of incubation. *Bacillus subtilis* β-amylase is valuable for maltose production, which can be hydrolyzed further by other groups of amylase for the production of high cassava glucose syrup used as sweeteners in the food industry.**

INTRODUCTION

Cassava is found to be a major staple food in West African countries while its peels continuously rot away at dumpsites. It has advantage over all other starch sources by maturing between six to twelve months. Cassava starch can be completely hydrolyzed to yield simple sugars which can be exploited and used as an alternative cheaper local sweetener in the food, laundry and pharmaceutical industries compared to the imported sugars and artificial sweeteners. Although a large-scale starch processing industry has emerged in the last century, there has been a shift from the acid hydrolysis of starch to the use of starch-converting enzymes in the production of maltodextrin, modified starches, or glucose and fructose syrups. Extracellular enzymes from bacterial sources are frequently used in industrial processes. Among these enzymes are the starch-degrading, amylolytic enzymes called amylases. The increased use of bacterial amylases has created the need to source for a Bacillus species that is capable of secreting β-amylase.

Amylase represents a group of enzymes of great importance to the food industry and other needs of life. They were also one of the first to be produced industrially from microbial sources. [1-2] In starch processing industries, immobilized cells were used to optimally exploit the amylase producing machinery of cells of which the β-amylase producing cells are employed for bioconversion of starch to maltose. [2, 3]

β-Amylase (α-1, 4 – glucan maltohydrolase) [E.C. 3.2.1.2] is an exo-enzyme that hydrolyses the α – 1, 4 –glycosidic linkages in polysaccharides so as to remove successive maltose units from non-reducing ends of the chains. [4,5] It is classified as a member of the 1-4 of glucosyl hydrolase family. [6] It cannot bypass the α-1,6 linkages, so its conversion of starch to β-maltose is incomplete. [4] β–amylase has been found in plants and also micro-organisms. Microbial amylases vary in characteristic as widely as the microbes producing them. The most studied microbes for the production of amylase are fungi and bacteria. [7]

β-Amylase is an important enzyme used in the food processing, brewing and distilling industries. [8] Its main form of introduction is in the preparation of syrups from starch via saccharification processes. [9] The practical interest of β-amylase centers on its capacity to produce maltose syrup from starch. [5,9] This is commercially done by a maltose- producing enzyme such as plant or microbial β-amylase. The maltose containing syrup is used in brewing and baking, soft drinks, canning and confectionery industries. Among the important functional characteristics of high maltose syrups are low hygroscopy, low viscosity in solution, resistance to crystallization, low sweetness, reduced browning capacity and good heat stability. These maltose-containing syrups

can be used as moisture conditioners, crystallization inhibitor, stabilizer, carriers, and bulking agents. [10] In this report, we describe the isolation of a bacterial strain from cassava waste that produces a starch-hydrolyzing β-amylase, and the purification and characterization of the enzyme.

MATERIALS AND METHODS

The research work was carried out between June 2008 and August 2010 at the Olabisi Olabisi Onabanjo University, Remo Campus, Ikenne, Nigeria. The bacteria were isolated from decomposing cassava peels at the dump site of a cassava milling factory in Okun-Owa, Ogun State, Nigeria.

Isolation of micro-organism from cassava peels.

One gram of cassava peel was weighed aseptically into 9 ml of sterile water and following serial dilution, 1 ml of 10^{-3} dilution was used as an inoculum on a sterilized 1% starch agar. [11] Using repeated streaking, the pure strain of bacteria isolate with largest amylolytic activity was isolated and taxonomically characterized following the method of Salle [12]. The amylase producing potential of the pure isolate was determined by monitoring their ability to hydrolyze starch and further characterizations were carried out following the standard methods of Harrigan and McCance; Buchanan and Gibbons; Collins *et al.*[13-15]

Enzyme extraction

The pure strain of isolate on the slant was inoculated aseptically on a 1% starch agar plate and incubated at 37 °C for 48 hours. The organism was then used as an inoculum on sterilized 1% starch buffered peptone water at pH 6.9 and incubated at 37 °C for another 48 hours. The solution was centrifuged at 2,900 rpm for 15 min at 20 ^{0}C. The supernatant was collected and the cell debris was discarded. The crude enzyme was assayed for enzyme activity and the protein concentration determined.

Cassava starch production (Substrate)

Cassava tubers were harvested, peeled, rinsed and fermented for 7days. The fermented cassava slurry was then sieved, sun dried and saved in a dry sterile tube.

Enzyme assay

β-Amylase activity was determined by measuring the rate at which maltose is released from starch which is measured by its ability to reduce 3, 5-dinitrosalicylic acid (DNSA). [16, 17] One unit of β-amylase activity was described as the amount of enzyme that will release 1mg of maltose per minute at 25°C. [17, 18] The substrate, 1% cassava starch was gelatinized in 0.016 M Sodium acetate buffer (pH 4.8). The reaction mixture consisted of 0.5 ml enzyme solution and 0.5 ml of the substrate, incubated for 4 minutes at 25°C and stopped by adding 1 ml of DNSA colour reagent. The mixture was heated in a water bath at 100°C for 5 min, cooled and diluted by adding 10 mL of distilled water. The reaction mixture was allowed to stand for 15 min at room temperature and the optical density read at 540 nm. A unit of amylase activity was expressed as:

$$\frac{\text{(Milligram of maltose released) x (Dilution factor)}}{\text{(Time of incubation) x (mg enzyme in reaction mixture)}}$$

Protein determination

The protein concentration of crude enzyme and various level of purification were determined using Gornall's Biuret assay [19]

Enzyme Purification

The enzyme secreted by the pure isolate was purified as described below.

Ammonium sulphate fractionation: The crude enzyme extract was subjected to ammonium sulphate fractionation. The volume of crude enzyme was taken and brought to 90% $(NH_4)_2SO_4$ saturation. The precipitated protein was left on ice for at least 2 hours, and was recovered by centrifugation at 7,000 rpm at 4 ^{0}C for 20 min. The precipitate was reconstituted with 0.016 M sodium acetate buffer, pH 4.8 containing 10% ammonium sulphate and 1% phenyl–methyl-sulfonyl-fluoride (PMSF) (a protease inhibitor).

Dialysis: The ammonium sulphate precipitated sample was dialyzed for 6 hours in 0.016 M sodium acetate buffer pH 4.8. The dialysis tubing was pre-treated by boiling in double-distilled water until it softens.

Ion-exchange chromatography: The dialysate was loaded on pre-treated DEAE-Cellulose column (2.5 x 40cm) which had been pre-equilibrated with 0.016 M acetate buffer, pH 4.8. Elution was carried out with the same buffer at a flow rate of 30 ml/hr. The bound proteins were eluted with 0.05 M NaCl in the same buffer (0.016 M acetate buffer, pH 4.8). [20] The fractions were monitored for protein concentration using Biuret solution [19] and assayed for β-amylase activity. The active fractions were pooled and saved as 70% ammonium sulphate precipitate.

Effect of temperature and pH

The optimum temperature of the enzyme was investigated by assaying the activity at different temperature which ranges from 30 ^{0}C to 100 ^{0}C and determining the residual activity. The optimum pH was also determined by assaying the enzyme at various pH values using 0.1 M of the following buffers: acetate buffer (pH 2.5-4.5), citrate-phosphate buffer (pH 5.0-6.0), phosphate buffer (pH 6.5-7.5) and Tris-HCl buffer (pH 7.0-9.0).

Determination of thermostability

The thermal stability of the enzyme was determined by incubating the enzyme solution at the temperatures between 40-80°C. At time intervals between 10-60 min, 0.5 ml of the enzyme solution was taken and added to 0.5 ml of the substrate (cassava starch) and the β-amylase activity was routinely determined as described above.

Determination of kinetic parameters

The kinetic parameters of the partially purified β-amylase enzyme were determined using aqueous 2% cassava starch solution as substrate. The enzyme was assayed routinely at varying substrate concentrations from 0.1-1.0%. The Michaelis-Menten constant (K_m) and the maximum velocity V_{max} were estimated from the Line-Weaver and Burk's plot. [21]

Determination of native molecular weight

The native molecular weight was determined on a calibrated Bio-Gel P100 column (2.5 x 100 cm). The molecular weight markers were Gamma–globulin (M_r 150 kDa; 5 mg ml^{-1}), Creatine Phosphokinase (CPK) (M_r 81 kDa; 5 mg ml^{-1}), Bovine Serum Albumin (BSA) (M_r 66 kDa; 5 mg ml^{-1}), Ovalbumin (M_r 45 kDa; 5 mg ml^{-1}), α-Chymotrypsinogen A (M_r 25 kDa; 5 mg ml^{-1}). The pooled fractions from the DEAE-Cellulose proteins were eluted with a 0.016 M acetate buffer, pH 4.8 at a flow rate of 10 ml h^{-1}. The void volume of the column was determined with blue dextran (2 mg ml^{-1}). The estimation and calibration were carried out according to the protocol described by Andrews (1964; 1965). [22, 23]

Determination of the hydrolysis Products

The hydrolysis products obtained on incubating 2 ml of enzyme and 2 ml of 1% pre-gelatinized cassava starch for 1 hour at room temperature were identified using thin-layer chromatography method of Ivor and Feinberg. [24] The standard sugars were D (+) Glucose (G), D (+) Fructose (F) and D (+) Maltose (M).

RESULTS

Taxonomical characterization of the microorganism

The most predominant and pure colony with amylase activity was identified as having a round, small and convex colony. The morphological, cultural and biochemical characterizations of the isolate gave the result in Table 1. From the results, it was concluded that the probable identity of the organism was *Bacillus subtilis.*

Enzyme purification

The summary of the enzyme purification table is shown in Table 2. β-Amylase was partially purified 2.77-fold from the crude extract. The ammonium sulphate protein precipitation step (Table 2) gave total and specific activity values of 74.25 U and 0.188 $U.mg^{-1}$, respectively, which were lower than the crude total and specific activities of 1140.30 U and 0.488 $U.mg^{-1}$. The specific activity of the partially purified enzyme (β-amylase) was 1.35 Umg^{-1}. The percentage yield achieved was 17.90%. The elution profile of the dialysate on the DEAE-Cellulose column is presented in Figure 1.

Effect of temperature, pH and determination of thermostability

The optimal temperature and pH of the *Bacillus subtilis* β-amylase were 70°C and 5.5 respectively (Figures 2 and 3). The thermal stability curve (Figure 4) gave a maximum activity of 9.75 U at 70 °C for 60 min of incubation.

Kinectic parameters of the partially purified enzyme

The kinetic constants V_{max} and K_m gave 1.136 ± 0.055 units min^{-1} and 2.496 ± 0.055% respectively as was estimated from the Line-Weaver Burk's plot (Figure 5).

Native molecular weight of the partially purified enzyme

The native molecular weight obtained by the gel filtration on BioGel P-100 gave one peak and was estimated to be 34.67 kDa as shown in Figure 6.

Determination of hydrolysis product

The hydrolysis product from the incubation of the partially purified and pre-gelatinized cassava starch was maltose (plate not shown).

TABLE 1 **Characterization of bacteria isolated from cassava peels.**

Characteristics	Test	Result
Morphological	Pigmentation	Creamish white
	Colony Morphology	Round, Small, Convex
	Motility	Positive
	Gram reaction	Positive
	Spore Formation	Positive
	Shape of Cell	Rod
Biochemical	Catalase	Positive
	Methyl Red	Negative
	Voges-Proskauer	Positive
	Starch Hydrolysis	Positive
	Nitrate Reduction	Positive
Sugar Fermentation	Glucose	Positive
	Xylose	Negative
	Lactose	Positive
	Sucrose	Positive
	Raffinose	Negative
	Fructose	Positive
	Galactose	Negative
	Arabinose	Positive
	Mannose	Positive

DISCUSSION

The secretion of alpha-amylase by *B. subtilis* has been extensively reported but this investigation shows that β-amylase is also secreted as an extracellular enzyme by the bacteria. The other microbial sources of β-amylase earlier reported include *Bacillus circulans* [25], *Bacillus licheniformis* [2], *Bacillus macerans* [26, 27], *Clostridium thermosulphurogenes* [28], *Bacillus megaterium* [29], *Bacillus coagulans* [30]. In general, these results extend fundamental understanding of biochemical similarity of β-amylase isolated from different sources. The same enzyme from *B. licheniformis* from soil and *B. subtilis* from waste water reported by Ajayi and Fagbade [2] gave activities of 0.36 and 5.64 U respectively while this work gave higher activities values of (as shown in Table 2) 2.715 U (crude enzyme) to 9.720 U (partially purified enzyme) and specific activities of 0.488 (crude enzyme) to 1.350 (partially purified enzyme). Data shown in Table 2 indicates that the ammonium sulphate precipitation step was inefficient in recovering β–amylase enzyme from the previous step.

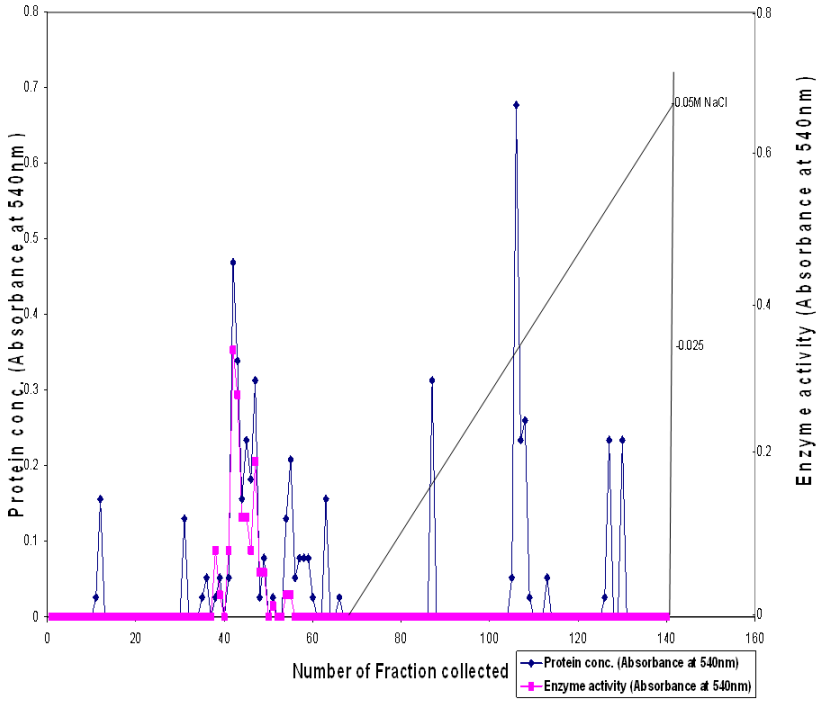

FIGURE 1 The Elution Profile of the *B. subtilis* β-amylase from the DEAE-Cellulose column ion-exchange chromatography.

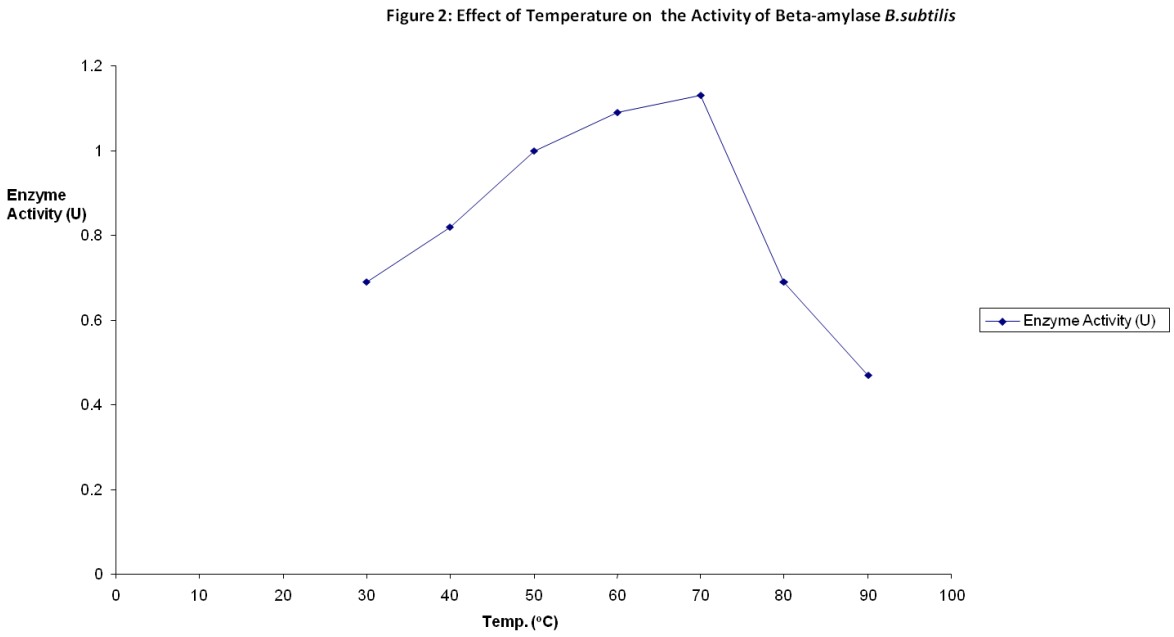

FIGURE 2 Effect of temperature on the activity of beta-amylase *B. subtilis*

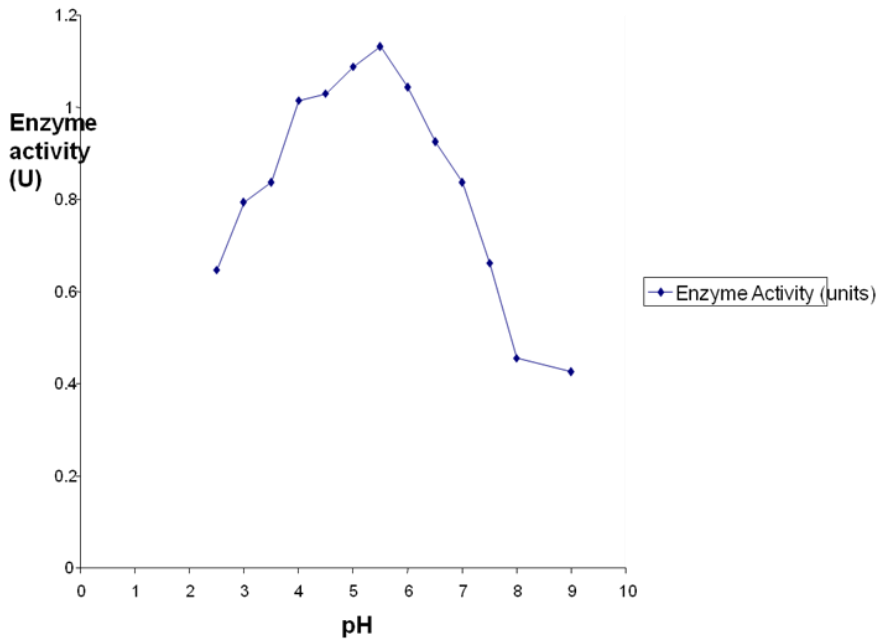

FIGURE 3 Effect of pH on partially purified *B. subtilis* β-amylase

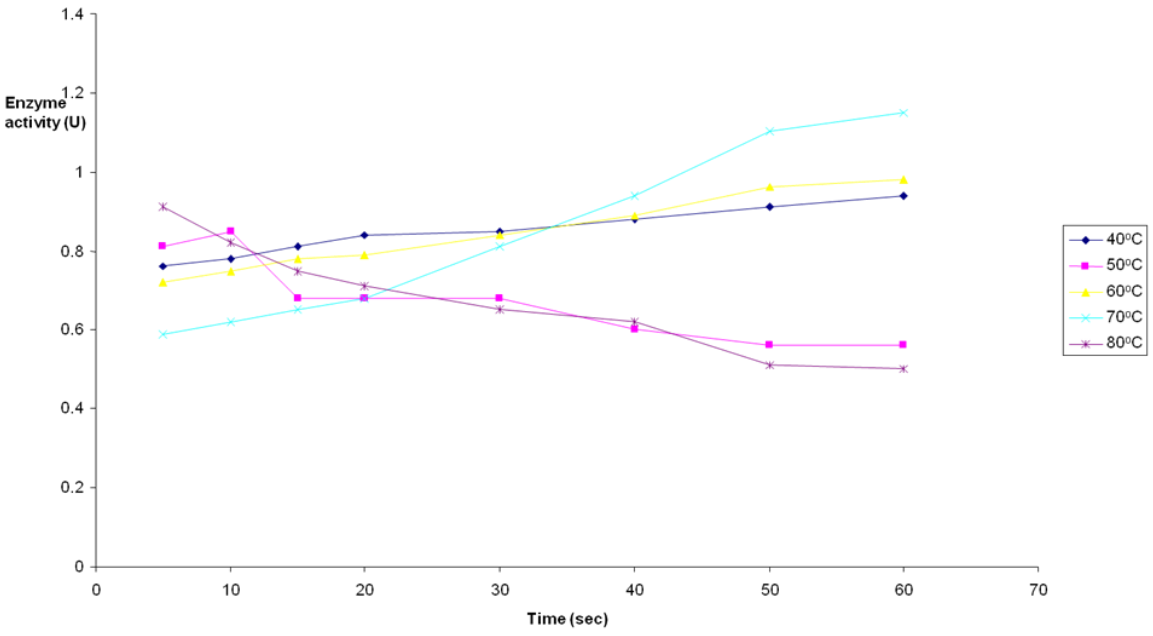

FIGURE 4 Thermostability Curve of Beta-amylase *B. subtilis*

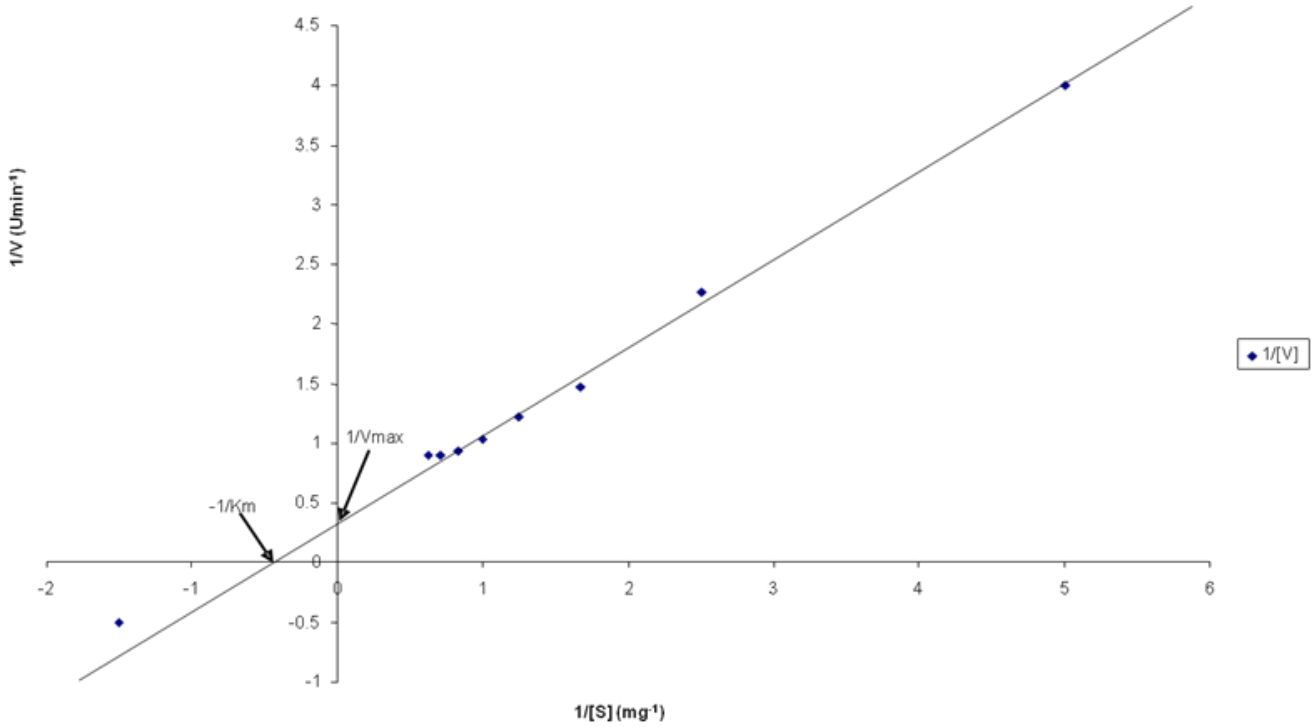

FIGURE 5 Lineweaver-Burk (Double reciprocal) plot of *B. subtilis* beta-amylase

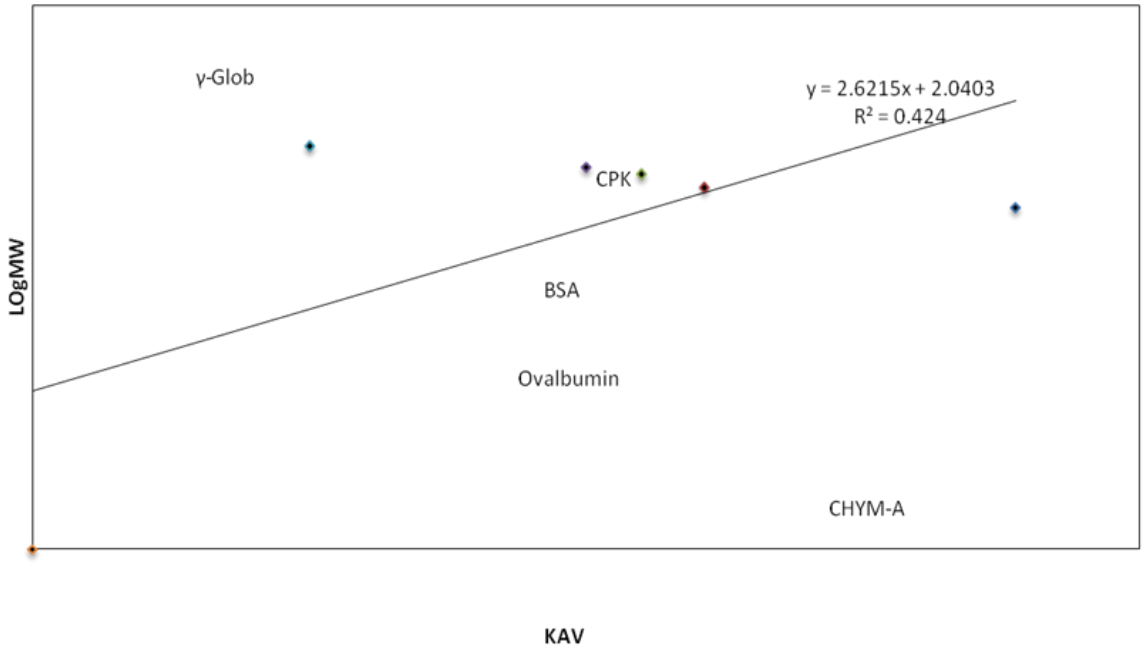

FIGURE 6 Native molecular weight of beta-amylase *B. subtilis* on BioGel P-100

TABLE 2 Purification Table of *Bacillus subtilis* β-amylase isolated from cassava peels.

Purification steps	Vol (mL)	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	500	1390.0	189.0	0.136	1.00	100
$(NH_4)_2SO_4$ fractionation	33	206.3	32.5	0.158	1.16	17.2
Dialysis	41	120.8	30.8	0.255	1.88	16.3
Ion-exchange chromatography	40	83.2 0	40.0	0.481	3.54	21.1

Further careful trials will be required to optimize the precipitation of the enzyme from the crude extract.

The optimum pH of 5.5 observed in this study is the same with those reported for purified β-amylase from different sources such as *Clostridium thermosulphurogenes* [4, 28] and barley, [31] with pH 5.5. The β–amylase secreted by *Bacillus subtilis* described here has an optimum temperature of 70 °C, which is lower than that of the same enzyme from *Clostridium thermosulphurogenes* with an optimum temperature of 75 °C [28]. The enzyme is thermostable at 70°C for 60 minutes which makes it suitable to withstand high temperatures employed in food and beverages industries especially malting in the brewing processes. The enzyme hydrolyses pre-gelatinized cassava starch giving maltose as the hydrolyzates on thin-layer chromatography.

The isolation of the *B. subtilis* β–amylase from cassava as a readily-available, cheaper, and local source of this enzyme in Nigeria is of significant economic importance. This could be exploited for industrial production of sweeteners such as cassava syrup, maltose syrup or glucose syrup in combination with other amylases (alpha-amylase and amyloglucosidase).

REFERENCES

[1] G. Reed, Enzyme in Food Processing (2nd Edition). Academic press, New York 10005 USA, (1975).

[2] A. O. Ajayi and E. O. Fagbade, Utilisation of corn starch as substrate for β-amylase by *Bacillus subtilis*, *African Journal of Biomedical Research*, 6(1) (2003) 37-42.

[3] R. R. Ray, S. C. Jana, G. Nanda, β- amylase production by immobilised cells of *Bacillus megaterium B6*, *J. Basic Microbiology*, 35 (2) (1995) 113-116.

[4] Dicko M. H., Searle-Van L., Beldman G. Onedrago O. G., Hilhorst R., Traore A. S.. Purification and Characterisation of β-amylase from *Curculigo pilosa*. *Applied Microbiol. Biotechnol*. 52 (1999) 802-805.

[5] M. H. Dicko, L. Searle-Van, R. Hilhorst, A. S. Traore, Extraction, Partial purification and Characterisation of β-amylase from the bulb of *G. Klattianus*. *Biosource Technology*. 73 (2000) 183-185.

[6] O. J. Ajele, Some physico-chemical properties of soyabean β-amylase, *Nigerian Journal of Biochemistry and Molecular Biology*, 12 (1997) 61-66.

[7] Fogarty W. M. And Kelly C.T, Recent advances in microbial amylases. In Microbial Enzyme and Biotechnology (eds, Fogarty, W.M. and Kelly, C.) (1980). pp 71-108. London, Elsevier *Applied science*.

[9] P. Boivin, Les enzmyes en brasserie In: Multon, J. L. (Eds), Enzymes en Agroalimentarie. *Colletion Science et Techniques Agroalimenaires*, Londres, Paris, New York (1997) pp 138-168.

[10] C. Badal, T. Saha, J. Zeikus, Improved method for preparing high maltose conversion syrups, *Journal of Biotechnology and Bioengineering*. 34 (1989) 299-303.

[11] M .M. Adeyanju, F. K. Agboola, B.O. Omafuvbe, O. H. Oyefuga and O. O. Adebawo A thermostable extracellular α- amylase from *B. licheniformis* isolated from cassava steep water. *Biotechnology* (2007) 6 (4): 473-480.

[12] A. J. Salle, Laboratory Manual on Fundamental Principles of Bacteriology. 6th Edition (1971). N. Y: McGraw-Hill Book Company.

[13] W. F. Harrigan and M.E. McCance, Laboratory Methods in Food and Dairy Microbiology, London: Academic Press. (1976) Pp 452.

[14] G. Buchanan and N. E. Gibbons, Bergey's Manual of Determinative Bacteriology 8th Eds Md. : Williams and Wilkins Co. (1974) pp.1094.

[15] C. H. Collins, and P. M. Lyne, Microbiol. Methods. 3rd Ed. (1970) Pp 454. Baltimore: University of Park Press.

[16] P. Bernfeld, Amylases alpha and beta. *Methods in Enzymol*. (1955) 1: 149.

[17] Murao S., Ohyama., and Aral M. (1979). β-Amylases from *Bacillus polymyxa*. No. 72. *Agric Biol. Chem*. 43 (4): 719-726.

[18] M. J. Bailey, A note on the use of dinitrosalicylic acid for determining the products of enzymatic reactions, *Applied Microbiology and Biotechnology*, 29 (1988) 494-496.

[19] Gornall A.G., Bardawill and David M.M., Determination of serum protein by means of the Biuret reaction. *J. Biol. Chem.* (1949) 177: 751-766.

[20] S. Matzui, S.Chibo And T. Shimomura, Purification and some properties of active β-Amylase in Rice. *Journal of Agriculture Biological Chemistry.* (1976) 41 (5): 841-847.

[21] H. Lineweaver; D. Burk. The Determination of enzyme constants. *Am. Chem. Soc.* (1934) 56: 658.

[22] P. Andrews, Estimation of Molecular Weights of proteins by Sephadex Gel Filtration. *Biochem. J.* (1964) 91:222-233.

[23] P. Andrews, Gel filtration behavior of proteins related to their molecular weights over a wide range. *Biochem. J.* (1965) 96: 595-606.

[24] S. Ivor and G. Feinberg, Paper and Thin Layer Chromatography and Electrophoresis. A Teaching Level Manual.2nd Edn., Shadon Scientific Co. Ltd., 62-65.

[25] D. E. Hensley, L.S. Karl, A.B. Joyce and A. L. Adolph β-amylase production by *Bacillus polymyxa* on corn steep-starch-salt medium. *Applied and Environmental Microbiology*. (1980) 678-680.

[26] F. G. Priest, Extracellular enzyme synthesis in the Genus *Bacillus*. *Bacteriological Reviews*.1977, pp 711-753

[27] A. H. Rose, Microbial Enzymes and Bioconversions. (1980). Academic Press, London, England.

[28] G-J. Shen, B. C. Saha, Y-E. Lee, L. Bhatnagar, J. G. Zeikus, Purification and Characterisation of a novel thermostable β-amylase from *Clostridium thermosulphurogenes*. *Biochem J.* (1988) 254: 835-840.

[29] M. Hoshino, Y. Hiroso, K. Sano, K. Mitsugi. Absorption of microbial β-amylase on starch. *Agric Biol. Chem*. (1975) 39 (12): 2415-2416.

[30] A. O. Ajayi and E. O. Fagbade Growth pattern and structure nature of amylases produced by some *Bacillus species* in starchy substrate. *African Journal of Biotechnology*. (2006). 5(5): 440-444.

[31] S. Gestler and F. Brick, Production of Thermotolerant from Barley; Biochemistry, β-Amylase. (1985). 229: 1153-1162.