PARTIAL PURIFICATION AND CHARACTERIZATION OF EXTRACELLULAR BACILLUS LICHENIFORMIS α-AMYLASE ISOLATED FROM DECOMPOSING CASSAVA (MANIHOT SPP) PEELS.

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ABSTRACT

Extracellular α-amylase was secreted by *Bacillus licheniformis,* an organism isolated from decomposing cassava peels and identified in this study with the characteristic features of the strain. The crude enzyme was precipitated at 70 % ammonium sulphate, followed by ion-exchange chromatography on DEAE-cellulose and gel-filtration on Bio-Gel P-100. The peak with the highest activity was pooled from latter chromatographic step and characterized afterwards. The specific activity of the enzyme rose from 1.01 to 104.8 U/mg with a yield of 46.32 % and 103.76 purification fold. The optimal pH and temperature of the enzyme were 6.50 and 50^0C respectively. The enzyme was observed to be thermostable at the 50^0C for 25 to 30 min. The kinetics revealed a sigmoidal curve at an increasing starch concentration with maximum velocity (Vmax) of 14.60±0.04 U/min; kinetic constant (k′) of 0.2818 ± 0.04 g/ml and binding sites greater than one (1) estimated from Hill plot. The native molecular weight of the enzyme was found to be 48kDa.

Key words: α-amylase, *Bacillus licheniformis*, ion-exchange chromatography, gel filtration, cassava peels.

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INTRODUCTION

Starch occurs mainly in the seeds, roots and tubers of higher plants (El Fallal *et al.,* 2012). Starch degrading enzymes like amylase have received great deal of attention because of their perceived technological significance and economic benefits. Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques (Satyanarayana *et al.,* 2005).

Amylases hydrolyze starch molecules to give diverse products including dextrins and progressively smaller polymers composed of glucose units (Windish and Mhatre, 1965). Hence they are used in a wide range of starch industries that is brewing, baking, starch liquefaction and distillery (Souza and Magalhaes). Evidences of amylase in yeast, bacteria and moulds have been reported and their properties documented (Buzzini and Martini 2002; Adeyanju *et al.,* 2007; Oyeleke and Oduwole, 2009). In spite of the wide

distribution of amylases, microbial sources are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization
(Pandey *et al.,* 2006, Burhan *et al.,* 2003).

Traditionally, amylases have been obtained through submerged fermentation (SmF) because of ease of handling and greater control of environmental factors such as temperature and pH. Several industrial enzymes and proteins have been reported to be extra cellularly secreted by bacteria most especially *Bacillus* species which are predominant industrial enzyme producer and are found widely distributed in the environment. This capacity of selected *Bacillus* strains to produce and secrete large quantities of extracellular enzymes has placed them among the most important industrial enzyme producers (Schallmey *et al.,* 2004). The Gram-positive bacteria of the genus Bacillus are industrially well-established microorganisms for the production of extracellular proteins (Degering *et al.,* 2010). *Bacillus licheniformis* is known to produce a vast array of valuable industrial enzymes among which include amylases, proteases, lipases, bacitracin and amino peptidase (Veith *et al.,* 2004; Voigt *et al.,* 2006), the activity made possible by being able to utilize polysaccharides such as starch, glycogen and amylose as carbon sources by hydrolysis.

The rate of hydrolysis of starch by α-amylase depends on many conditions such as temperature, pH, metal ion, nature of substrate presence of calcium ions and other stabilizing agents (Burhan *et al.,* 2003). In spite of the wide distribution of α-amylase, microbial sources are used for the industrial production. However, microorganisms producing amylases have successfully replaced chemical processing methodology in different industries (Pandey *et al.,* 2000; Vengadaramana, 2013).

In our previous study (Adeyanju *et al.,* 2007), we isolated the thermostable α-amylase enzyme from cassava steep water but the peels could also be another viable source of amylolytic organisms while it is more or else regarded as waste and allowed to rot away at the dump sites; we therefore investigated α-amylase enzyme from the decomposing peels by isolation and characterization of the bacterium with highest alpha-amylase activity while also purifying and characterizing the enzyme secreted by the organism for possible use in starch hydrolysis.

Materials and Methods

The organism used in this study was collected from the dump site of a cassava processing factory in Ikenne- Remo Ogun State, Nigeria and taxonomically characterized at the Microbiology Laboratory of Olabisi University of Olabisi Onabanjo University. 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). Glass distilled water was used for all preparations. All media and other chemicals used were of analytical grade while equipments 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 121°C for 15 min under pressure while all glass wares used were sterilized in hot air oven at 180°C for 3 hours. Inoculating loop was sterilized by flaming.

Culturing of Sample and Identification of Amylase Producing Bacteria

Into 9.0 mL sterile water was added 1.0 g of the decomposing cassava peels (sample) and with gentle swirling for about 5 min, the liquid was decanted and 1.0 mL was used as inoculum on sterile 1.0 percent starch agar plates (Adeyanju et al., 2007). The inoculated plates were then incubated at 37°C for 18 hours. The Gram's reaction and cell morphology of the isolate on agar plate were examined from appropriately stained heat fixed smear following the method of Salle (1971) and Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Enzyme Secretion and Extraction

The enzyme secretion was carried out using submerged fermentation. 18 hour old bacterial culture was inoculated into 1.6 L of Horikoshi

medium II (Horikoshi, 1999; Ahmed *et al.*, 2011); with the addition 0.01 percent of phenyl methyl sulfonyl fluoride (PMSF), a protease inhibitor. The flasks were incubated at 37°C in an orbital shaker at 150 rpm for 48 hours with an initial pH of 7.0. The contents of the flasks were centrifuged at 4,000 rpm for 10 minutes. The supernatant (crude enzyme) was collected and the pellet was discarded. The crude enzyme was assayed for enzyme activity and the protein concentration determined.

Enzyme and Protein Assays

α–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) using the modified Bernfield (1955) method. One unit of alpha amylase activity was described as the amount of enzyme that releases 1 µg of maltose per minute at 25°C. 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 50°C and the reaction stopped by the addition 1.0 ml DNSA colour reagent. The mixture was heated in a water bath at 50°C 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 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 Gornal *et al.,* 1949 using bovine serum albumin as standard.

Enzyme Activity (U/min)= $\frac{\text{µg of maltose released X Dilution Factor}}{\text{µg of protein in reaction mixture x incubation time.}}$

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 over night 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 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 stored at 4°C with the addition 1 % PMSF.

Ion–Exchange Chromatography on DEAE –Cellulose

About 30g of DEAE–Cellulose powder was pre-treated 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 Bio-Gel-P100

Bio-Gel P-100 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 Bio-Gel P-100 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°C for characterization studies.

Effect of Temperature and Thermostability Studies

The effect of temperature on the activity enzyme was routinely investigated at different temperature values ranging from 40°C 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 that had also been equilibrated at the same 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. The assay was carried out according routinely afterwards.

Determination of Kinetic Parameters

The kinetic constants K′ and Vmax 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). Vmax was estimated from the plot of velocity (U/min) versus substrate concentration (% Starch) while the Hill constant, K′ and Hill coefficient, n (number of binding sites) were estimated from the Hill plot, Log v/Vmax-v against Log [S].

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, - Globulin (mol. wt 150 k Da), Bovine Serum Albumin (mol. wt 66 k Da) Carbonic Anhydrase (29 k Da) and Lysozyme (mol. wt 19 k Da). The void volume was determined using blue dextran.

Results

Characterization and Identification of the Isolated Bacterial Strain Fig. 1 shows the photograph of the bacterial amylase-producing organism streaked on the starch agar plate and Fig. 2 is the photomicrograph of the organism after Gram staining. The morphological characteristics of the organism revealed creamish-white, Gram positive short rods and other characteristics shown in Table 1. The bacteria isolate was identified as Bacillus licheniformis according to the method described in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons, 1974).

Enzyme Purification

The results of the purification procedures were summarized in Table 2 yielding α-amylase with specific activity of 104.80 Umg-1 of protein with a yield of 46.32%. The elution profile of the dialysate on the DEAE-Cellulose column (Figure 3) showed more than one peak of amylase activity but the one with highest activity was pooled and purified further. The elution profile of the post DEAE-Cellulose on Bio-Gel P-100 gel filtration column is presented in Figures 4. Two major peaks were noted in this later figure and the one with the highest peak was (pooled from Fraction number 10 to 22).

Properties of the Purified α-amylase Isolated from Decomposing Cassava Peels.

The maximum α-amylase activity was obtained at 50oC. A reduction in enzyme activity was observed at values above 50°C (Figure 5) after this temperature, the activity was decreased drastically and enzyme was completely inactivated. The stability of α-amylase to temperature is shown in Figure 6. α-amylase was observed to be stable at 50ºC for 25 to 30 min. A sharp decrease in stability was observed as the incubation time increased beyond 30 min. The optimum pH was 6.5 (Figure 7). The graph of velocity versus substrate concentration gave a sigmoidal curve (Figure 8) and gave a Vmax value of 14.60±0.04 U/min. The kinetic constant (K') of 0.2818 ± 0.04 g/ml was obtained with the binding sites of greater than one (1) estimated from the Hill plot. The native molecular weight of the partially purified enzyme is shown in Figure 9, giving an estimated value of 48kDa from the plot of partition coefficient against Logarithm of molecular weight.

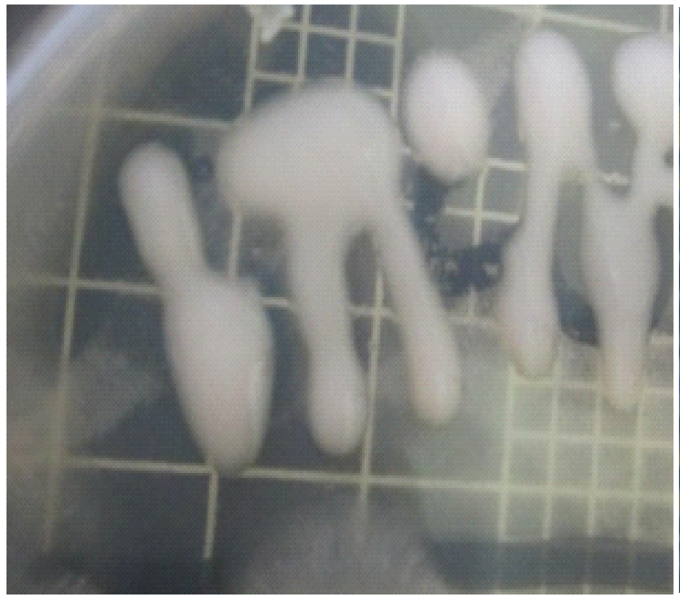

Fig. 1: Photograph of the bacterial amylase-producing organism streaked on the starch agar.

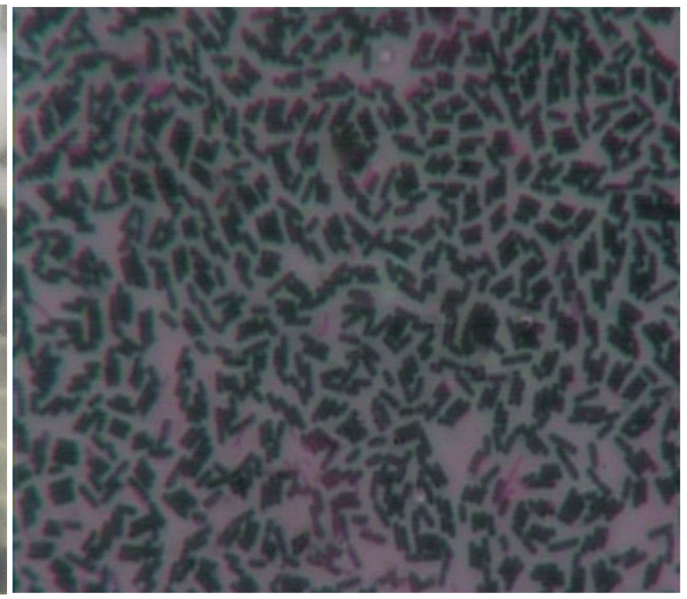

Fig. 2: Photomicrograph of *B. licheniformis*.

ÇĂľ'ǿ ﻭ: Morphological and Biochemical characteristics of bacteria isolated from cassava peels.

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Table 2: Summary of Purification Table of *Bacillus licheniformis* α-amylase Isolated from Decomposing Cassava Peels.

fication	Volume (mL)	Total Protein (Mg)	Total Activity (U)	Specific Activity (U/Mg)	Yield (%)	Fold
le Enzyme	1520	6809.60	6840.00	1.01	100	1.00
$)_2SO_4$ ionation	81	1655.64	4495.50	2.70	65.72	2.67
.E-Cellulose led Fraction)	43	43.86	3457.20	78.80	50.54	78.02
Gel P-100	36	30.24	3168.00	104.80	46.32	103.76

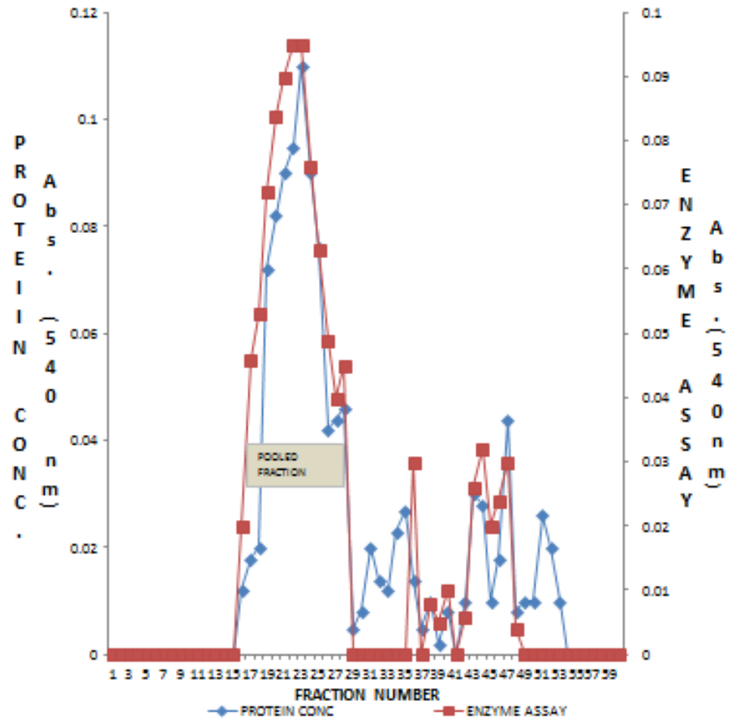

Figure 3: The Elution profile of the Bacillus licheniformis amylase from the DEAE-Cellulose column ion-exchange chromatography.
Abs-Absorbance; Protein Conc.-Protein Concentration

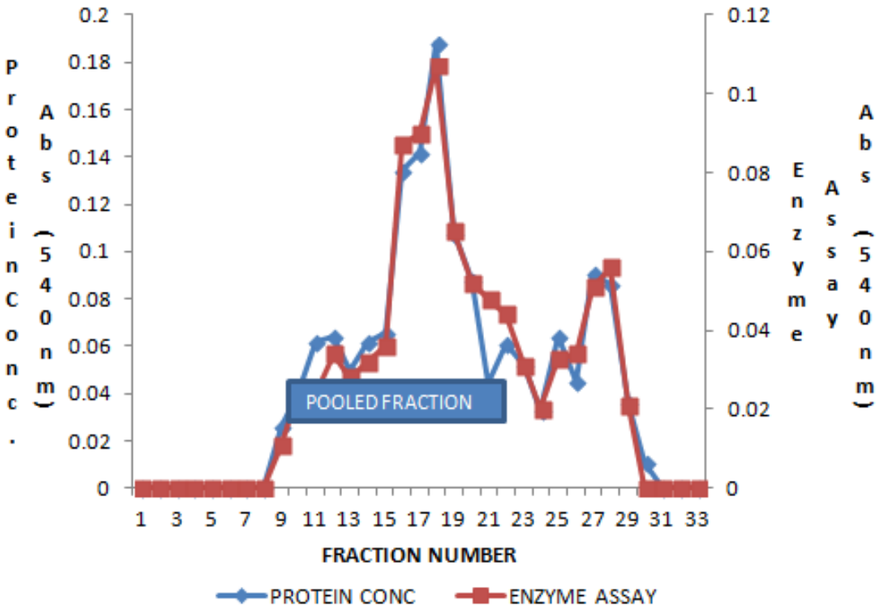

Figure 4: The Elution profile of the *B. licheniformis* α-amylase of the post ion-exchange fraction on BioGel P-100 gel filtration column.
Abs-Absorbance; Protein Conc.-Protein Concentration

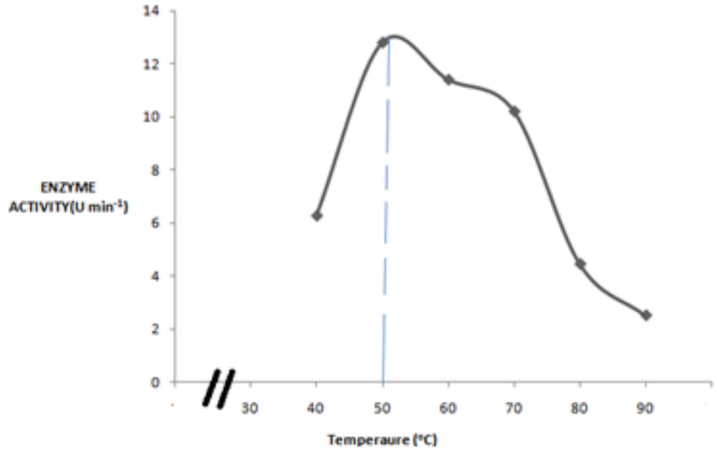

Figure 5: Temperature Profile of *Bacillus licheniformis* alpha-amylase Isolated from Decomposing Cassava Peels

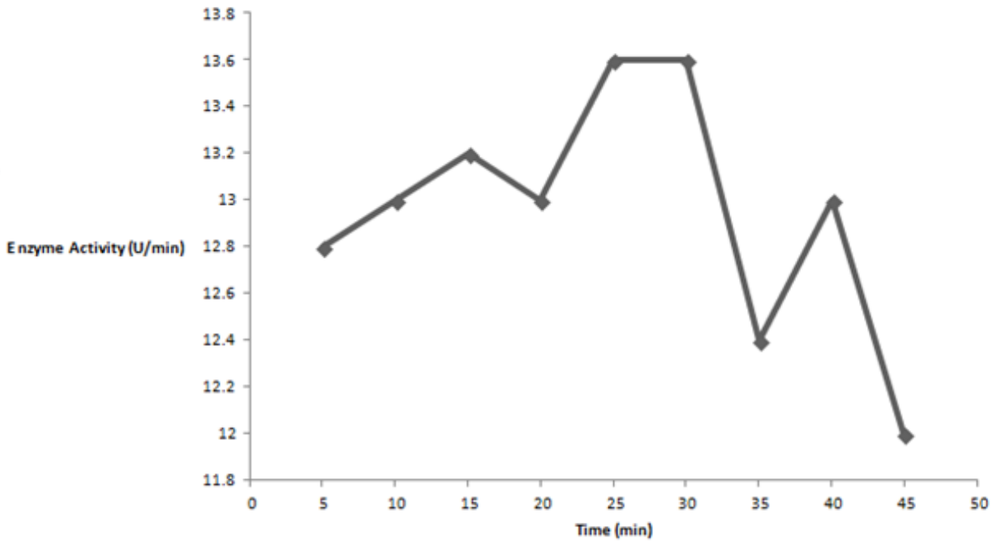

Figure 6: Thermal Stability Curve of the *Bacillus licheniformis* α-amylase at 50°C.

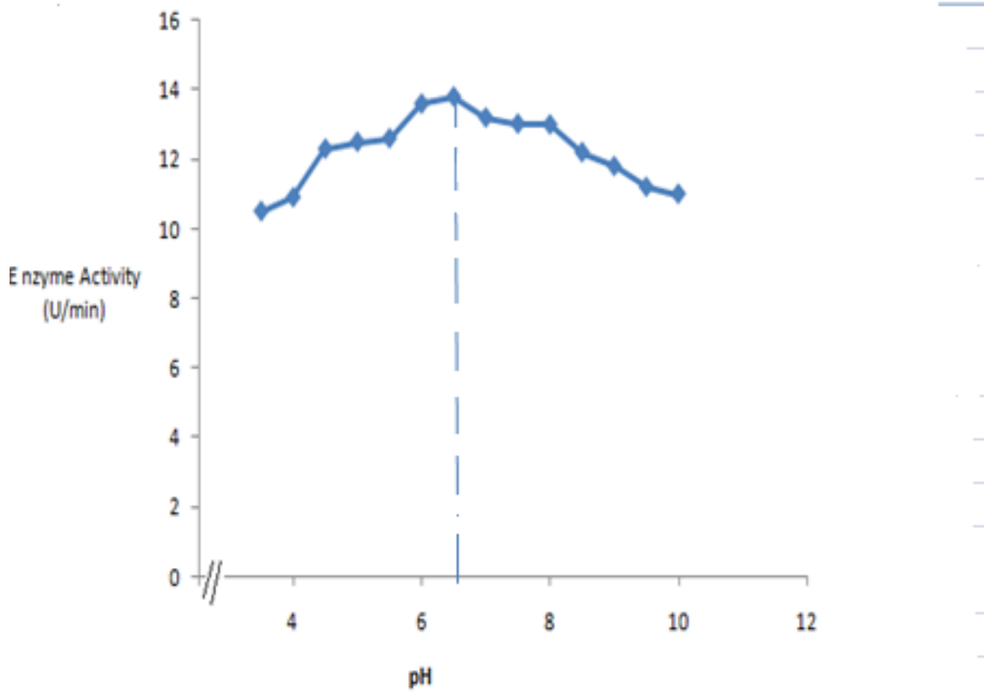

Figure 7: pH Profile of *Bacillus licheniformis* α-amylase

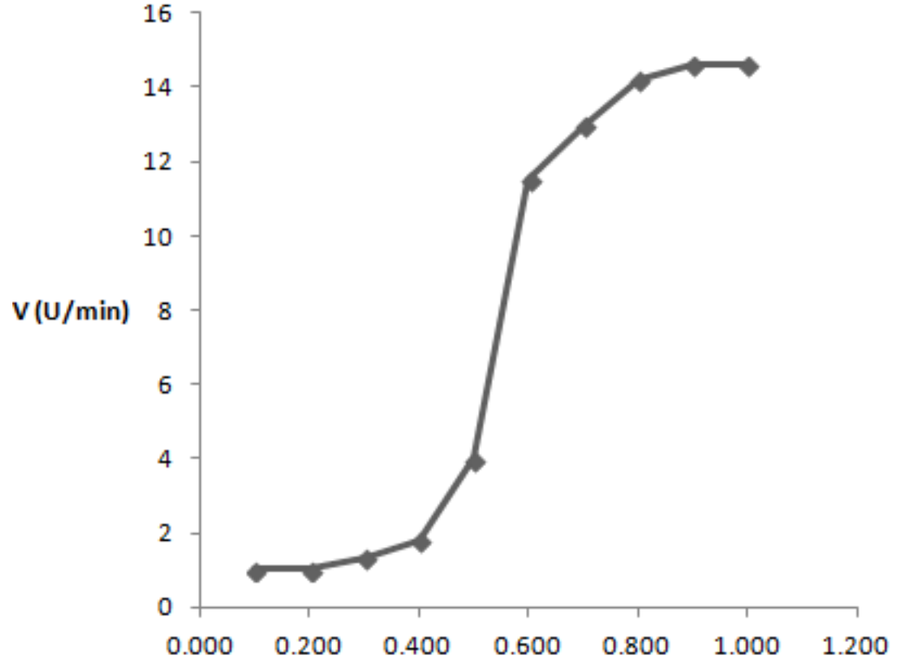

Figure 8: Plot of V (U/min) against [S] (% Starch of the *Bacillus licheniformis* α-amylase

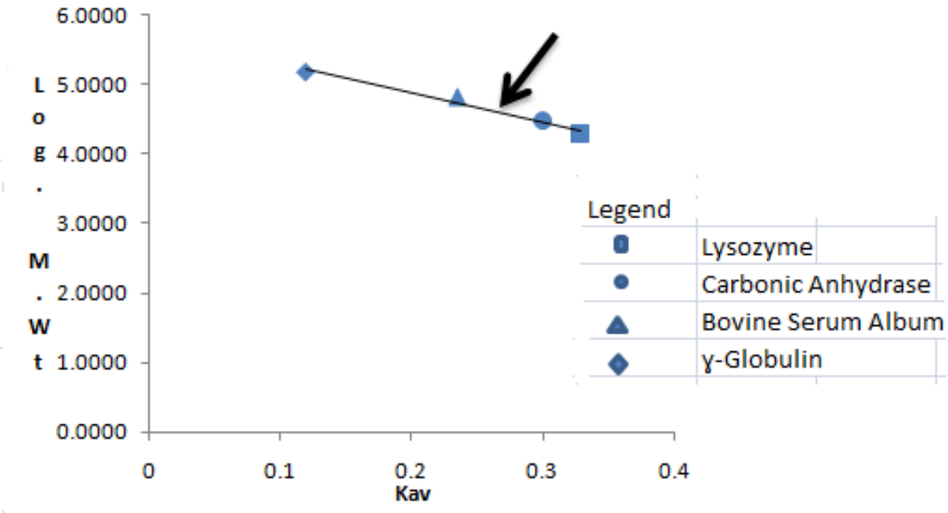

Figure 9 : Plot of Partition Coefficient (Kav) against Logarithm of Molecular Weight of *Bacillus licheniformis* α-amylase

Discussion

In this research, we were able to isolate Bacillus species with the highest amylolytic activity thriving on the decomposing cassava peels as Bacteria belonging to the genus *Bacillus* represent the most important strains for the industrial production of secreted proteins (Schallmey *et al.,* 2004). Utilization of bacterial strains specifically from genus Bacillus is gaining momentum because of their ability to resist and survive under harsh industrial conditions (Ghani *et al.,* 2013). Generally, microorganisms thrive on plant litter and soil because they harbor starch hydrolyzing enzymes which are employed 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). *Bacillus* spp. and the related genera produce a large variety of extracellular enzymes, of which amylases are of particular significance to the industry e.g., *B. cereus* (Rhodes *et al.,* 1987), *B. circulans* (Siggens, 1987), *B. subtilis* (El-Banna *et al.,* 2007), *B. licheniformis* (El-Banna *et al.,* 2008). Gram positive bacteria are industrially well-established microorganisms for the production of extracellular proteins (Degering *et al.,* 2010). Thus *Bacillus* isolated was found to be Gram-positive another novel characteristic of the organism characterized in this study.

Quite a number of researchers had reported isolating organisms with amylolytic activity from starch compost such as cassava mash waste, cassava farms, cassava steep water, buried potato pieces respectively (Pandey *et al.,* 2000; Tatsinkou *et al.,* 2005; Adeyanju *et al.,* 2007; Siddique *et al.,* 2014). The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and easy manipulation of microbes to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990). Among bacteria, *Bacillus sp*. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis, B. stearothermophilus, B. licheniformis and B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications (Sivaramakrishnan *et al.,* 2006). *Bacillus licheniformis* is referred to as an organism with great industrial potential producing a number of valuable industrial enzymes among which include amylases, proteases, lipases, bacitracin and amino peptidase (Veith *et al.,* 2004).

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,* 2012). *Bacillus licheniformis* has been reported to survive harsh environmental condition and is stable at temperature higher than the usual (Claus and Berkeley, 1986), 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 Elayaraja *et al.,* 2011 who reported optimum temperature of 50°C for *B. firmus* isolated from potato peels. Higher activity temperatures of 62°C was obtained by Zare Mirakabadi *et al.,* 2012 while 90°C was obtained from our previous study for *B. licheniformis* isolated from cassava steep water (Adeyanju *et al.,* 2007) both for *B. licheniformis* α-amylase. Its thermostablity at the optimum temperature of 50°C for 25 to 30 min. also conformed to the work of de Carvalho *et al.,* 2008 where thermophilic *Bacillus sp* strain SMIA-2 was stable at temperatures ranging from 40-50°C though for 1 h and but the isolated enzyme was found to be more active as it retained 100% activity at 50°C unlike the B. licheniformis α-amylase from soil that retained almost 50% of its maximum activity at 50°C (Zare Mirakabadi *et al.,* 2012). Thermostable α-Amylases 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 to iso-maltose is minimized (Konsoula and Liakopoulou-Kyriakides, 2007). pH optimum for B. licheniformis α-amylase has been reported to be in the acidic to neutral range (Pandey *et al.,* 2000; Sun *et al.,* 2010; Bozic *et al.,* 2011). The pH optimum of 6.5 obtained for B. licheniformis α-amylase is in agreement with Oyeleke and Oduwole (2009) and Daniel *et al.,* (2010) who stated in their report that most bacterial enzymes function between a pH range of 6 and 8; the exact value of 6.5 was reported by Liu *et al.,* 2014 for *B. licheniformis* α-amylase WT strain.

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 70 %. The purification steps in this study was similar to the works of Shih and Labbe, 1995 and Adeyanju *et al.,* 2007. The specific activity of the enzyme in each step increased, with the specific activity after ammonium precipitation being 2.70 U/mg and after gel filtration the specific activity was 104.80 U/mg, a value higher than the one reported for the same organism from cassava steep Adeyanju *et al.,* (2007). 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.

Despite wide difference of microbial α-amylases characters, their molecular weights are usually in the same range of 40-70 k Da (Gupta *et al.,* 2003). Therefore the native molecular weight of 48 k Da obtained falls within this range. It was noticed that *B. licheniformis* α-amylase obtained in this work obeys sigmoidal kinetics (Fig. 9). As earlier reported, it may appear that either the enzyme is oligomeric or it forms aggregates. Seigner *et al.* (1985) and Adeyanju *et al.,* (2007) have reported that α-amylase from porcine pancreas and cassava steep showed sigmoidal responses. The "Hill plot" (not shown) obtained in this study is linearization of the Hill Equation (Hill, 1910) with the slope being n (number of binding sites) and intercept on x-axis as kinetic constant, which is the affinity of the enzyme for starch (substrate). It is pertinent to note that human salivary amylase and porcine pancreatic amylase have six and five subsites, respectively (Brayer *et al.,* 2000; Kandra, 2002; Ramasubbu *et al.,* 2005) whereas other amylases, including α-amylase and maltogenic amylase, vary from seven to ten subsites. (Kandra *et al.* 2002) though we estimated the binding sites to be greater than one (1) in this study, a somewhat similar value as obtained in 2007. But, the K' value of 0.2818±0.04 g/ml obtained is an indication of stronger affinity for the substrate.

Conclusion

This organism is thus somewhat thermo-tolerant and produces a thermostable α-amylase which could be used in heat stable reaction and can be optimized for use in the industry in production of malt syrup, sweeteners, glucose syrup among others, from starch.

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