

Purification and Characterization of α -Amylase from *Bacillus subtilis* Isolated from Cassava Processing Sites

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Abstract

This study was designed to purify and characterize of α -amylase from pure strain of *Bacillus subtilis*. The crude α -amylase was purified by ammonium sulphate precipitation, then loaded on DEAE Sephadex A-50 ion exchange chromatography and gel filtration. The effect of pH, temperature and metal ions were investigated on the purified enzyme. The single protein band on SDS-PAGE suggested that the enzyme was homogenous. Two different activity peaks were observed in ion exchange chromatography designated pool A and pool B with the 8% and 4% yield, 15.93 and 6.44 purification fold and specific activity 2.55 $\mu\text{mol}/\text{min}/\text{mg}$ and 1.03 $\mu\text{mol}/\text{min}/\text{mg}$ respectively. The two fractions revealed the same optimum pH 7.0 for the α -amylase activity while the enzyme was relatively stable at pH 4.0 and 7.0 between 20 to 40 minutes and 60 to 80 minutes for pool A and pH 8.0 between 40 and 100 minutes for pool B. At 40°C, optimum temperature was reached, and amylase activity was maintained at 75% and 70% temperature stability between 60 to 80 minutes for pool A and B, less than 20%, the residual activity at 60°C and 70°C was recorded. The incubation of α -amylase with Na^+ and Zn^{2+} ions enhanced/activate the enzyme activity correspondingly, Al^{3+} and K^+ ions exhibited varied degree of inhibition while Ca^{2+} and Hg^{2+} ions caused total inhibition on α -amylase activity. The ability of purified α -amylase from *Bacillus subtilis* under wide range of temperatures and pH suggests its applications in industries and bioremediation of effluent discharge on food processing sites.

Keywords: *Bacillus subtilis*; Cassava processing site; α -amylase; Purification; Characterization

Introduction

Globally, indiscriminate dumping or disposal of wastes to the environment affects both macro-and-microorganisms causing ecological imbalance and displacement from their niche. The survival of microorganisms depended on several factors such as biotic, and abiotics. Soil stands as major reservoir of different microorganisms. Biodegradation of wastes on/in soil by the action of microorganisms could prompt a safer environment [1]. Microorganisms isolated from soil stand as potential source of industrial enzymes due to their abundance in nature. However, utilization of these enzymes such as amylases, cellulases, linamarases, mannanase and pectinase etc in our industries are limited due to the poor exploration. Amylases (EC 3.2.1) is a hydrolytic enzyme that catalyze the hydrolysis of amylose and amylopectin from starch, as well as starch derivatives such as dextrin and oligosaccharides liberating reducing groups [2]. Amylase constitute a class of industrial enzymes having approximately 25%-33% of the enzyme in the enzyme market [3,4]. α -amylase can be obtained from several sources, such as plants, animals and microorganisms [5,6]. Commercial application of amylase produced from genus *Bacillus spp* has been widely used in baking and bread industry, textile and leather, laundry and detergent industry, pulp and paper, pharmaceuticals, feed processing and agriculture [7-9]. Due to technological advancement, α -amylases applications have been extended in many fields such as clinical and analytical chemistry, medicine, brewing and food industry for the starch liquefaction and

saccharification, and biogeochemical cycle of carbon [7,10,11]. Enzymes from genus *Bacillus spp* such as *Bacillus subtilis*, *Bacillus stearothermophilus*, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* are of special interest in our industrial applications as the sole producer of amylase, its commercial production and ability to withstand high temperatures [7,12]. However, the high activity of exhibited by this enzyme produced from *Bacillus subtilis* at wide pH and temperatures suggest it applications for various industrial purposes and in bioremediation of soil polluted with effluents discharge on food processing sites.

Materials and Methods

Bacterial isolates

The bacterium (*Bacillus subtilis*) used in this study was isolated from cassava processing sites. The bacterium was isolated using standard microbiological methods under optimum cultural conditions.

α -amylase assay: α -amylase activity was determined as described by Okolo et al. [13] with slight modification. The reaction mixture comprising of 2.0 ml of 1% corn starch prepared in 0.5 ml of 0.05 M sodium phosphate buffer (pH 7.0) and 0.5 ml of the supernatant of the crude enzyme extract was used to assay for α -amylase.

The blank (control tube) contained 0.5 ml of 0.05 M sodium phosphate buffer (pH 7.0), 0.5 ml of 1% starch solution and 1 ml sterilized distilled water. Both the experimental and control tubes were incubated at 30°C for 10 minutes. At the end of incubation, the tubes were removed from the water bath (Lamfield Medical England Model

DK-600), and the reaction was terminated by adding 1 ml of 3,5-dinitrosalicylic acid (DNSA) reagent tube. The tubes were incubated for another 5 minutes in a boiling water bath for colour development and cooled rapidly. The activity of the reaction mixture was measured against blank at 540 nm. The amount of liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method of Miller. One unit of α -amylase was defined as amount of enzyme producing 1 micromole of glucose equivalent per minute under the experimental conditions.

Protein determination: The protein estimation of the crude extract was determined by Bradford method and Bovine Serum Albumin (BSA) was used as standard Bradford.

Purification and Characterization of α - amylase from *Bacillus subtilis*

Purification of α -amylase from *Bacillus subtilis*: The purification of crude extract (enzyme supernatant) obtained after cold centrifugation was done in three stages. In stage one, 400 ml of the supernatant of crude enzyme was precipitated by adding 240 g ammonium sulphate to obtained 60% ammonium sulphate concentration. The concentrates were then cold centrifuged at 6000 rpm for 30 minutes, the precipitate obtained was washed with 100 ml phosphate buffer (pH 6.0) and then dialyzed in the same buffers. In stage two, the partially purified α -amylase obtained was purified on ion exchange column chromatography containing DEAE (diethylamineethyl) Sephadex A-50 (2.0 \times 2.5 cm, Pharmacia). The eluates obtained from stage two were washed with 300 ml ion-free water, followed by 200 ml 0.01 M Tris-HCl buffer (pH 8.0). The gel was eluted with NaCl. The absorbance of each of the fraction at time interval was measured. In stage three, 2.5 ml of partially purified α -amylase was loaded onto a column chromatography (2.5 cm in diameter and 30.0 cm high) using Sephadex G-150 (Pharmacia). Phosphate elution buffer at 50 mM pH 6.0 was applied with the flow rate 20 ml/hour. A fraction of 5.0 ml was collected at interval of 30 minutes and the highest α -amylase activity was determined with corn starch as a substrate, at 280 nm (Jenwey 6305). Fractions with α -amylase activity were pooled and concentrated in glycerol solution at 30°C [14].

Characterization of purified α -amylase from *Bacillus subtilis*

Effect of pH on α -amylase activity and stability: Different buffers (0.05 M): Glycine-HCl (pH 3.0-4.0), sodium acetate buffer (pH

5.0-6.0), sodium phosphate buffer (pH 7.0), Tris-HCl (pH 8.0), glycine-NaOH buffer (pH 9.0) and 1% corn starch as substrate were prepared. The assay was carried out according to the standard assay procedure. The pH stability of the purified enzyme was determined at different pH buffers ranged from 5.0 to 8.0 at room temperature for different time interval up to 2 hours. Thereafter, relative α -amylase activity was assay.

Effect of temperature on α -amylase activity and stability: α -amylase activity was assay by incubating the enzyme reaction mixture at different temperature, 30°C to 90°C for 120 minutes in 0.05 M sodium phosphate buffer (pH 7.0) and 1% corn starch. The temperature stability was determined by measuring the residual activity at 60°C. After incubation, samples were collected at 20 minutes and thereafter residual activities were evaluated under standard assay conditions.

Effect of metal ions on α -amylase activity: A stock solution of 10 mM for different metal ions was prepared. Two milliliters of the prepared solution was mixed with 2 ml of enzyme solution. The mixture was incubated for 5 minutes at room temperature. Zero point five milliliters (0.5 ml) of the mixture were collected and α -amylase assay was carried out according to the standard assay procedure.

Results and Discussion

Summary of purification

Table 1 shows the α -amylase purification processes. The homogeneity of the purified α -amylase was obtained in three steps with 16% and 7% fold, approximately total protein recovery of 8% and 4% with specific activity 2.55 μ mol/min/mg and 1.03 μ mol/min/mg respectively of pool A and pool B. The purification fold increased due to desalting of the enzyme. The homogeneity of the crude enzyme could result from the fact that the isolate is from a known source. Purification of amylase from *Bacillus subtilis* by centrifugation on DEAE-Cellulose and gel filtration Sephadex 75 column with 26 purification fold and 10% yield has been reported [15]. Mukesh et al. [8] reported purified α -amylase from *Bacillus* sp. MN123 with 27.41% yield and 2358 U/mg specific activity. The percentage yield 13.4%, 16.3 fold and specific activity 19.8 mM/min/mg of purified xylanase from *Bacillus cereus* was reported [16].

Step	Vol.	PC	EA	TP	TA	SA	Yield (%)	Fold
Crude extract	500	7.65	1.2	3825	600	0.16	100	1
ASP (40 - 60%)	21.5	6.11	4.65	131.37	99.98	0.76	16.66	4.75
Dialysis against buffer	25	5.62	4.16	140.5	104	0.74	13.91	4.63
Ion exchange (A 50)								
Pool A	12.16	4.16	6.22	50.59	75.64	1.49	12.61	9.31
Pool B	8.91	3.98	4.01	35.46	35.73	1.01	5.95	6.31
Gel (G-100)								
Pool A	12.56	1.46	3.72	18.34	46.72	2.55	7.79	15.93

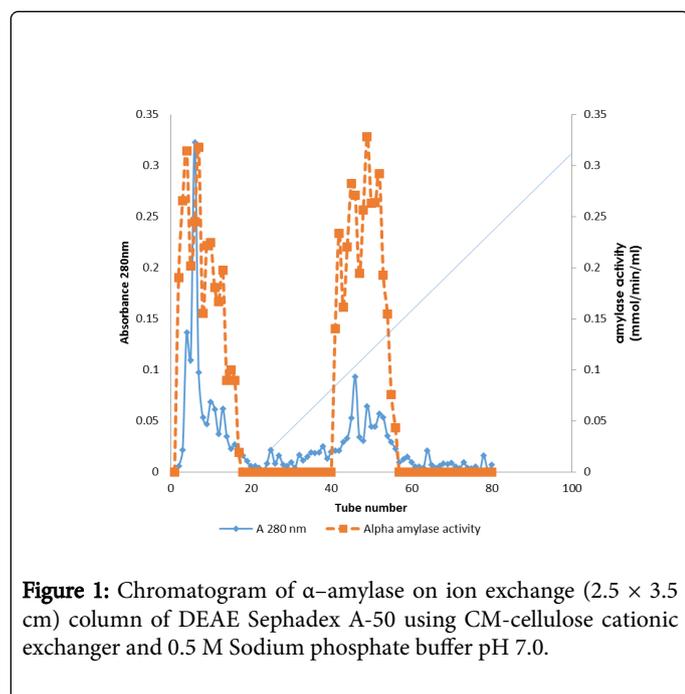
Pool B	10.72	1.95	2.01	20.9	21.55	1.03	3.59	6.44
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Table 1: Purification table of α -amylase produced from *Bacillus subtilis*. CP=crude protein (mg/ml); TP=total protein (mg); EA=Enzyme activity (micromole/min/mg); SPA=specific enzyme activity (micromole/min/mg); TA=total activity (micromole/min/ml); ASP=Ammonium sulphate precipitation.

The high specific activities of peak A (2.55 micromole/min/mg) and B (1.03 micromole/min/mg) than that of the crude and ammonium sulphate precipitates could be attributed to increase in the level of purity of the enzyme as compared to the crude and the precipitates, starch-hydrolyzing enzymes filtrate isotypes, the effect of synergy on the enzyme activity of the filtered enzymes when compared to the crude [17]. The increase in specific activities for both enzyme after gel filtration could be ascribed to the purification procedure which enhance the specific activity of the desired enzyme after the purification procedure [17].

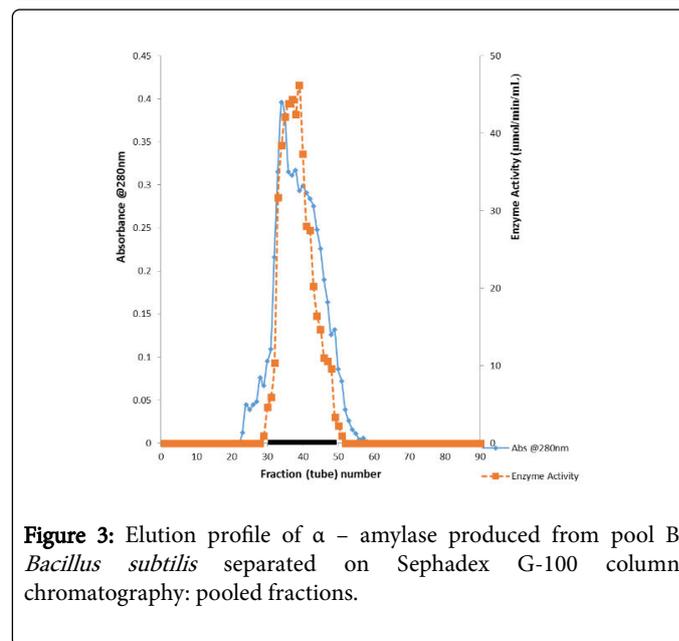
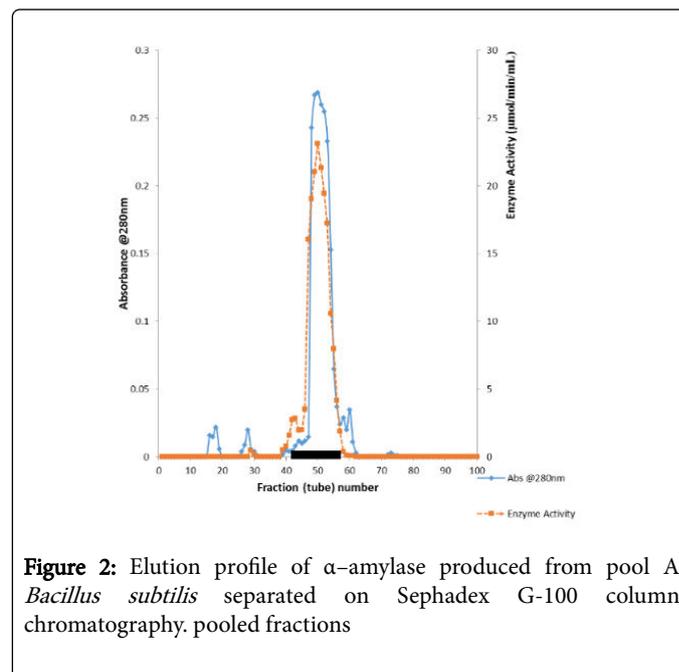
Elution

The elution pattern of the α -amylase enzyme on Sephadex A-50 gel filtration of the precipitated enzyme, two enzyme activity designated peaks loop A and loop B were observed showing that this enzyme might have isotypes (Figure 1).



α -amylase activity was detected in fraction tubes 1.00 to 18.00 and 40.00 to 57.00, between fraction tubes 19.00 and 39.00 no enzyme activity was detected. The fractionation tubes (pool A and pool B) with α -amylase activity were separately pooled and further purified separately on Sephadex G-150 (Figures 2 and 3). The highest enzyme activity of the partially purified α -amylase from pool A was attained at fraction tube 50 with specific activity $23.13 \mu\text{mol/min/mg}$ while the pool B was attained at fraction tube 39 with enzyme activity $42.64 \mu\text{mol/min/mg}$ respectively. The two forms of amylase activity resulting from the elution process might be due to their molecular weight and catalytic functions [18]. The result is however similar to the findings of

Alexander et al. [19] who reported three forms of purified α -amylase, Amyl I, Amyl II and Amyl III from *Aspergillus awamori*.



Characterization of α -amylase

Figure 4 shows the effect of pH on purified α -amylase activity while the pH stability of the purified α -amylase from pool A and pool

B are shown (Figures 5 and 6). The α -amylase from pool A and pool B increased progressively up pH 4 with specific activity 39.17 $\mu\text{mol}/\text{min}/\text{mg}$. The optimum activity was attained at pH 7.0. The relative activities of pool A and pool B at pH 6.0 was reduced by 34% and 47% and pH 9.0 was reduced by 10% and 48% respectively when compared with the value obtained at pH 7.0. The stability of the enzyme was checked at different pH ranged from 5.0 to 8.0 at room temperature for two hours. The α - amylase activity obtained from pool A was relatively stable between 20 to 40 and 60 to 80 minutes of incubation at pH 4.0 and 7.0 while α -amylase activity obtained from pool B was stable between 40 to 100 minutes of incubation at pH 8.0 respectively. pH of the growth medium plays an important role by inducing morphological change in the nature of microorganism and their enzyme secretion. The decrease in pH beyond optimum might be due to the concomitant alteration in the conformation of the enzyme protein caused by changes in pH of its environment resulting in changes in the three dimensional structure of the enzyme active sites and substrates binding speed to produce maximum product. pH 6.8-7.2 optimum for purified amylase from *Bacillus subtilis* has been reported [20]. Roy and Rowshanul [16] reported pH 6.0 optimum for purified xylanase from *Bacillus cereus*. It was reported that the stability of most plant enzymes decreased significantly at pH values below 4.0 and above 7.5, whereas, the majority of the corresponding microbial enzymes are rather stable at pH 7.0 [21]. Mukesh et al. [8] reported α -amylases stability over a wide range of pH 4 to 11. The optimum pH 7.0 and above for α -amylase activity from *B. subtilis*, *B. licheniformis*, *B. amyloliquefaciens* and thermophilic *Anoxybacillus flavithermus* has been reported [16,22-26] pH 8.0 optimum has been reported for amylase activity from *Thermus* sp., *Bacillus* KSM-K38 and *Bacillus* spp [27,28] Wide range (pH 3.5 to 12) optimum pH for α -amylases has been reported [7,29-32]. The result obtained from this study agrees with the findings of Agüloğlu Fincan and Bukhari and Rehman [32,33] who reported pH 7.0 optimum for purified α -amylase from *Bacillus subtilis* from local environment.

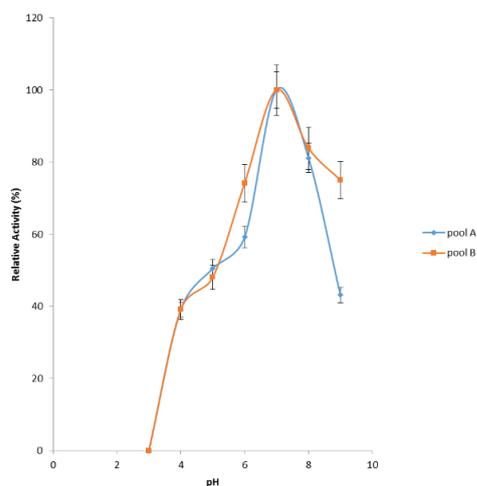


Figure 4: Effect of pH on activity of purified α -amylase.

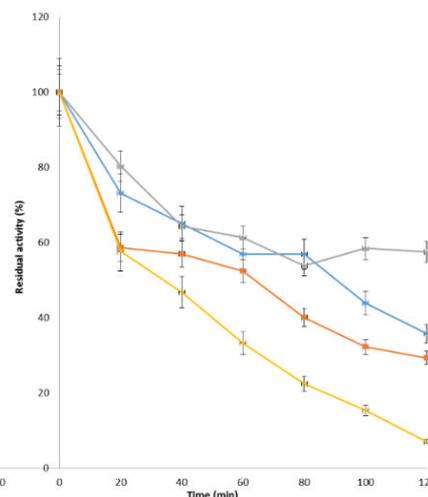


Figure 5: Effect of pH on the stability of purified α -amylase for pool A.

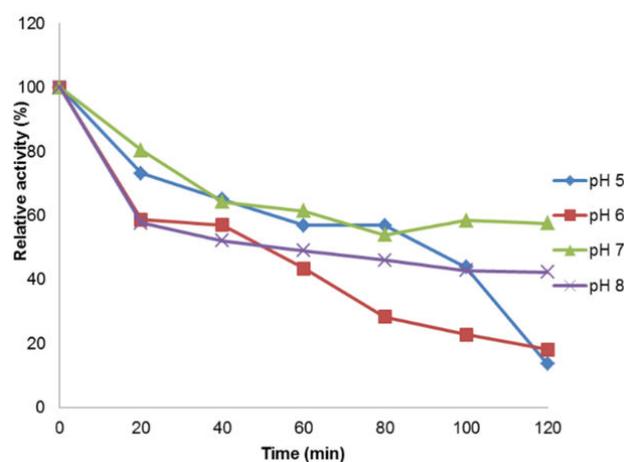


Figure 6: Effect of pH on the stability of purified α -amylase for pool B.

Effect of temperature on α -amylase activity and stability: Figure 7 shows the effect of temperature on purified α -amylase activity while the temperature stability of the purified α -amylase from pool A and pool B are shown in Figures 8 and 9. The α -amylase activity increased with increase progressively with incubation temperature until an optimum percentage relative activity was reached at 40°C and 60°C for pool A and pool B respectively (Figure 7). The purified α -amylase activity from pool A and pool B were relatively thermostable at 40°C and 50°C between 60 and 80 minutes and 40 and 60 minutes before a decline was observed (Figures 8 and 9).

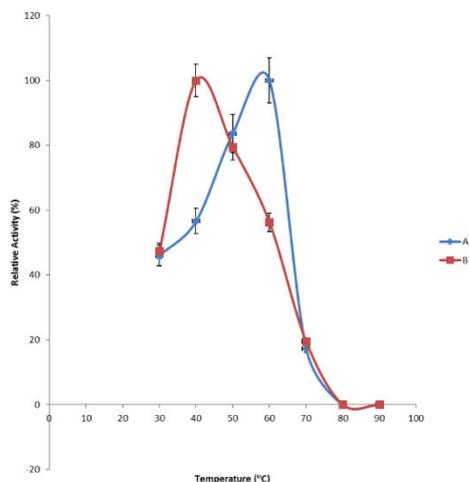


Figure 7: Effect of temperature on the activity of purified α -amylase.

Interest in thermostable amylases has increased tremendously, since resistance to thermal inactivation has become a desirable property in many industrial applications [34]. Enzymes are mostly proteins with a labile nature. Inactivating agents such as heat impair their native conformation so affecting their catalytic functions. It has been reported that most amylases *Bacillus* spp have an optimum temperature of around 40–70°C [35].

The increase in temperature beyond optimum might cause the denaturation of the enzymes. An optimum temperature 55°C of α -amylase from thermophilic *Anoxybacillus flavithermus* has been reported [26]. Roy and Rowshanu [16] reported optimum temperature 45°C for purified xylanase from *Bacillus cereus*. Findings on thermal stability amylase enzyme from bacteria belonging to genus *Bacillus* has been reported [25]. Averagely, the optimum temperature obtained from this study agree with the findings of Demirkan, Ponnuswamy and Prakash and Mukesh et al. [8,15,31] who reported 50°C optimum for purified α -amylase and cellulase from *Bacillus* spp from a paddy field.

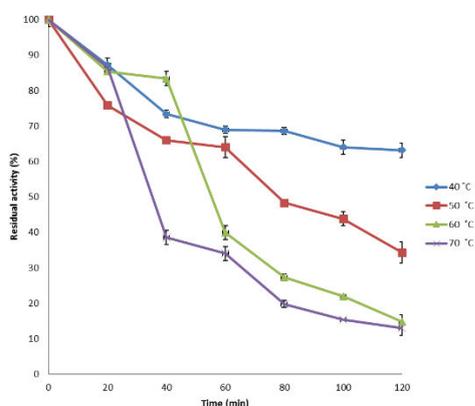


Figure 8: Effect of temperature on the stability of purified α -amylase for pool A.

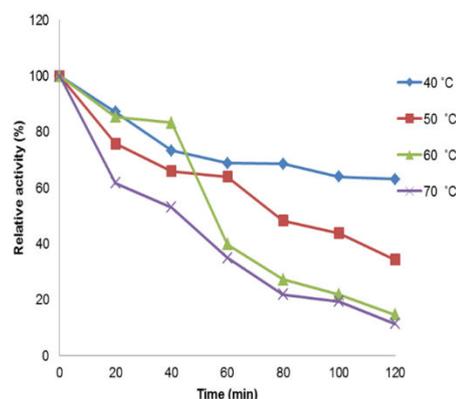


Figure 9: Effect of temperature on the stability of purified α -amylase for pool B.

Effect of metal (ions) on α -amylase activity: Table 2 shows the effect of concentration of metal (ions) on purified α -amylase activity. The metal ions exerted activation and inhibition on the α -amylase activity. The α -amylase activity was stimulated/activated by Na^+ and Zn^{2+} ions correspondingly, Al^{3+} and K^+ ions exhibited varied degree of inhibition while Ca^{2+} and Hg^{2+} ions caused total inhibition on α -amylase activity. The salt tolerance test is important in saccharification of starch and in treatment of effluent with high salinity containing starch or cellulose residues in pollution control mechanism [35,36]. Metallic ions can either show the inhibitory or stimulating effect on the activity of enzymes depending on the concentration of the metallic salt solution. Most amylases are known to be metal ion-dependent enzymes, particularly with regard to divalent ions like Ca^{2+} , Mg^{2+} , Mn^{2+} and Zn^{2+} [37]. The effect of this metal ions activating the enzyme activity might be due to the presence of chloride in the surrounding soil environment. The inhibition of α -amylase by Hg^{2+} might indicate the importance of the enzyme function [10]. The inhibition of enzyme activity by metallic ions could be due to competition between the exogenous cations and the protein-associated cations, resulting in decreased metalloenzyme activity [38]. Oboh and Ajele [39] and Mohapatra et al. [40] in their previous studies have reported metallic chlorides as most potent activators of amylase. Demirkan [15] also reported inhibition of purified amylase activity from *Bacillus subtilis* by metal ions such as Hg^{2+} , Mn^+ and Cu^{2+} while Na^+ , Zn^+ and Mg^{2+} enhance the enzyme activity. The result obtained from this study contradict Gupta et al. and Mukesh et al. [8,10] who reported enhancement of α -amylase activity from *Bacillus* sp. MNJ23 by calcium and cobalt due to their high affinity and metalloenzymes nature. Stability of α -amylase activity by Ca^{2+} ions at high temperatures has been reported [41]. Enhancement of amylase activity by metallic ions might be due to their ability interact with negatively charged amino acid residues such as aspartic and glutamic acid [42], thereby stabilizing and maintaining the enzyme conformation. Researchers have reported inhibition of amylase activity by Ag^+ , Hg^{2+} , Cd^{2+} , Cu^{2+} , Pb^{2+} , Fe^{2+} , Ni^{2+} , Mn^{2+} and Zn^{2+} ions [10,37,43]. The result obtained from this study agrees with the findings of Igarashi et al. [44] who reported strong inhibition 100% (0% relative activity) of Hg^{2+} ions on alpha amylase from *Bacillus* specie.

Metal	Residual activity (%)
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	Pool A	Pool B
Al ³⁺	37.61	63.54
Na ⁺	147.71	213.54
Ca ²⁺	0	0
K ⁺	21.10	97.92
Hg ²⁺	0	0
Zn ²⁺	156.88	277.08
Control	100	100

Table 2: Effect of metal ions on purified α -amylase.

Conclusion

The results from this study revealed that *Bacillus subtilis* isolated from cassava processing sites exhibited the tendency of producing amylase two conspicuous iso-enzymes from the same medium. Thus, this can be employed in agro-processing industries in starch-utilizing industries and food, textile, pharmaceuticals, biofuel, removal of soil pollutant and decontamination of soil debris.

These results suggest that the activity of the enzyme is fairly higher in alkaline pH, making this enzyme attractive for the detergent industry.

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