Purification and Some Properties of a Thermostable α-Amylase Produced from *Bacillus subtilis* Isolated from the Soil

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Abstract

This work reports the isolation, purification and some properties of a thermostable α-amylase producing Bacillus subtilis isolated from the soil. Soil samples were collected and screened for thermophilic bacterial strains with amylase activity and to examine the amylase heat tolerance potentiality. The isolate was Gram positive, motile rod, bearing terminal endospore. The optimum temperature of amylase activity was at 50°C. Maximum enzyme production occurred at pH 7.0. Corn starch produced the best enzyme activity of 1.82 U/mg protein and was found to be the best carbon source followed by soluble starch (1.21 U/mg protein). Among the five nitrogen sources studied, peptone caused the production of highest enzyme activity of 1.79 U/mg protein, followed by casein (1.38 U/mg protein). Ammonium sulphate proved to be the less suitable nitrogen source for enzyme production. Calcium chloride stimulated enzyme production (2.0 U/mg protein) more than other salts. These characteristics of Bacillus subtilis suggests its promising characteristics for various biotechnological applications.

Key words: α-amylase; *Bacillus subtilis;* Thermophile; Enzyme purification

Introduction

The potential of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Abu *et al*., 2005). Microbial species exist in many environments like extremes of temperature, pH, chemical content and pressure. This existence of microbes is due to certain genetic and or physiological adaptations (Aguilar, 1996). Thermophiles are the organisms which are adapted to grow optimally at high temperatures. The enzymes from thermophiles are known as thermophilic enzymes and these enzymes find a number of commercial applications because of their thermostability and thermoactivity. One of the most attractive attributes of thermophiles is that they produce enzymes capable of catalysing biochemical reactions at temperature higher than those of mesophilic organisms.

Amylases are enzymes which utilize and hydrolyse starch as substrate. On the basis of how amylase breakdown starch molecules and produce glucose, these are classified as α-amylase (which breaks down the bonds at random manner), β-amylase (which acts on the glucose-glucose bonds and remove two glucose units at a time and produce maltose and amyloglucosidase. β-Amylase breaks the non reducing end of the straight chain and produce glucose. Amylase producing industries need a temperature tolerating amylase-producing bacterial strains which are able to produce thermostable amylase, because temperature and pH controls are critical during some stages of production. Amylase can be derived from various sources such as microorganisms (bacteria and fungi), plants and animals. Microbial production of amylase is more fruitful than that of other sources like plants or animals because microbial amylase is fast, cost efficient, easy and moderate for obtaining enzymes of desired characteristics. In general, the enzymes get denatured and lose their activities at temperatures over 50-60^{0}C. However thermostable enzymes allow a higher operational temperature which is advantageous because of higher reactivity, higher stability, higher process yield, lower viscosity and fewer contamination problems (Mozhaev, 1993).

Starch is a carbohydrate source consisting of two molecules amylase and amylopectin. Amylose is formed from chains of glucose linked α-1,4 and amylopectin is formed from α-1,4 linked chains of glucose with 1,6-linked branch points. The amylases are enzymes that work by hydrolyzing the straight chain bonds between the individual glucose molecules that make up the starch chain. A single straight chain starch is called an amylose. A branched starch chain (which can be considered as being built from amylose chains) is called an amylopectin. These starches are polar molecules and have different ends.

Thermostable enzymes therefore have highly attractive and increasing attention because of their potential use in biotechnological processes. Besides, these enzymes also help in ascertaining the major attributes and mechanisms of low proteins achieve extreme thermostability. The most wide spread applications of thermostable α-amylase are in the starch industry. Bacteria and fungal amylases, and in particular the enzymes from the *Bacillus* species, are of special interest for large scale biotechnological processes due to their remarkable thermostability.

The microbial enzymes meet the industrial demands; a large number of them are available commercially and have almost replaced chemical hydrolysis of starch processing industry (Pandey *et*

al., 2000). The major advantage of using microorganisms for the amylase production is economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics. Alpha amylase has been derived from several fungi, yeasts, bacteria and actinomycetes, however, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. Mode of action, properties and product of hydrolysis differ, some what and depend on the source of enzyme. Microorganisms isolated from soil sources are used for enzymes production at industrial level, although the potential for synthesis of several novel enzymes by terrestrial microorganisms have been recognised. The physical and chemical parameters like temperature, pH, incubation time, duration, salt concentration affect the enzyme production and enzyme activity strongly, so optimization of these parameters are very useful for good production of enzyme by microorganisms. Screening of soil microorganisms with higher amylase activity could attract the discovery of novel amylases suitable for new biotechnological and industrial applications (Mohapatra *et al*., 2003). Many microorganisms produce amylase enzyme, most commonly used amylase producing bacteria for industrial production are *Bacillus subtilis*, *Bacillus licheniformis, Bacillus amyloliquefaciens* (Ray *et al*, 2008). Among Bacteria, *Bacillus* species are widely used for thermostable α-amylase production to meet industrial needs. *Bacillus subtilis*, *Bacillus stearothermophillus, Bacillus licheniformis*, and *Bacillus amyloliquefaciens* are known to be good producers of thermostable α-amylase and these have been widely used for commercial production of the enzyme for various applications (Canganella *et al*., 1994). Therefore, the isolation of thermophilic bacteria from natural sources and their identification are very important in terms of discovering new industrial enzymes. In keeping with this view, the soil around cassava processing plant could serve as a good source for new thermophilic microorganisms with novel industrially important properties, so this area was selected for study. The aim of this present research is to isolate a thermostable amylase enzyme-producing *Bacillus subtilis*, to purify the enzyme and to investigate its properties.

Materials and Methods

Sample collection: Soil samples were collected from cassava processing mill around Beach Junction, Nsukka into sterile conical flasks. The pH of the sample was measured with a hand-held pH meter and recorded.

Isolation of bacteria: Soil sample was serially diluted and plated out on Nutrient agar (Oxoid, Ltd. UK) plates. The plates were incubated in an incubator at 55ºC for 24 hours. The bacterial isolates were further sub-cultured by streaking on fresh agar plates. Pure cultures were maintained at 4ºC and identified based on the taxonomic schemes given in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

Screening for amylase production: Bacterial isolates were screened for amylase production by starch hydrolysis test on starch agar plates. Pure bacterial isolates were streaked on the starch agar plate and incubated at 55ºC for 24 hours. After incubation, 1% freshly prepared potassium iodide solution was flooded on the plates. Presence of a clear zone around the bacteria colony indicated hydrolysis of starch.

Medium: The medium for microbial cultivation designated Medium M contained the following: 2% soluble starch (Merck, Darmstadt); 0.5% peptone (Oxoid), 0.2 % Na_2HPO_4 and 0.1% KH_2PO_4. The final pH was adjusted to 6.3 using 0.2 M NaOH. The medium was sterilized at 121ºC for 15 minutes. The inoculum was grown for 24 h in a Gallenkamp orbital incubator at 100 x g at 30ºC. The cells were collected by centrifugation using Gallenkamp Junior centrifuge at 2515 x g for 15 minutes, washed twice with 0.1 M phosphate buffer (pH 6.3) and diluted to an optical density of 0.1 measured in a Spectrumlab 23A spectrophotometer at 600 nm. All media (100 mL) contained in 500 mL conical flasks were inoculated with 1mL of this standard suspension and incubated in a Gallenkamp orbital incubator at 100 x g.

Enzyme assay: Amylase activity was assayed by incubating the enzyme solution (0.5 ml) with 1% soluble starch (0.5 ml) in 0.1 M Phosphate buffer (pH 6.3). After 30 minutes, the reaction was stopped by the addition of 4 ml DNS reagent then heated for 10 min in boiling water bath and cooled in a refrigerator. Absorbance readings were used to estimate the units of enzyme activity from glucose standard curve. One unit of activity was defined as the amount of enzyme that released 1µg of glucose from starch per minute under the assay condition.

Purification of enzyme: Enzyme supernatant fluid was brought to 45% saturation with $(NH_4)_2\ SO_4$ and was recovered by centrifugation at 2515 x g for 15 min. The supernatant was precipitated with cold acetone (30%) followed by centrifugation at 2515 x g for 10 minutes. Ammonium sulphate was further added to 65% saturation followed by centrifugation. The enzyme solution was dialyzed against 0.1 M Phosphate buffer (pH 6.3).

The influence of pH on enzyme activity: The effect of pH on activity of the alkaline α- amylase was determined by using buffer solutions of different pH (acetate buffer, 3.0-5.0; potassium phosphate buffer, 6.0-7.0; Tris-HCl buffer, 8.0-9.0) for enzyme assay. The buffers were used at a concentration of 0.1 mol/l. The pH activity profile of the enzyme was determined by incubating 0.5 mL of the enzyme contained in test tubes with 0.5 mL of 1 % (w/v) soluble starch (Merck) prepared in buffers of different pH values at 55ºC for 2 h. The reaction was terminated by adding DNS reagent and the enzyme activities were determined.

The influence of temperature on enzyme activity: The influence of temperature on enzyme

activity was studied by incubating 0.5mL of the enzyme solution contained in test tube and 0.5 ml of 1 % soluble starch (Merck) solution prepared in 0.1M Phosphate buffer (pH 6.3) for 2 h at various temperatures (35, 40, 45, 50, 55, 60 and 65ºC) in a thermo static water bath (Kottermann, Bremen, Germany). The reactions were stopped by adding DNS reagent. The enzyme activity was therefore determined.

Gel Electrophoresis: Polyacrylamide gel Electrophoresis (PAGE) was carried out as described by Laemmli (1970). The gel casting apparatus was assembled and 12% resolving gel solution prepared. The comb was then carefully inserted, after which the stacking gel solution was loaded taking care not to introduce air bubbles around the comb and the stacking gel allowed to polymerize completely. The glass and gel sandwich were then removed from the casting base, and the sandwich placed into the electrophoresis tank taking note of the terminals. The bottom of the electrophoresis tank was then filled with electrophoresis buffer to the fill level mark on the tank. The gel was then stained after this process with Coomasie Brilliant Blue R stain, and destained until the protein bands were seen. Equilibration of the gel was done in 500 mL of the storage solution for an hour and the molecular weights of the proteins determined.

Assay procedures: Protein content was estimated by the method of Lowry *et al.*, (1951) using bovine serum albumin (Sigma-Aldrich) as a standard. Reducing sugar was determined by the dinitrosalicylic acid (DNS) method of Miller (1959) using 50-200 µg glucose as standard.

Results and discussion

The effects of various carbon sources on α-amylase production from *Bacillus subtilis* was evaluated by the addition of various carbon sources namely, sucrose, xylose, soluble starch, corn starch and potato starch at 1% concentration. Among these sources of carbon, corn starch caused the production of the best enzyme activity of 1.82 U/mg protein. It was closely followed by soluble starch with 1.21 U/mg protein enzyme activity while potato starch caused the production of the lowest enzyme activity (0.76 U/mg protein) (Fig 1). The effects of different carbon sources suggested that the isolated α-amylase is an inducible enzyme and its rate of induction depends on the type of carbon source present in the medium for microbial growth and enzyme production. The isolated enzyme was able to degrade raw starches and would be useful in many food industries (Singh *et al.*, 2012).

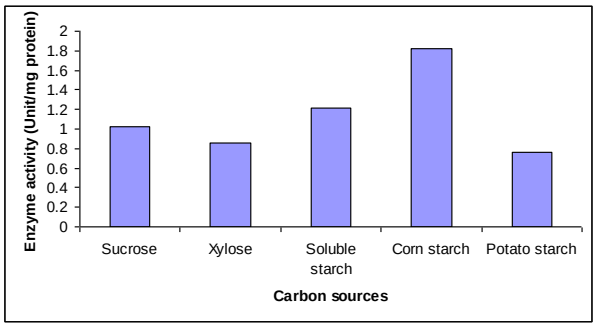

Fig 1: Effects of carbon sources on α-amylase activity

The effect of nitrogen sources was investigated on the production of α-amylase enzyme by *Bacillus subtilis* and is shown in Fig. 2. Among the five nitrogen sources studied, peptone caused the production of highest enzyme activity of 1.79 U/mg protein, followed by casein (1.38 U/mg protein). Ammonium sulphate proved to be the least suitable nitrogen source with an enzyme activity of 0.77 U/mg protein. It has been shown that organic nitrogen sources have a superior stimulating effect on amylase production than inorganic nitrogen sources (Hewitt and Solomons, 1996). Various other organic nitrogen sources have also been reported to support maximum α-amylase production by *Bacillus* species (Rasooli *et al*, 2008).

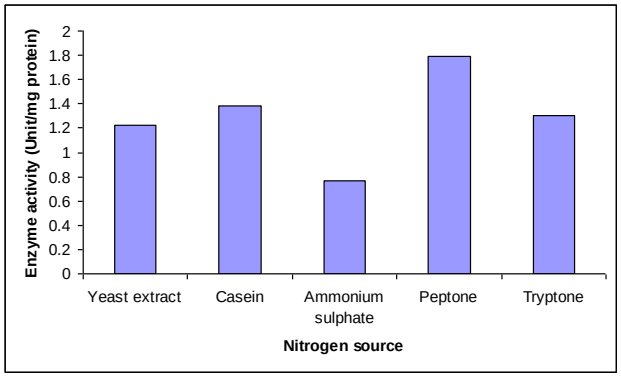

Fig 2: Effects of nitrogen sources on α-amylase activity

The effect of metal salts on α-amylase production from *Bacillus subtilis* was studied by adding different metal salts namely, $CuSO_4$, $ZnSO_4$, $MgSO_4$, $FeSO_4$, and $CaCl_2$ in the medium at 0.01% concentration (Fig. 3). Calcium chloride supported best enzyme activity of 2.0 U/mg protein, followed by magnesium sulphate (1.50 U/ mg protein). Iron sulphate, copper sulphate and zinc sulphate had inhibitory effects on α-amylase production. Most α-amylase are known to be metalloenzymes, therefore supplementation of salts of certain metal ions provide good growth of bacteria and thereby better enzyme production. Ca^{2+} had significant effects on the metabolism and physiology of bacteria and that was also found to be effective on amylase production (Deshpande and Cheryan, 1984).

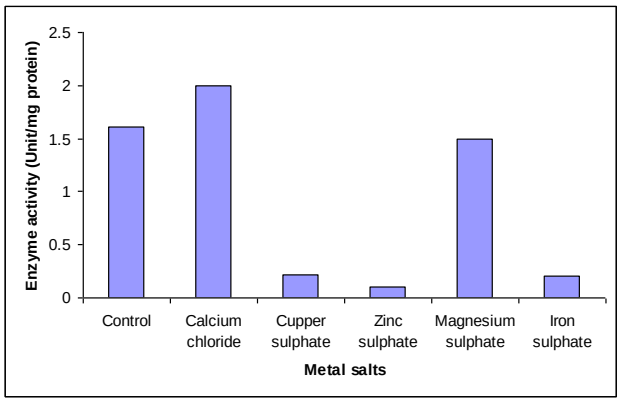

Fig 3: Effects of metal ions on α-amylase activity

Enzyme activity was investigated at various pHs (Fig. 4). Optimum pH for amylase production was pH 7.0 after which there was a decline at pH 8.0 and 9.0, with enzyme activities of 1.8 U/mg protein and 1.42 U/mg protein respectively. The effect of pH on α-amylase activity indicated that the amylase is active in the wide pH range of 3.0 – 9.0. This suggests that enzyme would be useful in processes that require a wide pH range from acidic to slightly alkaline. Different organisms have different pH optima, and decrease or increase in pH, on either side of the optimum value results in poor microbial growth (Nwokoro and Odiase 2015). These results suggest that there is a stimulation of enzyme synthesis at neutral pH and that the higher enzyme production at this pH was a result of increased cell growth. The pH of 6 and 7 have been reported for normal growth and enzyme production in *Bacillus* strain isolated from the soil (Mishra and Behera 2008). The pH values of media used for α – amylase production were 7.0 (Singh *et al*., 2012); 9.0 (Burhan *et al.,* 2003); 8.0 (Shanmughapy 2009). Amylase production by *Streptomyces aureofaciens* 77 (Shatta *et al*., 1990), *Streptomyces erumpens* MTCC 7317 (Kar and Ray, 2008) and *Streptomyces rimosus* (Yang and Wang, 1999) increased gradually as initial pH values ascend from 5.0 to 7.0.

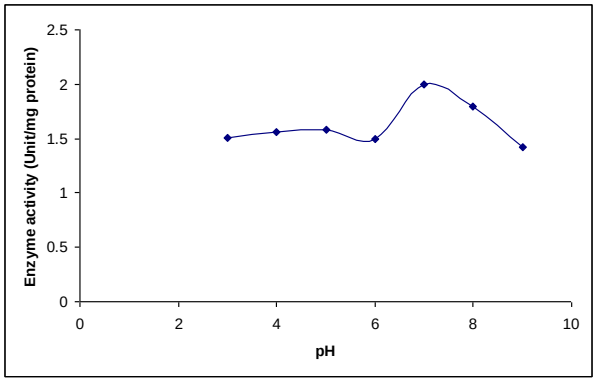

Fig 4: Effects of pH on α-amylase activity

Temperature had a considerable effect on the activity on α-amylase by *Bacillus subtilis* (Fig. 5). The results showed that enzyme activity increased with temperature and had highest activity at 50ºC. At 60ºC there was a decline and at 65ºC, a very sharp decline was observed. When the temperature was increased, from 40ºC, a gradual rise in enzyme activity was discovered and was highest at 50ºC, after which a decline was observed with further increase in temperature. At 40ºC, the enzyme activity was 1.26 U/mg protein. At 45ºC this increased gradually to 1.30 U/mg protein. Enzyme activity was observed to be 1.41U/mg protein at 50ºC which was the highest activity. Temperatures used for α - amylase production were 55ºC (Igarashi *et al.,* 1998); 50ºC (Boyer and Ingle, 1974); 60ºC (Kim *et al.,* 1996) and 40ºC (Zhao *et al.,* 2008).

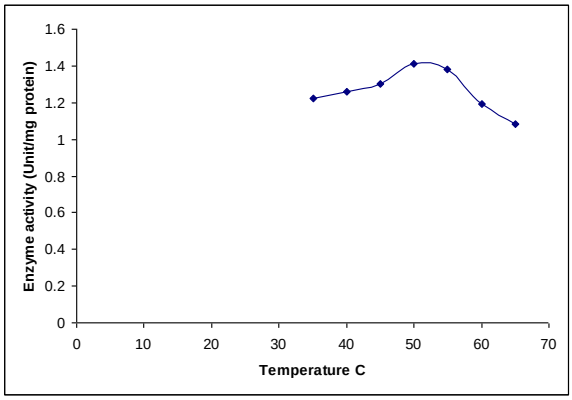

Fig 5: Effects of temperature on α-amylase activity.

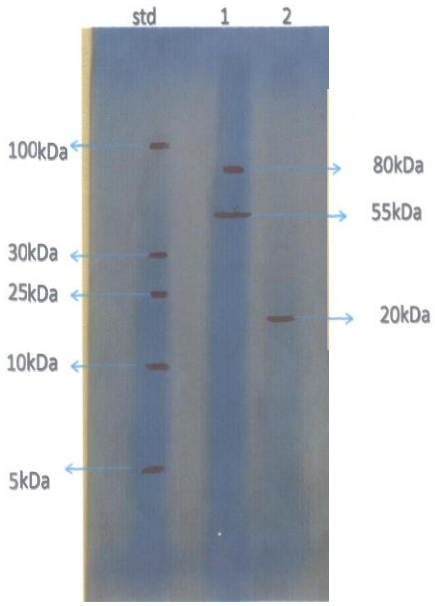

Fig 6: SDS-PAGE analysis: Lane std contains the molecular mass weight marker; Lane 1 contains crude enzyme and Lane 2 contains purified alpha amylase.

Relative molecular mass of 20 kDa was observed for α-amylase (Fig 6). This is very close to the molecular weight of 28 kDa for a thermostable raw starch-digesting α-amylase from *Geobacillus thermoleovorans* subsp. *stromboliensis* subsp. nov. (Finore *et al*., 2011).

Conclusion

This study reports biological production of thermostable α-amylase by soil bacteria *Bacillus subtilis* isolated from cassava processing site. The bacterium produced high levels of thermostable α-amylase with characteristics such as ability to grow under extreme temperatures which makes it suitable for application in starch processing and other food industries. Supplementation of the medium with salts of certain metal ions, provided good growth of the bacterium and thereby better enzyme production. Enzyme production was found maximum at a temperature of 50ºC and pH 7.0. Among these sources of carbon, corn starch caused the production of the best enzyme activity of 1.82U/mg protein. Potato starch caused the production of the lowest enzyme activity (0.76 U/mg protein). Peptone caused the production of highest enzyme activity of 1.79 U/mg protein, followed by casein (1.38 U/mg protein). Ammonium sulphate proved to be the less suitable nitrogen source for enzyme production.

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