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PRODUCTION OF A THERMOSTABLE A-AMYLASE AND ITS ASSAY USING *BACILLUS LICHENIFORMIS* ISOLATED FROM EXCAVATED LAND SITES IN IBADAN, NIGERIA.

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ABSTRACT
Screening for amylolytic properties from obtained isolates was carried out on starch agar plates while production and characterization of α-amylase was carried out using submerged fermentation. The isolated organism was identified as Bacillus licheniformis. Physiological studies on the isolate showed that temperature of 55ºC and pH 7 was optimum for growth of the organism while fructose (1%), peptone (0.5%), pH 7 and temperature of 60ºC supported optimum amylase production. Crude characterization of α-amylase revealed optimum amylolytic activity at pH 7 and a temperature of 70ºC. The isolated Bacillus licheniformis produced thermostable α-amylase with characteristics suitable for use in starch processing other industries.
Keywords: Bacillus licheniformis, thermostable α-Amylase, Excavated land, Characterisation

INTRODUCTION

The role of enzymes in many processes has been known for a long time. With better knowledge and purification of enzymes, the number of applications has increased, and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged. Thermostable enzymes are stable and active at temperatures which are even higher than the optimum temperatures for the growth of the microorganisms (Saboto *et al*., 1999).

Thermostable enzymes are gaining wide industrial and biotechnological interest due to the fact that their enzymes are better suited for harsh industrial processes (Leuschner and Antranikan, 1995). One extremely valuable advantage of conducting biotechnological processes at elevated temperatures is reducing the risk of contamination by common mesophiles. Allowing a higher operation temperature also has a significant influence on the solubility and bioavailability of organic compounds and thereby provides efficient bioremediation. Other advantages of carrying out industrial processes at elevated temperatures include higher reaction rates due to a decrease in viscosity and an increase in diffusion coefficient of substrates and favorable equilibrium displacement in endothermic reactions (Mozhaev, 1993; Krahe *et al*., 1996; Kumar and Swati, 2001). Such enzymes could also be used as models for the understanding of thermostability and thermoactivity, which is also useful for protein engineering (Haki, and. Rakshit, 2003).

The starch industry is one of the largest users of enzymes for the hydrolysis and modification of useful raw material (Poonam and Dalel, 1995). The starch polymer, like other polymers, requires a combination of enzymes for its complete hydrolysis. These include α-amylases, glucoamylases or β-amylases and isoamylases or pullulanases. Excavation can be defined as the act of making hollow, by cutting, scooping, or digging out a part of a solid mass (Chao-Hui *et al*., 2010). It can also mean the process of removing rock or earth from a solid, broken, or unconsolidated layer by means of an excavator, bulldozer, scraper, or similar machine.

Thermophilic bacteria are widely distributed in nature (Allan and Frank, 1894). Members of this aerobic spore-forming genus *Bacillus* are commonly isolated from many types of soil at a range of depths and altitudes, and under various climatic conditions (Stabb *et al*., 1994; Von *et al*., 1999). *Bacillus licheniformis* α-Amylase (BLA) is among the most extensively studied thermostable natural enzymes used in starch technology and in biotechnological processes. Although it is encoded by a non-thermophilic bacterium, it remains active for several hours at temperatures over 90°C under conditions of industrial starch hydrolysis (Lee *et al*., 2006).

Thermophilic organisms have been known to enhance different industrial processes involving production of different metabolites both primary and secondary better than mesophiles. Therefore, the isolation of some thermophiles within our indigenous environment will be of benefit to different biotechnological processes. This is believed will shorten production time as well as improve quality of the end product.

MATERIALS AND METHODS

Selection, isolation and identification of bacterial strain:

Soil samples were collected from two excavated land sites; of which one of the sites is located at Ibadan toll gate, and the last sampling site is from the Ratcon excavation site (along new Ibadan-Ilorin road, Nigeria).

Excavated land sites were sampled without bias with a soil sampler (auger). The samples were transported to the laboratory in sterile containers for further analysis.

Trypticase soy agar and nutrient agar were used for the isolation of bacteria from soil samples. The media were prepared according to the manufacturers' instruction. After homogenization for 10minutes, the media was then sterilized at 121°C for 15minutes in an autoclave. Soil samples were heated at 50°C for 10 minutes after which 10 fold Serial dilutions was carried out on samples and then higher dilutions were plated. The bacteria obtained were identified by standard microbiological techniques based on their morphology, colour, arrangement of vegetative cell, possession of spores and biochemical examination.

Pure bacterial isolates were screened for amylase production on starch agar plates containing 1% soluble starch, 0.2% yeast extract, 0.5% peptone, 0.1% $MgSO_4$, 0.1% NaCl, 0.02% $CaCl_2$ and 2% agar at pH 7.0 (Iraj *et al.*, 2008). The cultures were inoculated by streaking on the plates and incubated at 55°C for 48h. Amylolytic isolates were selected by flooding the agar plates with Gram's iodine solution.

Preparation of Inoculum

One to five loopful from a consistently growing 24hours old slant culture of the chosen isolates was aseptically washed in 10ml sterile distilled water and mixed thoroughly to obtain a uniform mix and dispersion of aggregate cells (Egharevba *et al.*, 2010).

Production of α-Amylase in Submerged Medium

Amylase production was carried out at 55°C in a basal medium containing soluble starch (1%), maltose (1%), $(NH_4)_2SO_4$ (0.2%), $CaCl_2$ (10^{-4}M), K_2HPO_4 (10^{-1}M), $MgCl_2.6H_2O$ (0.02%), pH 7. The medium was inoculated with 2% (v/v) of an overnight culture of the isolate and incubated at 55°C in incubator. After 24hours, samples were harvested and the cells were separated by centrifugation (10,000 Revolution /20 min) at 4°C in a Hitachi Centrifuge type cr21g ii. The supernatant was collected in a clean Erlenmeyer flask and then used for enzyme assay and characterization studies.

α-Amylase Assay

The activity of α-amylase was assayed by incubating 0.5 ml enzyme with 0.5ml soluble starch (1% w/v) prepared in 0.1 M sodium phosphate buffer (pH 7.0). After incubating the mixture at 55°C for 60 min the reaction was stopped by the addition of 2ml of 3-5-Dinitrosalicylic acid reagent (Bernfeld, 1951) and absorbance was measured in a Labda 25 UV/visible Spectrophotometer at 600nM . One unit (U) is defined as the amount of enzyme which releases 1 mMol of reducing end groups (Maltose)min^{-1} in 0.1 M sodium phosphate buffer (pH 7.0) with 0.5% (w/v) soluble starch as substrate at 55°C.

Optimization of Culture Conditions for α-Amylase production

The basal medium was supplemented with yeast extract, casein, KNO_3, peptone and urea, each at a concentration of 0·5% (w/v). The effect of carbon source was also investigated by supplementing the basal medium with glucose, sucrose, fructose, lactose and galactose each at a concentration of 1% (w/v). In all cases, the broth was inoculated with 2% (v/v) of an overnight culture of the isolate and α-Amylase assay was determined using a Labda 25 UV/visible Spectrophotometer at 600nM after centrifugation (Iraj *et al.*,2008).

Effect of pH on α-Amylase Production and activity

The effect of varying pH values (pH 5.0 – pH 8.0) on α-Amylase production was investigated by preparing the production media at different pH while the pH optimum of the enzyme was determined by varying the pH of the assay reaction within pH 5.0-8.0 using the following buffers (0.1M) sodium acetate (pH 5.0-5.5) and sodium phosphate (pH 6.0-8.0).The residual enzyme activity was measured using a Labda 25 UV/visible Spectrophotometer at 600nM.

Effect of Temperature on α-Amylase production, activity and stability

The effect of varying temperatures (40°C - 80°C) on α-Amylase production was investigated by incubating the production media at different temperatures while the temperature optimum of the enzyme was determined by varying the temperatures (40-80°C) of the enzyme incubation. The effect of temperature on α-amylase stability was determined by measuring the residual amylolytic activity after 24 h of pre-incubation in 0.1 M sodium phosphate buffer (pH 7.5), at temperatures ranging from 30-100°C.

RESULTS AND DISCUSSION

The isolated *Bacillus* sp was characterized based on cultural, biochemical and morphological appearances and it was confirmed to be *Bacillus licheniformis* (Table 1).

Members of this genus have endospores that are resistant to chemical and physical agents. The developmental cycle of endospore formation, have been reported by Rahman *et al.* (2003) and Kenneth, 2009. Endospore formation universally found in the group could be responsible for their survival in different soil environment regardless of the soil temperatures. α-Amylase produced by thermostable *Bacillus* specie in this study could be one of the mechanisms for survival as their ability to produce such enzyme explains their survival and growth in various substrate-dependent environments.

Figure 1 shows the effect of different carbon sources on the production of α-amylase. Among the different carbon sources supplemented in the production medium, fructose gave the highest α-amylolytic activity, although a slight reduction in amylolytic activity was seen when galactose was used as a supplement but lactose was seen to repress amylase activity.

This suggested that fructose was not a repressor of α-amylase enzyme in this study unlike the observed catabolic repression by glucose and fructose in *Bacillus coagulans* reported by Babu and Satyanarayana, (1993). The result of this study is in accordance with reported α-amylases from *B. thermooleovorans* (Narang and Satyanarayana, 2001) and *B. cereus* (Anto *et al.,* 2006).

Figure 2 shows the effect of different nitrogen sources on the production of α-amylase. When different nitrogen sources was supplemented in the production medium, peptone gave the highest α-amylase activity, although slight reduction in amylolytic activity was observed when urea and potassium nitrate (KNO_3) were used as supplements. Addition of nitrogen sources has been reported to have an inducing effect on the production of various enzymes including α-amylase (Pedersen and Nielsen, 2000). Similar observations were noticed in case of amylase production by Lin *et al.* (1998); Bajpai and Bajpai (1981); Gangadharan *et al.* (2006) and Saxena *et al.* (2007).

Table 1: Cultural and morphological identification for *Bacillus licheniformis*

Characteristics	***B. licheniformis***
Spores	+
Catalase	+
Anaerobic growth	+
Voges Proskauer	+
Gram Reaction	+
Acid from	
Glucose	+
Arabinose	+
Mannitol	+
Fructose	+
Galactose	-
Glycerol	+
Lactose	-
Sucrose	+
Hydrolysis of	
Caesin	+
Starch	+
Utilization of citrate	+
Nitrate reduced to nitrite	+
Formation of indole	-
Growth at	
40	+
45	+
50	+
55	+
60	+
65	+
70	-
Growth at pH	
4	+
5	+
5.5	+
6	+
6.5	+
7	+
7.5	+
8	+
Growth at NaCl	
0.2µM	+
0.4 µM	+
0.6 µM	+
0.8 µM	+

KEY: + = Positive, - = Negative

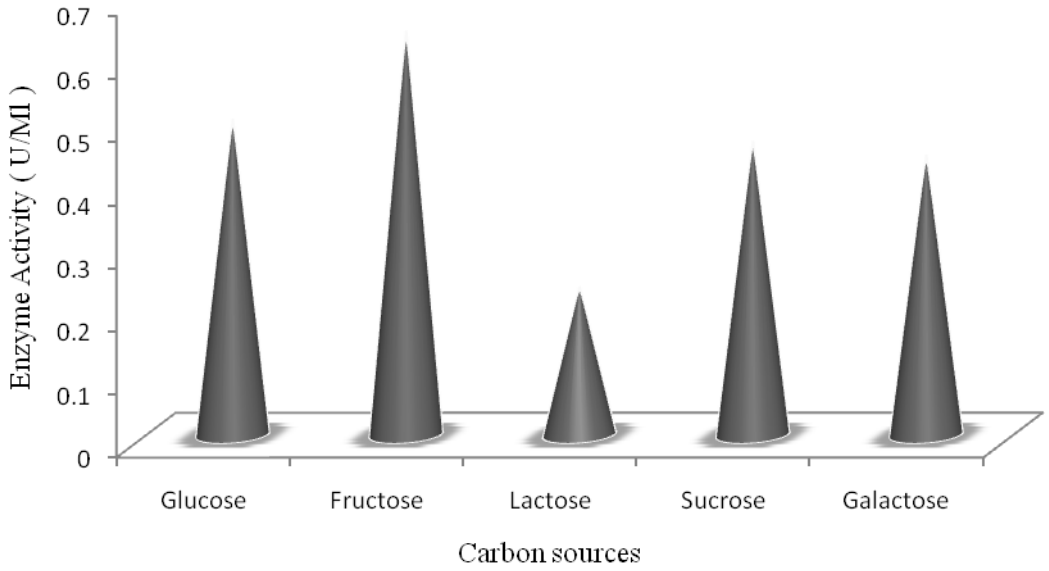

Figure 1: Effect of various carbon sources on α-amylase production

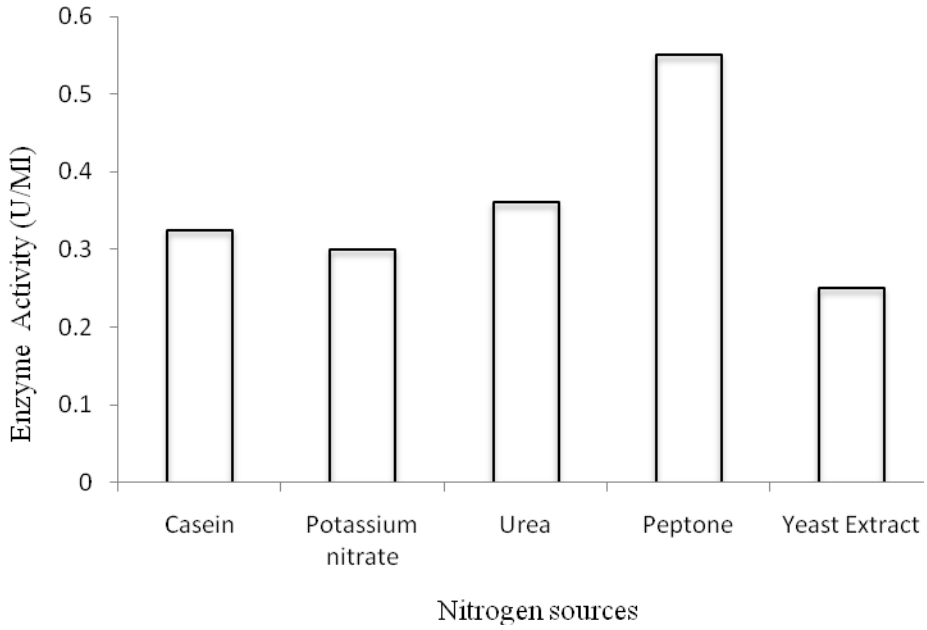

Figure 2: Effect of various nitrogen sources on α-amylase production

Amylolytic activity gradually increased as the pH of the production medium was adjusted from pH 5 to pH 7 (Figure 3). Amylolytic activity was highest when the pH of the production medium was at pH 7. pH of the growth medium plays an important role by inducing changes in enzyme secretion. In this study, optimum enzyme activity at pH 7.0 could be as a result of enhanced bacterial cell growth at that pH, although pH 5.0 to 8.0 supported amylase production. Similar findings have been reported by Bajpai and Bajpai (1989) for growth and production of amylase by *B. licheniformis* TCRDC-B13 and Haq *et al.* (2005) for production of amylase by *B. subtilis.*

The effect of pH on the activity of α-amylase is also shown in Figure 3. The activity of amylase increased at a steady pace as the pH of the substrate increased from pH 5 to pH 7.Highest amylase activity was observed at pH 7, although Vihinen and Mantsala (1989) reported that the pH optima of α-amylases vary from pH 2-pH 12, the result of this study is in accordance with Iraj *et al.* (2008) who reported that *B. licheniformis* Shahed-07 had an optimum activity at pH 7.0.

Bacillus licheniformis was capable of maximum amylase production at 60°C (Figure 4); this implies that temperature plays a major role in amylase production affecting the growth of the organism and production of metabolites. Metabolites are produced when favorable conditions are available. These results are in accordance with the report of Lin *et al*. (1998) and Fred and George (1925). As temperature of the reaction mixture was increased (from 30ºC-70ºC), there was also a steady increase in amylolytic activity such that the highest amylase activity was observed at 70ºC, but a further increase to 80ºC led to decrease in amylolytic activity. The increase in amylase activity with temperature can be attributed to increase in the collision between substrate and enzyme and further increase in temperature beyond 70ºC destabilized the 3-Dimensional structure of the enzyme resulting in its denaturation. This agrees with the report of Carlos *et al*. (2000) and Sodhi *et al*. (2005) who reported a reduction in enzyme activity at temperatures above 60°C. The optimal temperature for amylase production and growth of the organism was observed to be different in this study. This agrees with the report of Lin *et al*. (1998); although Teodoro and Martins (2000) reported that optimum temperatures for amylase production and growth were same for some *Bacillus* spp.

The stability of α-amylase to temperature is seen in Figure 4. α-Amylase was observed to be stable over a temperature range of 30ºC to 70ºC. A gradual increase in stability was observed as the temperature was increased gradually from 30ºC to 70ºC, with the enzyme been more stable at 70ºC although, amylase stability slightly decreased as the temperature was further increased to 80ºC This agrees with the report of Asgher *et al.* (2007) who reported that α-amylase was highly stable for 1 hour at 60˚C and 70˚C, while at 80˚C and 90˚C, 12% and 48% of the original activities were lost, respectively. Iraj *et al*. (2008) also reported reduction in enzyme activity at temperatures above 70ºC. The stability of the enzyme could be due to its genetic adaptability to carry out its biological activities at a higher temperature (Allan and Frank, 1894). This thermostability is an important factor for the use of amylolytic enzymes in starch-processing industries (Iraj *et al*., 2008).

When the starch concentration was varied (Figure 5), there was a sharp increase in amylase activity when starch concentration was increased from 1% to 2%, but a gradual increase in amylase activity was observed as the starch concentration was further increased to 3%, although amylolytic activity was observed to be highest at 5% substrate (starch).

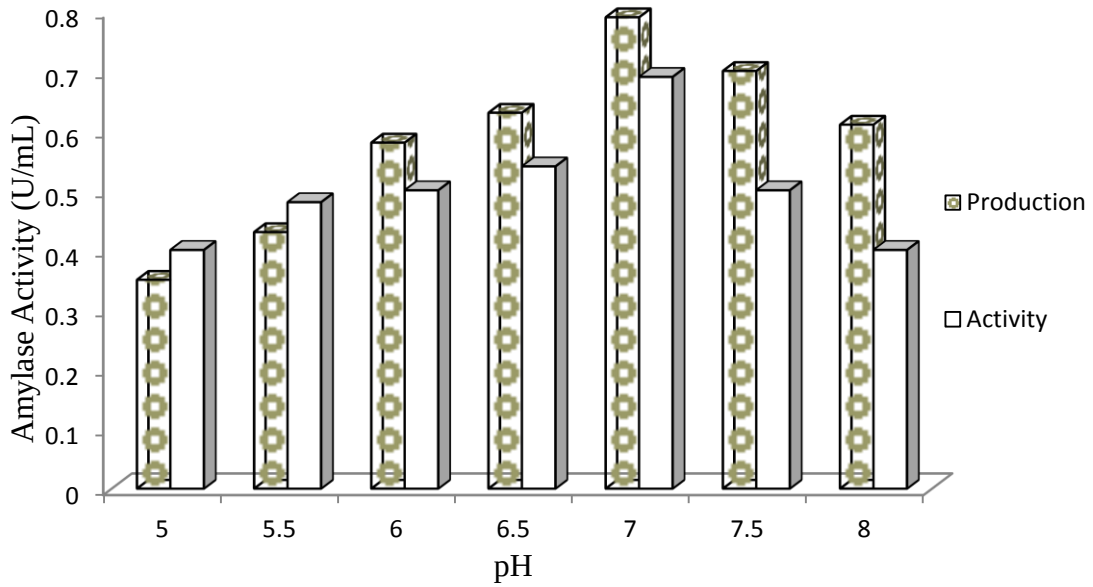

Figure 3: Effect of pH on the production and activity of α-amylase

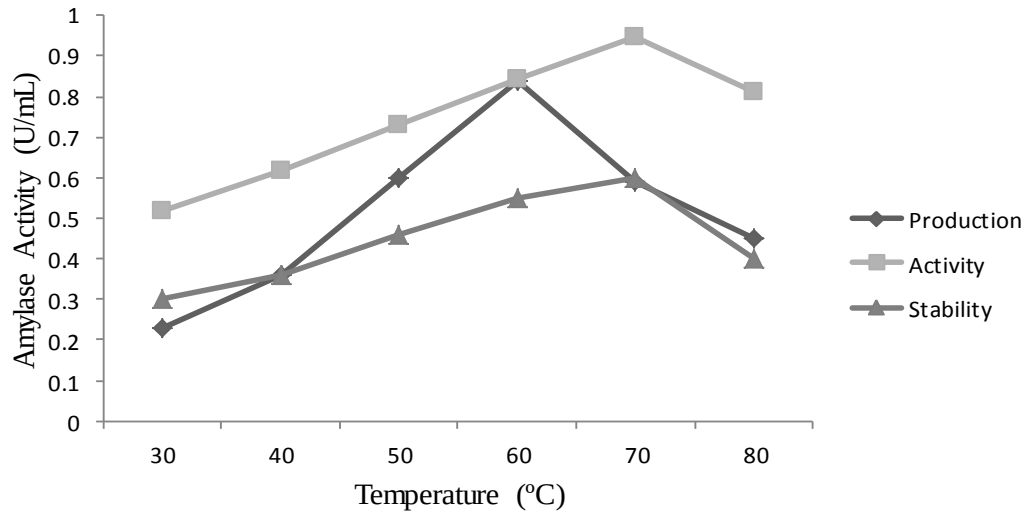

Figure 4: Effect of temperature on production, activity and stability of α-amylase

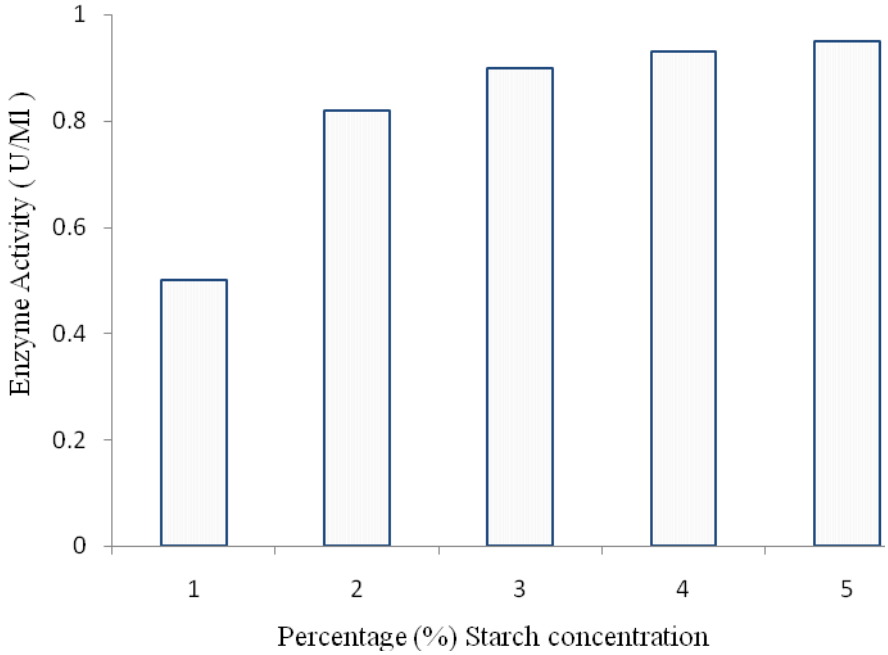

Figure 5: Effect of substrate concentration on α-amylase production of *B. licheniformis*

In conclusion, the findings of this work indicate that *B. licheniformis* produced thermostable α-amylase with characteristics suitable for starch processing and other industries. It is expected that further optimization will yield enhanced enzyme production for commercial processes.

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