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# Screening for the Production of Thermostable Amylase from Bacterial Isolates Recovered From Raw Honey.

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**ABSTRACT**

Twenty sample of bottled raw honey were analyzed for the presence of thermostable amylase producing bacteria by streaking 0.1ml aliquots of suitable dilutions on Nutrient Agar in duplicates. The plates were incubated at $37^0$C for 24 hours. The isolates were sub-cultured and stored in slants for further use. Amylase production was detected by streaking on Starch agar plates and incubating at $55^o$C for 48 hours after which the plates were flooded with iodine solution. The amylolytic activity was confirmed by clear zone around the bacterial colonies. Amylase production was carried out in a basal medium containing soluble starch. *Bacillus licheniformis* was capable of maximum amylase production at $40^o$C at pH 7.0 (254µ/ml). Amylase production was highest in fructose (190µ/ml) while peptone was found to be the most suitable nitrogen source for the enzyme activity (17µ/ml). Thermostable α-amylase produced by *Bacillus lichenformis* had characteristics which makes it suitable for industrial applications and starch processing.



## Introduction

Enzymes act as biocatalysts for a wide variety of chemical reactions. Microbial enzymes are generally the most suitable for commercial applications. Amylase can be divided into α-amylase, which cleaves the bonds in the interior of the substrate (endoamylase) and β-amylases which act on the reducing extremities of the substrate molecules. Amylases have been reported to occur in microorganisms, although they are also found in plants and animals (Aro *et al.*, 1992). α-amylases are enzymes that break the bonds of polysaccharides that have ten (or) more unit of D-glucose united by α-1,4 glucosilic linkage (Kashyap *et al.*, 2002, Pandey 2003). The attachment occurs in a non-selective form (as endo enzyme) on different points of the chain simultaneously, so that the first hydrolysis products are oligosaccharides of 5 to 7units of glucose. The amylase producing bacteria (such as *Bacillus subtilis, B. Cereus, B. Lichenformis, B. Amyloloquefaciens* and *B. Megaterium)* and fungi (such as *A. Niger, Pennicillum, Cephalosporium, Neurospora and Rhizopus*) are major amylase producing microorganisms. Microorganisms such as yeasts, fungi, bacteria, actinomycetes and algae are effective producers of amylase (Pandey, 2003). The industrial use of enzymes often requires enzymatic reaction to be conducted at higher temperatures, generally under those conditions productivity improved with less microbial contamination. Therefore, thermostable enzymes have been the heat of numerous studies involving in the elucidation of thermal denaturation mechanism and development of rational strategies for the enhancement of enzyme thermostability (Santos *et al.*, 2003).

Honey, a natural sweet substance produced by honey bees from nectar or blossoms or from the secretion of living parts of plants or excretion of plants can be considered as a reservoir of microbes that withstand the concentrated sugar level in honey. Honey amylases have the potential of degrading food starch and cause viscosity loss. The osmophilic feature of honey could hypothesise the presence of moderate halophilic bacteria with new properties. Recently, a considerable attention has been given to enzymes produced by moderately halophilic microorganisms and their biotechnological potentials (Ventosa *et al.*, 1998, Xue *et al.*, 2008). Enzyme with optimal activity at high salt concentrated are useful for many harsh industrial processes where concentrated salt solutions otherwise inhibits many enzymatic conversion (Amoozegar *et al.*, 2003, Hutcheon *et al.*, 2009).

### 1.1 Justification of Study

Enzymes are bioactive and biological catalyst that aids the rate of reactions. Hence, production of thermostable amylase enzyme from bacterial isolates recovered from raw honey will be a useful research which its application will be widely utilized in the food industry. Also the ability of producing an enzyme that can withstand moderate and high temperature will be an interesting contribution to the relevant industrial processes. Hence, this work was aimed at screening of raw honey samples for thermostable α-amylases producing bacteria.

## 2.0 Materials and Methods

### 2.1 Sample collection.

Twenty bottles of raw honey samples were purchased from National Root Crops Research Institute, Umudike, Abia State and taken to the laboratory in a cooler containing ice cubes. The samples were analyzed with two hours of collection of the samples.

### 2.2 Isolation of bacteria.

Serially diluted raw honey samples were inoculated by streaking 0.1ml aliquots of suitable dilutions on Nutrient Agar

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in duplicates. The plates were incubated at 37ºC for 24hours. The isolates were sub-cultured and stored in slants for further use.

**2.3 Demonstration of Amylase Activity**

This was carried using the method described by Balkan and Ertan (2007). The isolated bacteria were streaked on Starch agar plates and incubated at 55ºC for 48 hours after which the plates were flooded with iodine solution. The amylolytic activity was confirmed by clear zone around the bacterial colonies.

**2.4 Identification of amylase-producing bacterial isolates.**

The bacterial isolates that demonstrated amylase producing activities were characterized based on cultural, morphological and biochemical tests (sugar fermentations). They were identified using Bergey's Manual of Systematic Bacteriology (Lacey and Cross, 1986).

**2.5 Production of α-amylase in submerged medium.**

Amylase production was carried out in a basal medium containing soluble starch (1%) maltose (1%), Ammonium sulphate (0.28), calcium chloride ($10^{-4}$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 55ºC in an incubator. After 24hours, samples were harvested and the cells were separated by centrifugation at 5000rpm for 20minutes in a centrifuge. The supernatant was collected in a clean Erlenmeyer flask and then used for enzyme assay and characterization studies.

**2.6 Amylase assay.**

This was carried out using the method of Bernfeld (1995). The activity of α-amylase was assayed by incubating 0.5ml enzyme with 0.5ml soluble starch (1% w/v) prepared in 0.1m sodium phosphate buffer (pH 7.0). After incubating the mixture at 55ºC for 60 minutes, the reaction was stopped by the addition of 2ml of 3-5-Dinitrosalicyclic acid reagent and the absorbance was measured in a UV visible spectrophotometer at 600m.

**2.7 Effect of temperature on α-Amylase production and stability.**

The effect of varying temperatures (35ºC-45ºC) on amylase production was investigated by incubating the production media at different temperatures while temperature optimum of the enzyme was determined by varying the temperatures (35ºC-45ºC) of the enzyme incubation. The effect of temperature of α-amylase stability was determined by measuring the residual amylolytic activity after 24 hours of pre-incubation in 0.1m sodium phosphate buffer (pH7.5) at temperatures ranging from 35 - 45ºC.

**2.8 Effect of pH on α-amylase production and activity**

The effect of varying pH values (5.0-10.0) 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 5.0-10.0 using the following buffers (0.1M) sodium acetate (pH5.0-5.5) and sodium phosphate (pH6.0-10.0). The residual enzyme activity was measured using a UV visible spectrophotometer at 600nm.

**2.9 Effect of culture medium on α-amylase production.**

The method described by Iruj *et al.*, (2008) was used. The base 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 in duplicate with 2% (v/v) of overnight culture of the isolate and α-Amylase assay was determined using a UV visible spectrophotometer at 600nm after centrifugation.

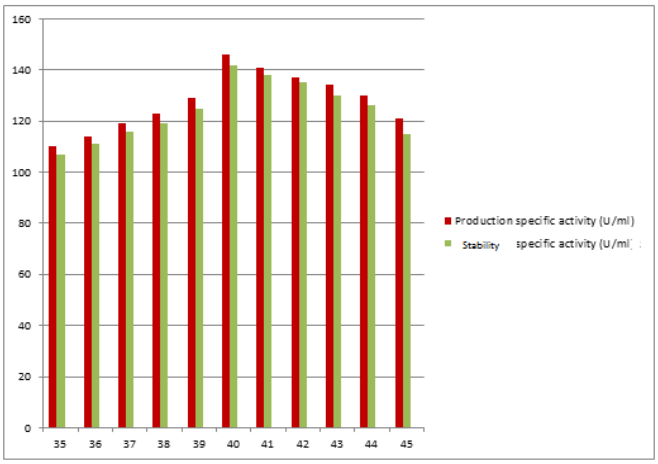


**Fig 1. Effect of Temperature on α-amylase Production and stability.**

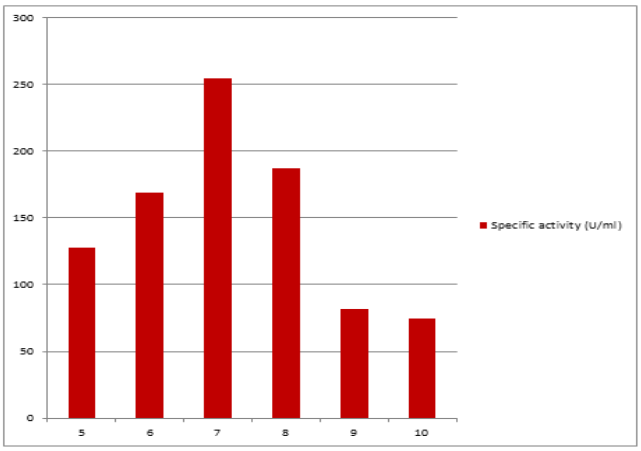


**Fig 2. Effect of pH on α-amylase production.**

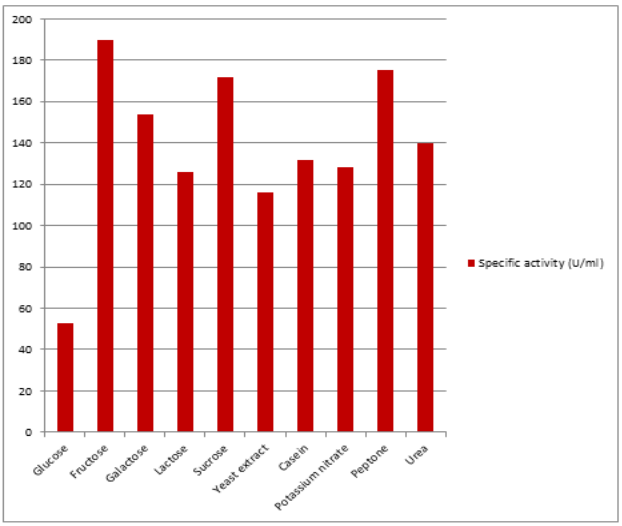


**Fig 3. Effect of culture medium on α-amylase production.**

**3.0 Result and discussion**

The research work shows that *Bacillus licheniformis* was capable of maximum amylase production at 40ºC, thus implying that temperature plays a major role in amylase production affecting the growth of the organism and production of metabolites. Metabolites are produced when favourable conditions are available. As temperature was increased from 33ºC to 40ºC, there was also a steady increase

in amylolytic activity and production such that the highest amylase activity was observed at 40°C, but a further increase to 41°C led to decrease in amylolytic activity and amylase production. The increase in amylase activity and production with temperature might be attributed to increase in collision between substrate and enzyme and further increase in temperature beyond 40°C possibly destabilized the 3-Dimensional structure of enzyme resulting in its denaturation. The optimal temperature for amylase production and growth of *Bacillus licheniformis* were the same. This agrees with the report of Teodoro and Martins (2000) who reported that optimum temperatures for amylase production and growth were same for some *Bacillus* spp. The stability of α-amylase to temperature was seen to increase as temperature was increased from 35°C to 40°C with the enzyme being more stable at 40°C, amylase activity slightly reduced. The stability of the enzyme could be due to its genetic adaptability to carry out it biological activity at higher temperatures. This thermostability is an important factor for the use of amylolytic enzymes in starch processing industries (Iraj *et al*., 2008).

The maximum amylase production was observed at pH 7.0 (254 µ/ml) and minimum amount of amylase production was observed at pH 10.0 (75 µ/ml). pH of the production medium plays an important role by inducing changes in enzyme secretion. In this study, the optimum enzyme production at pH 7.0 might be attributed to enhanced cell growth at this pH. However, it was observed that amylase production occurred at the pH range between 5.0-10.0, although a decrease started from pH 8. Ellaiah *et al.,* (2002) stated that at high pH, the metabolic action of a bacterium may be suppressed and thus it inhibits enzyme production. Optimum pH of 7.0 for amylase production has been reported by Haq *et al.,* (2005) and Sivakumar *et al.,* (2012) in *Bacillus subtilis a*nd *Bacillus cereus* respectively.

Among the different carbon sources supplemented in the production medium, the maximum amylase production was recorded in fructose (190µ/ml) and minimum amylase production was recorded in glucose (53µ/ml). This suggested that glucose repressed amylase activity. A similar observation was made by Sivakunar *et al.,* (2012) for amylase activity of *Bacillus cereus* while Heseltine *et al.,* (1996) observed that glucose repressed the production of amylase in the hyperthermophilic bacterium: *Sulfolobus solfataricus*. The highest *α*-amylolytic activity observed due to supplementation with fructose agrees with the findings of Narang and Satyanarayana (2001) and Anto *et al.,* (2006) for amylase activity of *Bacillus thermodeovorans* and *Bacillus cereus* respectively.

In the present study, peptone was found to be the most suitable nitrogen source for *Bacillus licheniformis* and the enzyme activity was 17µ/ml. The lowest amylase production was observed in yeast extract (116µ/ml). The nitrogen sources are of secondary energy needs for the organism and play important roles in the growth of the organism and production. Addition of Nitrogen sources has been reported to have an inducing effect on the production of various enzymes inducing α-amylase (Pedersen and Nielsen, 2000). Similar observations were made by Gangaharan *et al.,* (2006) and Saxena *et al.,* (2007). The highest amylolytic activity recorded for growth medium supplemented with peptone was not surprising because Ramachandran *et al.,* (2007) reported the peptone gave an increase in enzyme yield in SSF using coconut oil cake as substrate. Teodore and Martins (2000) also reported that peptone and yeast extracts favoured the growth and synthesis of amylase for *Bacillus* spp.

**Conclusion and Recommendation**

The findings of this study showed that thermostable α-amylase was produced from *Bacillus lichenformis.* The enzyme also had characteristics which makes it suitable for industrial applications and starch processing. We thus recommended that further research be carried out on the effect of higher temperature ranges (above 45°C) on amylase production and stability from other organisms. This could be very useful in the development of biotechnological processes.

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