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Research Article

## THERMO STABILITY STUDY OF CRUDE AMYLASE FROM BACILLUS ISOLATE

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### Abstract

An amylolytic strain was collected from rotten potato and its activity evaluated. The isolated strain was cultivated for amylase production in shake flasks at  $35 \pm 2^\circ\text{C}$  and the fermentation pattern was studied. Optimum temperature for maximum enzyme synthesis was observed at  $35^\circ\text{C}$ , when initial pH of fermentation medium was adjusted to 5.5. Maximum extracellular amylase activity of 7.9 U/mL and the maximum intracellular activity of 320 U/mL was recorded. Although maximum biomass was present at 12.6 g/L but highest growth rate was observed between 08 to 40h with maximum at 36h. The extracellular amylase present in the broth was partially purified with an overall yield of 44% through purification procedure of ammonium sulphate precipitation. After completed extraction and partial purification and stabilization, the stability of enzyme was observed in a range of temperature and pH between  $60^\circ\text{C}$ - $90^\circ\text{C}$  and 2-8 pH respectively. Maximum enzyme activity was demonstrated at  $90^\circ\text{C}$ , and pH of 5.5 and 6.5. The thermo stability of the amylases of this *Bacillus* species was comparable to that of amylases from other microbial sources.

**Key word:** Bacillus sp; Amylolytic strain; amylase activity; thermos table amylase.

### Introduction

Although  $\alpha$ -Amylase can be obtained from several sources, such as plants, animals and microorganisms such as; fungi, yeasts and bacteria. The microbial amylases have a broad spectrum of industrial applications as they are more stable than when prepared with plant and animal  $\alpha$ - amylases (Tanyildizi *et al.*, 2005). Microbial production of amylases has major advantage over other sources (plant and animal) because of the economically bulk production capacity and the fact that metabolic pathways of microbial system are easily modified to obtain enzymes of desired characteristics and quality. Since demand of this enzyme in industry is at large scale therefore to meet the growing demands in the industry it is necessary to improve the performance of the microbial system and thus increase the yield without increasing the cost of production (Gangadharan *et al.*, 2008). The various production parameter, particularly physical (temperature, agitation, aeration) and chemical parameters, play very vital role in the improvement of fermentation processes due to their impact on the economy and practicability of the enzyme production process (Srivastava *et al.*, 1986). The growth and enzyme production of the organism are powerfully influenced by medium composition thus optimization of media components and cultural conditions is the principal task in an industrial biological process (Bolton *et al.*, 1997). *Bacillus* species are heterogeneous forms of organisms and

they are very easy-going in their adaptability to the environmental conditions. Various factors influence the nature of their metabolic process and the enzyme produced. Medium composition and concentration greatly affect the growth and production of extracellular amylase in bacteria (Lévêque *et al.*, 2000). A large variety of extracellular enzymes are produced by the genus *Bacillus*, some of which such as the amylases are of noteworthy in industrial application. Among these enzymes produced by *Bacillus* sp., the thermostable varieties are more acceptable with respect to industrial point of view. Thermostable  $\alpha$ -amylases have had many commercial applications for several decades. These enzymes are used in the textile and paper industries, food, adhesive, and sugar production (Marchal *et al.*, 1999; Mahon *et al.*, 1999; Niehaus *et al.*, 1999; Chand *et al.*, 1999; Kandra *et al.*, 2003). However, due to rapid progress in biotechnology, the amylase application has expanded in several fields such as clinical, medicinal and analytical chemistry, as well as in brewing and distilling industries (Gupta *et al.*, 2003; Pandey *et al.*, 2000; Souza *et al.*, 2010; Mamo *et al.*, 1999). Bearing in mind the industrial importance of amylase, the purpose of the present investigation was to screen *Bacillus* species isolated from rotten potato in order to study their suitability with regard to  $\alpha$ -amylase production.

## Material and Methods

### Soil Samples Collection

Soil samples were collected from starchy wastes of flour markets and mills using aseptic polythene bags. Samples were preserved in refrigerator at 4 °C till further processing of soil sample.

### Isolation and Partial Identification of Strain

The strains were isolated from soil samples according to the method of Min *et al.* (1999). The soil sample was at first enriched on a liquid medium made of 1% soluble starch, 0.5% peptone (w/v), 0.5% yeast extract (w/v), at pH 3.0. 1g of soil was mixed with the medium placed in tubes, and incubated at 25 to 28 °C for 3 days with shaking. 0.2% of the enriched liquid was then transferred onto sterilized Petri dishes (Min *et al.*, 1999), containing 1% soluble starch, 2% agar (solidifying agent). To avoid any fungal contamination, the medium was supplemented with ketokenazole (150 mg/l). The Petri dishes were sealed with parafilm and incubated at 25 to 28 °C for 48 to 72 h until starch digesting bacterial colonies appeared. The colonies were purified several times on agar plate. Partial identification of bacillus species were done on the basis of Morphological characteristics of isolates.

### Amylolytic Activity of *Bacillus sp.*

To conform amylolytic activity of bacillus sp. an equal volume of cells of each isolate was loaded in a 2 mm hole made in solidified agar aseptically. The agar plate was incubated for 24 h at 30°C, after which a 4% lugol solution was added to the medium. The diameter of halo formed after addition of lugol was measured which is an indication of amylolytic activity of the strain. The most active strain was selected and preserved for further experimental process.

### Enzyme Productivity of *Bacillus sp.*

Isolated species was propagated at 35±2°C for 24 h, in 50 ml of 8% starch medium placed in 250 ml flask, under shaking. The effect of carbohydrate source on the productivity of the strain was studied by running the propagation on different starch media: commercial soluble starch, corn, cassava, rice, wheat and potato. In addition, the propagation was undergone at different starch concentrations (1 to 15%, w/v), different temperatures (20 to 55°C) and different pH (5 to 8) using the most productive fermented medium. The initial pH of the medium was adjusted using 0.1 molar solution of sodium hydroxide and sulphuric acid. At the end of each fermentation batch, broth was centrifuged at 10,000 rpm for 10 min., and the supernatant collected as crude enzyme extract. A portion of the crude extract was used to determine the enzyme productivity of the strain through measurement enzyme activity, while the remaining extract served for partial purification and characterisation of amylase.

### Partial Purification of Amylase

A solution of 60% (w/v) sodium sulphate was added to the clear supernatant and enzyme recuperated by centrifugation at 10,000 rpm. The enzyme precipitate was then suspended in phosphate buffer 0.007 M, pH 5.0. The purified extract was used to determine enzyme activity and properties.

### Enzyme Assay

Amylase activity both in crude and partially purified extracts was assayed using soluble starch as substrate, according to the method of Keleke *et al.* (1998). The amylase activity was defined as the amount of soluble starch hydrolysed by 1 ml of enzyme extract in 60 min. The optimal temperature for activity was determined by assaying activity between 20 and 100 °C. Thermo stability of the amylase was performed by maintaining the enzyme solution in water bath at different temperature (20 to 100 °C) for 30 min, then cooling and running the activity assay. Measurement of optimum pH for amylase activity was carried out by running the activity assay between the pH range of 2.0 to 10.5, using 0.05 M Na<sub>2</sub>HPO<sub>4</sub>-citrate, tris-HCl and glycine-NaOH as buffer solutions. The pH stability was determined by incubating partially purified enzyme in water bath at 70 °C and measuring the residual activity. All measurements were undergone at least in triplicate.

## Results and Discussion

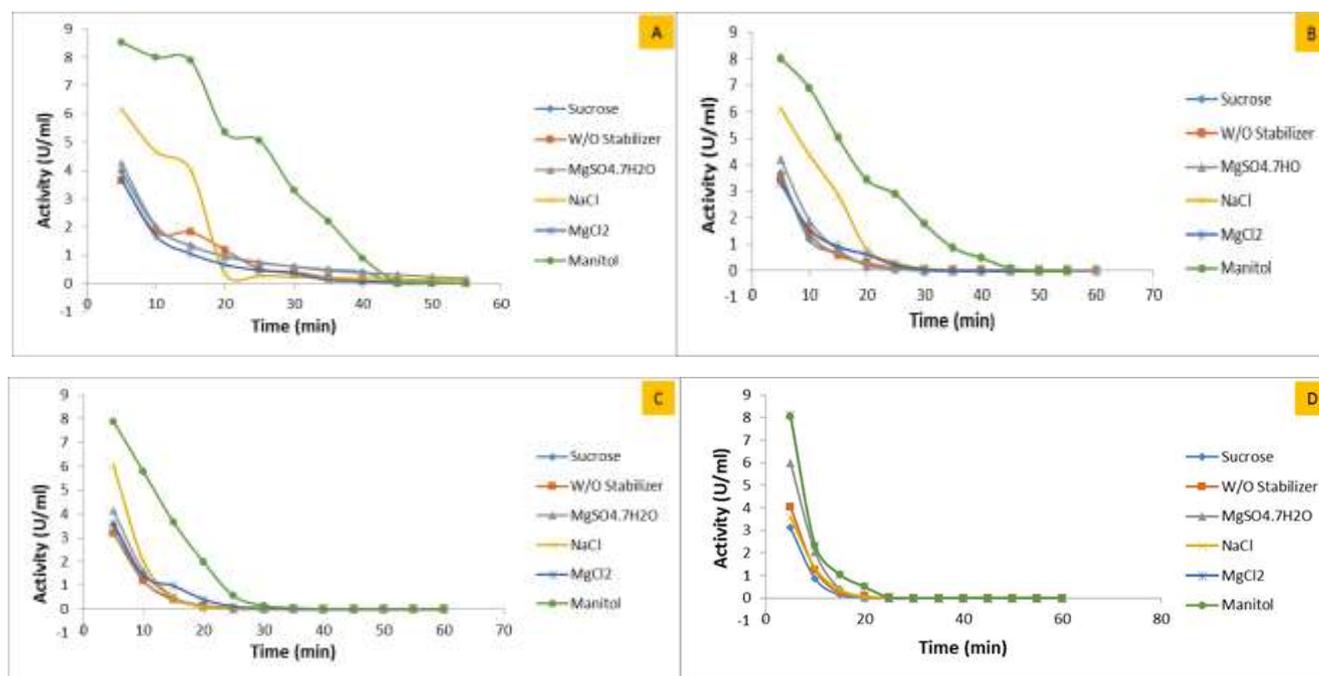
### Effect of Stabilizer

Initially a stock solution of 0.01M of each stabilizer was prepared. The stabilizer used were MgSO<sub>4</sub>.7H<sub>2</sub>O, NaCl, MgCl<sub>2</sub>, sucrose and Mannitol. 2ml of stabilizer solution was mixed with 3 ml of enzyme solution.

### Enhance Thermo Stability of the Amylase

Without stabilizer, amylase shows very low stability at different temperatures. When it was treated with sucrose, MgSO<sub>4</sub>.7H<sub>2</sub>O, NaCl, MgCl<sub>2</sub> and Mannitol, for 1hour, its stability was increased. Mannitol gives maximum stability to amylase enzyme. Stability of amylase decreases with respect to temperature and incubation time. According to experiment it was observed that mannitol provides higher stability for longer time. Hence it was concluded that mannitol is good stabilizer for amylase enzyme (Fig. 1 A-D).

The enzyme was observed stable at a temperature range between 60 and 90°C, above a sharp declination in stability was found. Highest activity was demonstrated at 90°C. The above enzyme stability profile is similar to the performance of amylase from some bacteria genus such as *Bacillus licheniformis* (Morgan and Priest, 1981) and does not fit for fungi and yeast amylases because they naturally are not thermo stable (Barbier, 1997), except if they have been genetically modified.



**Fig. 1:** Effect of temperature on the enzyme activity (U/ml); **A.** at 60 °C; **B.** at (70 °C); **C.** at (80 °C) and **D.** at (90 °C)

The effect of pH on amylase activity was optimized at stability range from pH 2.0 to pH 8.0 after 30 minutes of incubation period at 60 to 90°C. Optimum pH for maximum activity of enzyme was 5.5. This may indicate a poly enzyme structure of the *B. sp.* amylase (Muralikrishna *et al.*, 2005). The above discussed property of the *B. species* amylase, particularly their thermo stability, explores experimental considerations, taking into account the natural medium where the yeast strain have been isolated. Bacterial fermentation is the main phenomenon-taking place in starchy soils of flourmills. The bacteria considered are usually thermo-resistant.

## Conclusions

In conclusion, work done and critical analysis of literature, shows that the enzyme is very sensitive to temperature. Therefore, the selection of optimum temperature is essential for the production of amylase. Production of amylase was maximum at temperature 37 °C. Further increase temperature in the resulted decrease in the activity of amylase. However, the temperature of the fermentation medium was found to be optimum at 37 °C. When temperature is altered below or above the optimum the activity is decreased or becomes denatured. Different organisms have different temperature optima and decrease or increase in temperature on either side of the optimum value results in poor microbial growth.

Among the different stabilizer (water, sucrose, NaCl, MgCl<sub>2</sub>·7H<sub>2</sub>O, Mannitol) used, mannitol was observed to be most effective stabilizer for amylase enzyme. The enzyme remained stable in presence of stabilizers, at a temperature range between 20 and 60 °C, above which the stability was rapidly declined. The maximum activity was displayed at

70 °C. In conclusion, without stabilizer amylase show very low stability but treated with different stabilizer for 1h. Amylase showed significant stability at different temperature and different time interval. (Based on Optical Density).

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