INTERNATIONAL JOURNAL OF SCIENCE AND NATURE

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BIOCHEMICAL CHARACTERIZATION OF THERMOPHILIC AMYLASE ENZYME ISOLATED FROM *BACILLUS* STRAINS

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ABSTRACT
This study was conducted to screen for thermophillic *Bacillus* strains with amylase activity and to examine the amylase heat tolerance potentiality. It was, also this study was aimed to determine the optimal culture conditions for growth and amylase production. Two strains were isolated from soil: *Bacillus sterothermophilus* and *Bacillus acidocaldarius*. Two growth media were prepared to determine the optimal condition for amylase production, one was complex media containing starch 1.0%, yeast extract 0.04%, $(NH_4)_2HPO_4$ 0.4%, KCl 0.1% and $MgSO_47H_2O$ 0.05%, and the other was semi-synthetic medium containing peptone 0.4%, $(NH_4)_2HPO_4$ 0.4%, $NH_4H_2PO_4$ 0.4% and KCl 1.0%. The two isolates grew better on complex medium than on semi synthetic medium and amylase production on complex medium was highly expressed ranging between 57.5-63.3 U/ml. Amylase activity was increased by increasing starch concentration. *Bacillus acidocalarius* compared to *Bacillus sterothermophilus* expressed a high extracellular amylase activity. The optimum temperature of amylase activity ranged between 50-70°C and the *B. sterothermophilus* amylase activity remained stable up to 70°C for 60 min.

INTRODUCTION

Amylase is an enzyme that catalyses the breakdown of starch into sugars. Amylase is glycoside hydrolases and act on α-1, 4-glycosidic bonds. Amylases [α-amylase, β-amylase and glucoamylase] are among the most important enzymes in present-day biotechnology. The enzymes of amylase family have great significance due to its wide area of potential application. Modern bread making techniques have included amylase enzyme into bread improver (Maton *et al*, 1993). The extent of amylase action and starch breakdown depends primarily up on the thermostability of amylase. The most notable and important differences between amylases from different source are their thermostability (Adams, 1997). Fungal amylase is quite labile, being destroyed rapidly at temperature above 60°C, while bacterial amylase is most stable and shows little inactivation at temperature up to 85°C. The composition and concentration of media greatly affect the bacterial growth and production of extracellular amylases (Srivastava and Baruah, 1986).

The objective of this study was to screen an amylase from thermotolerant *Bacillus* strains with the potentiality as bread additive and to overcome the current amylase. The optimal culture condition for bacterial growth and amylase production by isolated strains were also investigated.

MATERIALS AND METHODS

Conventional, isolation and identification of thermophilic *Bacillus*

Bacillus strains were isolated from soil samples taken from rhizosphere of potato crop field. Soil samples were suspended and heated at 80°C for 15 min. Five dilutions were made from the soil suspension then inoculated in nutrient agar medium by spread plate method and aerobically incubated at 65°C for 24 hours. Colonies were purified and morphologically and biochemically checked.

Amylase production in basal media with various starch concentration

The isolates were grown in basal media containing peptone 0.4%, $(NH_4)_2HPO_4$ 0.9%, $NH_4H_2PO_4$ 0.2%, $MgSO_47H_2O$ 0.5% and KCl 1.0% then the medium was suplmented with different concentrations of starch (0, 0.025, 0.05, 0.1, 0.3 and 0.5%). pH of the medium was adjusted to 7.0 before autoclaving, and the inoculated media were incubated at 37°C for 4 days. The yield of amylase was estimated in extracellular fluid after removal of the bacterial cells from the culture broth by centrifugation at 10000 xg for 10 min.

Amylase production in complex medium and semi-synthetic media

Amylase was produced also in a complex medium containing starch 1.0%, yeast extract 0.04%, $(NH_4)_2HPO_4$ 0.4%, KCl 0.1% and $MgSO_47H_2O$ 0.05%, and semi-synthetic medium containing peptone 0.4%, $(NH_4)_2HPO_4$ 0.4%, $NH_4H_2PO_4$ 0.4% and KCl 1.0%.

Amylase Assay

Amylase activity was determined by the method of Miller (1959). The assay mixture contained 0.05 ml of soluble starch in sodium phosphate buffer (pH6.9) and 0.5 ml enzyme solution. The reaction was performed at 25°C for 4 min and stopped by addition of 1 ml of 3.5-dinitrosalicylic regent. The enzyme activity was obtained from a calibration curve prepared by the same procedure with D-glucose as the standard. One unit of amylase activity is defined as the amount of enzyme that releases the amount of reducing sugar equivalent to 1 µmol of glucose per min under assay conditions.

$$\text{Unit/ml} = \frac{\text{micromole maltose released}}{\text{ml enzyme in reaction mixture x 4}}$$

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Determination of pH optima

Substrate solution was prepared in sodium phosphate buffer at pH 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0 or 10.0. Buffers were supplemented with 0.006 M sodium chloride. The substrate solution contained 1% starch. A half milliliter of enzyme preparation was preincubated in a water bath at 25°C for 5 min and enzyme reactions were initiated by adding 0.5 ml of substrate solution. Then the mixture was incubated at 25°C for 5 min, reactions were terminated by adding 1 ml DNS reagent and the mixture incubated in boiling water for 5 min and the activity of enzymes were determined.

Determination of temperature optima

For determination of the optimum temperature, preparations of enzymes and substrate solutions were prepared as described above, however, the pH of the mixtures corresponded to determined optimum pH of the respective enzyme. The mixtures were incubated for 5 min at 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85 or 90°C, and then the amylase activity was measured.

Determination of thermostability

The amylase preparations were preincubated at 60, 65, 70, 75, 80 and 90°C for 30 and 60 min. After preincubatation the samples were cooled, and they were reincubated at 25°C, and the residual amylase activities were estimated.

RESULT AND DISCUSSION

Conventional isolation and identification of thermophilic Bacillus

Two strains were isolated form soil samples and were selected for their high amylase expression after initial testing of a large number of isolates in growth medium containing starch. Table 1 shows the results of morphological and biochemical tests of isolates, which were done according to Bergey's manual (Brenner, *et al*. 1986).

Amylase production in various media

Several of media were formulated for maximal production of extracellular amylase by a thermophilic *Bacillus sterothermophilus* and *B. acidocaldarius* strains. Among various media used for high amylase yield, the complex media with starch concentrations of 1.0, 3.0 and 5.0% has expressed a high amylase activity ranging from 56.8, 48.1 and 45.0 and 62.4, 42.4 and 39.5 U/ml respectively for *Bacillus sterothermophilus* and *B. acidocaldarius* respectively (Figure 1). Of the different concentrations of starch, 1.0 % starch was found to be optimal for amylase production for both strains. As in this study increasing the concentration of starch was found to stimulate amylase formation in *Bacillus* sp. by several workers (Srivastava and Baruah, 1986; Aiyer, 2004 and Qader *et al*., 2006) and in Aspergillus niger (Rezaei *et al*., 2009).

TABLE 1. Results of morphological and biochemical tests of isolates

Test	Strain 1	Strain 2
Gram's test	+	+
Spores forming	+ (central endospore sporangium swollen)	+ (central endospore sporangium not swollen)
Hydrolysis of gelatin	+	+
Production of iodole	-	-
Reduction of nitrate	-	-
Hydrolysis	+	+
Hugh and Liefson	+	+
VP	+	+
Oxidase	+	+
Catalase	+	+
NaCl		
2%	+	+
5%	+	+
7%	-	-
10%	-	-

From the biochemical tests, two isolates proved to be *Bacillus sterothermophilus* and *B. acidocaldarius*.

Amylase was produced also in the complex medium and in the semi-synthetic medium to examine the optimal culture conditions for production of thermostable amylase by isolated *Bacillus* strains; it found that complex medium expressed a high amylase activity ranging from 57.5 and 63.3 U/ml for *Bacillus sterothermophilus* and *B. acidocaldarius* respectively compared to the semi-synthetic medium. The amylase activities ranged from 32.55 and 28.8 U/ml for *Bacillus sterothermophilus* and *B. acidocaldarius* respectively (Fig.2). The result of amylase production indicated that the composition and concentration of media greatly affected the growth and production of extracellular amylase in bacteria; this is in agreement with Otludil *et al.* (2005), Ensari *et al.* (2006) and Tanyildizi *et al.* (2007).

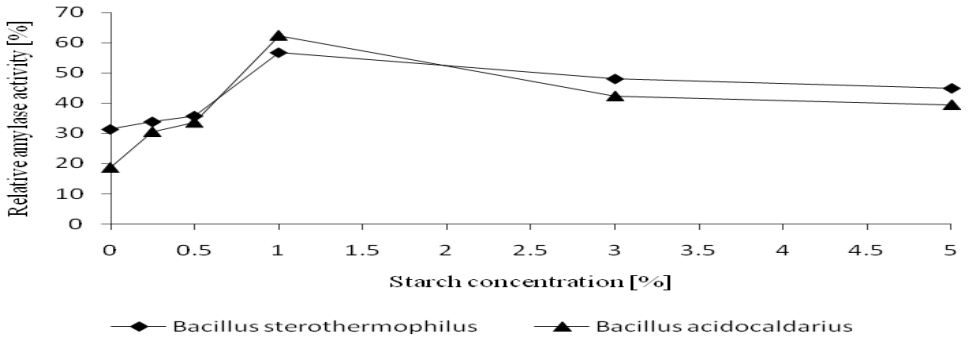

FIGURE 1. Amylase activity of *Bacillus sterothermophilus* and *B. acidocalsarius* amylases produced in basal media with different concentrations of starch.

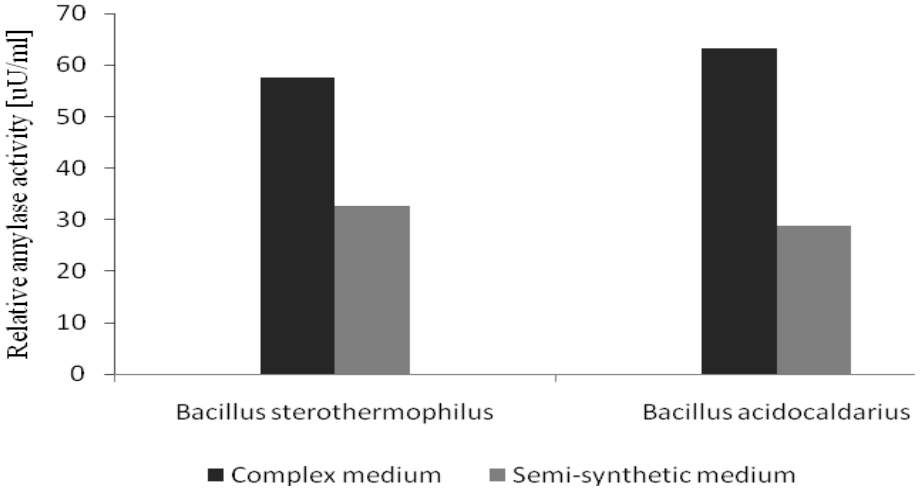

FIGURE 2. Amylase activity of *Bacillus sterothermophilus* and *B. acidocalsarius* amylases produced in complex and semi-synthetic media

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Determination of pH optima

The pH activity profile (Fig. 3) of *Bacillus sterothermophilus* shows an optimum at pH 7 compared to the *B. acidocaldarius*, with an activity optimum at pH 6. The relative activities of *Bacillus sterothermophilus* at pH 9 and 10 were about 1.5 and 4.5 times higher than those of the *B. acidocaldarius*. Similar findings have been reported by Qatar *et al*. (2006), who stated that the optimum pH of *Bacillus* sp. AS-1 was around 7.5, while it disagreed with results of Rezaei *et al*. (2009), who found that the optimum temperature of Amylase produced from *Aspergillus* was pH 3.0.

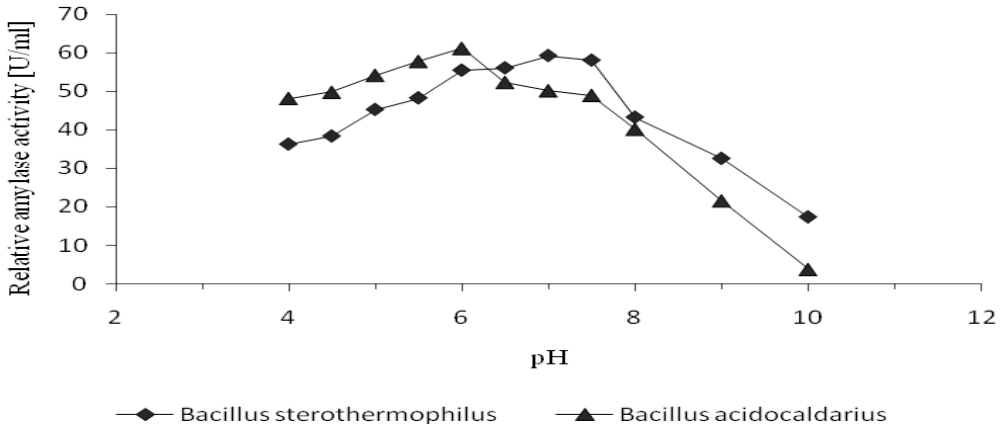

FIGURE 3. pH profile of amylase preparation.

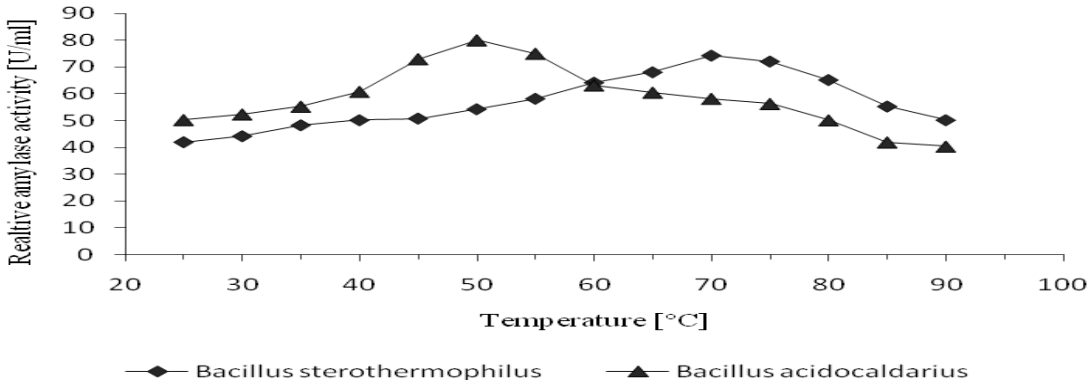

FIGURE 4. Temperature profile of amylase preparations.

Determination of temperature optima

Temperature profiles of different phytases were measured by incubating the enzymes at different temperatures (Figure 4). The results indicated that the amylase from *Bacillus sterothermophilus* exhibited their maximum activities at higher temperatures (70°C) than the amylase

produced from *B. acidocaldarius* (50°C), this is similar to the findings by Qatar *et al*. (2006).

Determination of thermostability

For the thermal stability estimation, the enzyme was pre-incubated at 60, 65, 70, 75, 80 and 90°C for 30 min and 60 min at the optimum pH, and the remaining activity was determined (Fig. 5 and 6). The amylase from *Bacillus sterothermophilus* was almost completely active up to 90ºC with a remaining average activity of 95%. On the other hand, more than 80% of the original activity of amylase from *B. acidocaldarius* was retained up to 80ºC after heat treatment for 30 min.

The main conclusions of this study are as follows: (1) The amylase from *Bacillus sterothermophilus* has a high potential for use as bread additive due to its biochemical properties; (2) Amylase from *B. acidocaldarius* has optimum pH around 6 and temperature optimum about 50°C; (3) A complex media with 1% starch is more efficient to produce amylase from the isolated *Bacillus* strains; (4) A 1% starch is an optimum concentration for amylase production.

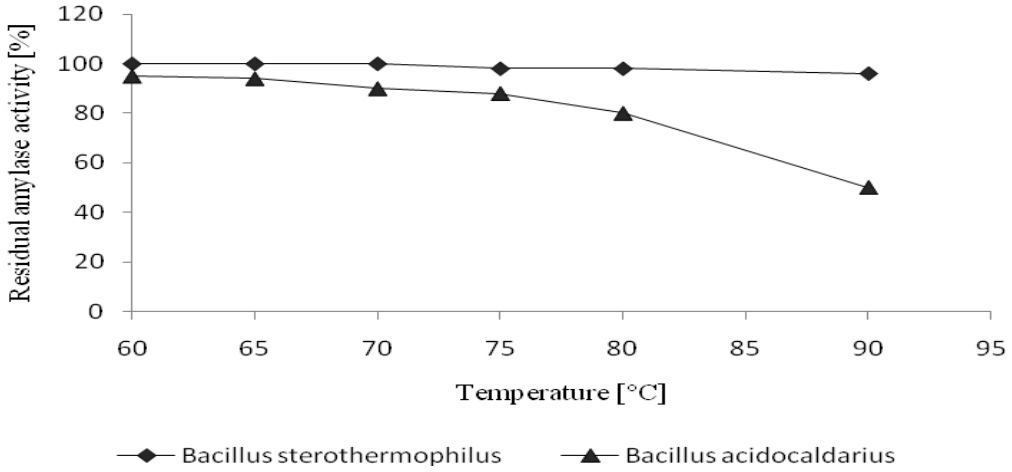

FIGURE 5. Residual enzymatic activity of amylase preparation after exposure at different temperatures for 30 min. The data expressed as percentage before heat treatment.

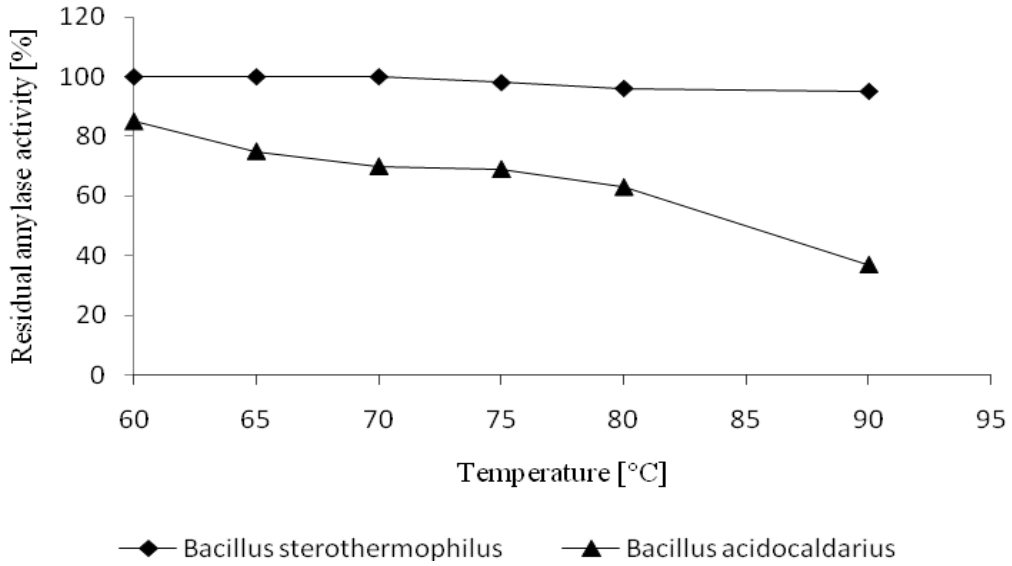

FIGURE 6. Residual enzymatic activity of amylase preparation after exposure at different temperatures for 60 min. The data expressed as percentage before heat treatment

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