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Isolation of a thermophilic bacterium to produce thermostable α–amylase

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ABSTRACT

This study was aimed at isolating a bacterium that can produce thermostableα- amylase. Bacterial strains were isolated from cow-dung (62 Nos.), rice broth (34 Nos.) and laboratory environment (126 Nos.). Among the bacterial strains high amounts of α-amylase producing three strains were selected and one of the strains isolated from the rice broth (strain RB_4) was the best and was identified to belong to Bacillus spp. The α- amylase produced by Bacillus RB_4 at 50°C was active at pH 7.0 and at 70, 85 & 95°C. Bacillus RB_4, showed highest growth (OD_{600nm}, 1.27) at 42°C and produced highest α-amylase activity at 50°C (20.0 Units mL^{-1}, 48h). Growth temperature of Bacillus RB_4 did not influence the temperature optimum of the enzyme. The enzyme lost its activity due to its entrapment in the precipitated calcium phosphate. Removal of calcium and phosphate ions by dialysis and addition of calcium (150 ppm) have improved the enzyme stability at 85°C. The isolated Bacillus RB_4 growing at 50°C produced α-amylase showing activity at 95°C and stable at 85°C.

Key words: α-Amylase, *Bacillus spp.*, growth, isolation, thermostable.

INTRODUCTION

α-Amylases have potential applications in a wide number of industrial processes such as food, fermentation, textile, paper, detergent and pharmaceutical industries [1]. α-Amylases should be active at high temperatures of gelatinization (100-110°C) and liquefaction (80-90°C) of starch to economize the process. Gelatinization of starch requires a high-energy input resulting in increased production cost of starch-based products. Hydrolysis of raw starch below gelatinization temperatures has gained importance in view of energy cost, effective utilization of natural resources and viscosity problems [2-6]. Thus thermostableα-amylase production becomes essential [7]. *B. subtilis* [8], *B. stearothermophilus*[9], *B. licheniformis* [10]and *B. amyloliquefaciens* [11] are known to be the producers of thermostableα-amylase and widely used for commercial α-amylase production. This study is aimed to search for thermophilic bacteria that can produce thermostableα-amylase.

MATERIALS AND METHODS

Materials and Media

The chemicals and media used were from standard sources.Nutrient broth ($25gL^{-1}$) with $2.0gL^{-1}$starch at pH 7.0 was used as the activation medium. The fermentation medium contained (gL^{-1}) starch, 2.0; $CaCl_2.2H_2O$, 0.01; $MgCl_2.6H_2O$, 0.01; $FeCl_2$, 0.01; K_2HPO_4, 2.5; KH_2PO_4, 10.0; peptone, 4.0; NaCl, 2.0 and $(NH_4)_2SO_4$, 4.0.

Estimation of α-amylase activity
Estimation of α-amylase activity and the unit of α-amylase activity are as described before [12].

Strain isolation and identification
Collection of strains from different sources and purification and Selection of α-amylase producers
Under sterile conditions cow dung (CD, 50g) and rice broth (RB, 50mL) were collected 50mL activation medium and incubated at 37^{o}C. By aerating the sterile flack containing activation medium at 50^{o}C for 3h, the third sample of the bacterial strains was collected from laboratory environment (LE). All three samples were plated on nutrient agar-starch (20gL^{-1}) plate and incubated at 50^{o}C for 24h. From the bacterial colonies developed, pure culture were obtained.

Collected bacterial strains were grown on nutrient agar -starch plates to develop the colonies and replica plates were taken. To the plates 5 mL iodine reagent (I_2 2.0 gL^{-1} and KI 20gL^{-1}) was added and excess solution was removed after 30 seconds. The diameters of the colorless (halos) areas of the iodine-nutrient agar – starch plates were measured.

Preparation of inocula
The bacterial strains, which, produced clear zones around the colonies, were selected and sub cultured. To 25mL of activation medium, 2 loops full of strains from selected bacterial colonies were inoculated and incubated at 45^{o}C for 18h in a shaker water bath (100rpm).

Selection of potential α-amylase producers
Among the α-amylase producing strains, which produced higher diameters of colorless halos were selected and incubated in fermentation medium at 50^{o}C in an orbital shaker (100rpm). The α-amylase produced was monitored.

Activities of α-amylases produced by selected strains at different temperatures
The effect of temperature on the activities of α-amylases from selected strains was determined in 0.01M phosphate buffer pH 7.0.

Identification of the species and genusof the selected organism
The species and genus of the selected strain were determined [13, 14].

Cultivation of the selected strains in fermentation medium and the activities of α-amylases produced by the selected strains at different temperatures
The fermentation medium was inoculated with the inocula of the different selected strains and incubated at 50^{o}C (100rpm). The activities (pH 7.0) of α-amylases produced by different strains were determined at different temperatures.

Cultivation of the selected best strain in fermentation medium
The fermentation medium was inoculated with the selected strain and incubated at 50^{o}C (100rpm). The α-amylase production and growth (OD at 600nm) were monitored.

Effect of different temperatures on the growth and α-amylase production by the selected strain
The selected strain was inoculated to fermentation medium and incubated at 30, 42 and 50^{o}C. The growth and α-amylase production were monitored.

Effect of temperature on the α-amylase produced at 42 and 50^{o}C
The α-amylase produced in the fermentation medium at 42 and 50^{o}C were taken and the enzyme activity at different temperatures (60, 70, 85, 90 and 95^{o}C) was determined.

Stability of dialyzed α-amylase in presence and absence of Ca^{2+}
α-Amylase (20mL) containing spent medium was taken and dialyzed against deionized water at 20^{o}C for 7 and 18h. Then to one portion (10mL) calcium (150ppm) was added and the other portion was used as the control (without calcium). The enzyme stability at 85^{o}C was monitored.

RESULTS AND DISCUSSION

Selection and identification of thermostable α-amylase producer

Collection and purification of bacterial strains

The bacterial samples from cow dung, rice broth and laboratory environment were collected. Here the activation medium contained starch as the carbon source, because the aim was to isolate the α–amylase producers. From the three different sources, it was possible to isolate 222 pure bacterial colonies. The total number of bacterial colonies obtained from cow dung (CD), rice broth (RB) and laboratory environment (LE) were 62, 34, and 126 respectively. Each colony was considered to be obtained from different strains. In previous studies different sources such as soil containing decaying materials [10], gruel of rice [10], soil receiving kitchen waste [10], bakery waste [10], flourmill waste [10], tea waste [10], Egyptian soil [15], soil [16], etc. have been reported as the sources to isolate thermostable α-amylase producing bacterial strains. In this study too, the bacterial samples were isolated from hot environment expecting them to be thermophilic and can produce thermostable α-amylase. Based on the above assumption among the isolated strains potential thermostable α-amylase producer was selected in the following experiment.

Selection of α-amylase producers

When the isolated bacterial strains are grown on nutrient agar –starch plates at 50^{o}C, the α-amylase producers would consume the starch present in the medium around the colonies for their growth. In nutrient agar-starch plates, the α-amylases produced by the organisms diffuse into the medium and hydrolyse the starch. Hence when the iodine reagent is added, clear zones are observed around the colonies due to the absence of starch. The diameters of the clear zones around the colonies are directly proportional to the amount of α-amylase produced. Thus larger the diameter of the clear zones, higher is the amylase producing ability. The diameters of the colorless halos obtained with different bacterial strains are given Table 1. From the results it can be observed that among the 62 bacterial samples obtained from cow dung, four stains (6.45%) were α-amylase producers and among the 34 bacterial strains from rice broth five strains (14.7%) were α-amylase producers. None of the bacterial samples obtained from the laboratory environment produced α-amylase. Therefore totally 09 bacterial strains were considered to select the potential α-amylase producers. The microorganisms isolated from cow dung and rice broth were from high environmental temperatures and hence they grew at 50^{o}C and produced α-amylase. The diameters of the clear zones obtained with the strains isolated from cow dung ranged from 2.2 to 2.4 cm (Table 1). Among the four strains, the strain CD_1 was selected as it gave the clear zone with highest diameter. The strains isolated from rice broth gave the clear zones with the diameter ranging from 2.00 to 2.7cm (Table 1). Among the strains RB_2 and RB_4 gave clear zones with higher diameters. Alkalophilic*Bacillus subtilis* CB-18 isolated from soil produced alkaline α-amylase and produced 0.7cm zone in starch agar plate [16], which is very small when compared to our results. The strains (CD_1, BR_2 and BR_4), which gave larger clear zones, were selected for further studies.

Selection of potential α-amylase producers

To select the best α-amylase producer, strains CD_1, RB_2 and RB_4 were grown in fermentation medium and α-amylase production by these three strains was determined. All three strains produced highest α-amylase activity at 48h while the strain RB_4 produced highest amount of α-amylase activity (20.0 UmL^{-1}) (Table 1). Hence the strain RB_4 was selected for further studies. The ratios between the diameters of the clear zones produced by strains CD_1, RB_2 and RB_4 were 1.0: 1.1: 1.13 while the ratios between the α-amylase activities produced were 1.0: 1.1: 1.5 respectively. Thus the organisms producing the highest diameter of the clear zones have produced highest titer of α-amylase activity, but the observed ratios of the clear zones and enzyme activities were not the same. This could be because the α-amylase produced by different bacterial strains might be of varying molecular mass or size and hence would have shown difference in diffusing property in the agar (solid) medium. In a study carried out in the same laboratory, among the 72 bacterial strains isolated from soil receiving bakery waste, the organism selected and identified as *Bacillus licheniformis*, had produced 7.0 (±0.21) UmL^{-1} of α-amylase activity at 24h [10], which is three times less than that produced by the strain RB_4. As the aim was to select the thermostable α-amylase producing strain, the effect of temperature on the activities of the α-amylases produced by the three selected strains were determined.

Activities of α-amylases produced by the selected strains at different temperature

The effect of temperature on the activities of α-amylases from the strains CD_1, RB_2 and RB_4 were determined (0.01M phosphate buffer pH 7.0) (Table 1). The α-amylases from the strains CD_1 and RB_2 showed temperature optimum at 70°C while that from strain RB_4 showed at 85°C. The α-amylase activity at 90, 95 and 99°C were 97.4, 89.6 and 79.0% respectively of that obtained at 85°C. Starch gelatinization needs the temperature above 100°C and hence the strain RB_4 producing the enzyme that showed the activity at 85°C was selected for further studies. Organism selected from soil receiving bakery waste and identified as *Bacillus licheniformis*, has produced α-amylase having optimum activity at 90°C and at pH 7.0 [10]. Maximum α-amylase activity in the temperature range of 50-70°C was reported [17]. The optimum temperature for the activities of crude and purified α-amylase from *Bacillus licheniformis* ATCC 6346 was 85°C [18].

Identification of the selected bacterial strain RB_4

Microscopic studies

Microscopic study was carried out to identify the genus of the strain. The strain RB_4 was stained as blue-violet in color, rod with spores indicating that it is a gram-positive rod. Strain RB_4 moved rapidly across the microscopic field with twisting and this indicated the true motility. The hanging drop method used here is a type of wet mount slide preparation that permits the observation of living, unstained cells in a fluid medium. Gram-positive motile non-branching spore forming rods belong to Family Bacillaceae [19, 20]. Thus the strain RB_4 might be belonging to the Family Bacillaceae.

Biochemical tests

Biochemical tests were carried out to confirm the genus of the strain and to identify the species. The strain RB_4 has shown good growth under aerobic condition but did not grow under anaerobic condition (in anaerobic jar). This indicated that the strain RB_4 is a strict aerobe. The strain RB_4 produced O_2 from H_2O_2.This showed that the strain RB_4 is a catalase producer. If the bacterium oxidizes tetramethyl-p-phenylenediaminedihydrochloride, it will turn purple, indicating that the organism can produce cytochrome oxidase. Absence of color change indicates that there is no cytochrome oxidase production. The strain RB_4 did not bring out the colour change. Therefore it does not produce cytochrome oxidase. Strain RB_4 was tested for its fermenting ability of lactose, sucrose and glucose. The strain inoculated slants showed pink red butt and yellow slope. These results indicated that the strain RB_4 is a glucose fermenter. When the strain RB_4 was inoculated to Mac Conkey agar medium, it did not change the colour to red. This indicated that the strain BR_4 does not ferment lactose.

Identification of the genus of selected strain RB_4

The colony of the strain RB_4 has circular form with entire margin, white in color, moist and shiny surface with convex elevation. After 40h of growth, the diameter of the colony was 1.5 to 2.0 mm. The strain produced opaque single colonies. Based on the identification studies so far carried out, it can be confirmed that the strain RB_4 belongs to Genus *Bacillus*. *Bacillus* is distinguished from the other endospore-forming bacteria on the basis of being a strict or facultative aerobe, rod-shaped, and (usually) catalase-positive. According to the identification studies based on the morphological, cultural and biochemical tests carried out so far, the strain RB_4 belongs to Genus *Bacillus* and named as *Bacillus* RB_4.

Cultivation of *Bacillus* RB_4 in the fermentation medium

The results showed that as the fermentation progressed, the α-amylase production also increased steadily leading to increased α-amylase activity to 20 U mL^{-1} at 48h. The growth of *Bacillus* RB_4 (OD_{600nm}, 1.10) was highest at 24h and the maximum α-amylase production was at 48h. The bacterial strain reached the stationary phase at 24h. In a previous study it has been observed that α-amylase was produced during the growth phase and not at the onset of the stationary phase [21].

Effect of different temperatures on the growth and α-amylase production by *Bacillus* RB_4

The fermentation was carried out at 30, 42, 50 and 60°C and the growth and α-amylase production were monitored. Maximum growth was obtained at 42°C (1.27, OD_{600nm}) and 24h while the growth at 30, 50 and 60°C were 1.08, 1.10 & 0.59 respectively and the highest α-amylase activity was obtained at 50°C (20.0 U mL^{-1}, 48h). At 30, 42 and 52°C the enzyme activities produced were 18.86, 19.82 and 9.98 UmL^{-1} (48h) respectively. Thus the strain *Bacillus* RB_4 is capable of growing and producing α-amylase activity up to 60°C but were 50% of those obtained at 50°C. *Thermoactinomyces vulgaris* isolated from Egyptian soil was able to grow at 55°C [15]. A thermotolerant*Fusarium*

sp. isolated from Eastern Nigerian soil was able to produce α-amylase at 50°C [22]. Thus the strain *Bacillus* RB_4 is having the character as expected and able to not only grow but also produce α-amylase at 50°C.

Effect of temperature on the α-amylase activity produced by *Bacillus* RB_4 at 42 and 50°C

The activities of the α-amylase produced at 42 and 50°C by *Bacillus* RB_4 were measured at different temperatures (60 – 95°C) and at pH 7.0. Both the enzyme samples showed highest activity at 85°C. Therefore the growth temperature of the strain does not have the influence on the temperature optimum of the enzyme activity. The temperature optimum and the kinetic properties of the enzymes produced by the same organism do not depend on the growth temperature of the organism. However by repeated heat shock or continuous exposure to high temperatures it may be possible to change the properties of the organism [23].

Stability of α-amylase

The stability of α-amylasein the supernatant of the spent mediumat 85°C was carried out in presence and absence of calcium (150ppm) (Figure 1). The enzyme lost 95 and 18% of the original activity in presence and absence of calcium at 30min of incubation. α-Amylase produced by *Fusarium* sp. isolated from Eastern Nigerian soil has retained 78% of the activity at 30min and at 70°C [22]. α-Amylase of *Bacillus licheniformis* retained 37.6% of its activity at 90°C at 30min and 10.4% of its activity at 1h, whereas at 80°C it retained 68.8% of its initial activity at 30 min and 59.1% of its initial activity at 1h [10]. When the crude α-amylase of *Bacillus licheniformis* ATCC 6346 was preincubated at 85°C and at pH 7.0, it lost no activity at 10 min [18]. With time, in the pre-incubated enzyme samples precipitation occurred. The decrease in enzyme activity with incubation time could be due the precipitation of calcium ions as calcium phosphate. This is because in the spent medium phosphate ions are present. Along with the calcium phosphate precipitate, the enzyme would have been entrapped and precipitated. Calcium has been the widely used additive to attain thermostability of α-amylase [24, 25]. In this studies removal of calcium by precipitation could have been the reason for decreased stability of the enzyme in the spent medium to which calcium was added. Hence it was decided to reduce the Ca^{2+} precipitation by reducing the concentration of PO_4^{3-} concentration by dialysis.

The enzyme dialyzed for 18h against distilled water to remove the ions (especially phosphate ions) at 20°C and used for stability studies and no enzyme activity was observed even with the added calcium ions. Loss of activity of α-amylase dialyzed for 18h could be due to the total removal of ions which is present along with the enzyme protein, which is important for the activity of α-amylase. Ca^{2+}activates amylolytic activity [16]. α-Amylases produced by *Bacillus* are metallo enzymes having calcium as a co-factor, which require calcium ions for their activity, structural integrity and stability [7] and the complete removal of calcium from enzyme produced by *Bacillus* species leaves an inactive protein which can be reactivated in full on the restoration of divalent cation [26]. Calcium is necessary for enzyme folding and enzyme stability. Secondary calcium binding sites have also been reported, which enhance the thermostability [7]. Dialysis against 0.01M EDTA caused 58% loss of activity and that was restored to 92% of the original activity by 0.04M Ca^{2+} [27]. When the purified α-amylase of *Bacillus licheniformis* ATCC 6346 was preincubated at 85°C and at pH 7.0, it lost 75% of its initial activity at 10min [18].

When the time for dialysis was decreased to 7h, the enzyme did not lose its activity. In the absence and presence of calcium (150ppm) the enzyme retained 32 and 73% of its original activity respectively at 3h of incubation at 85°C (Figure 1). Themostability of amylases were evaluated with varying concentrations from 0.2 to 0.5M concentrations of calcium chloride and the highest activity obtained was with 0.3M calcium chloride at 55°C [28]. Stability of α-amylase was improved at 80°C with 0.1% (w/v) $CaCl_2.2H_2O$ [29]. In presence of 1mM calcium, no loss of activity was observed at 60min, 85°C and pH 7.0 [30]. Thus like other *Bacillus*α-amylases this *Bacillus* RB_4α-amylase has also showed increased stability in presence of calcium ions and also established that the enzyme needs ions for its activity. Further prolonged dialysis removes the calcium, which is attached to the protein and makes the enzyme to irreversibly lose its activity.

Table 1: The diameter of the colourless (halos) zones obtained around the bacterial colonies at 48h of incubation after the addition of iodine solution; highest α-amylase activity produced and optimum temperatures of the α-amylases obtained from different strains

Source	Strain	colorless zoneDiameter (cm)	α-Amylase Production* (UmL^{-1})	Optimum temperature (°C)
Cow dung	CD_1	2.40	20.0	70
	CD_2	2.30	ND	ND
	CD_3	2.35	ND	ND
	CD_4	2.20	ND	ND
Rice Broth	RB_1	2.50	ND	ND
	RB_2	2.65	18.1	70
	RB_3	2.30	ND	ND
	RB_4	2.70	15.6	85
	RB_5	2.00	ND	ND

**Highest activity produced at 48h.*
ND- Not determined.

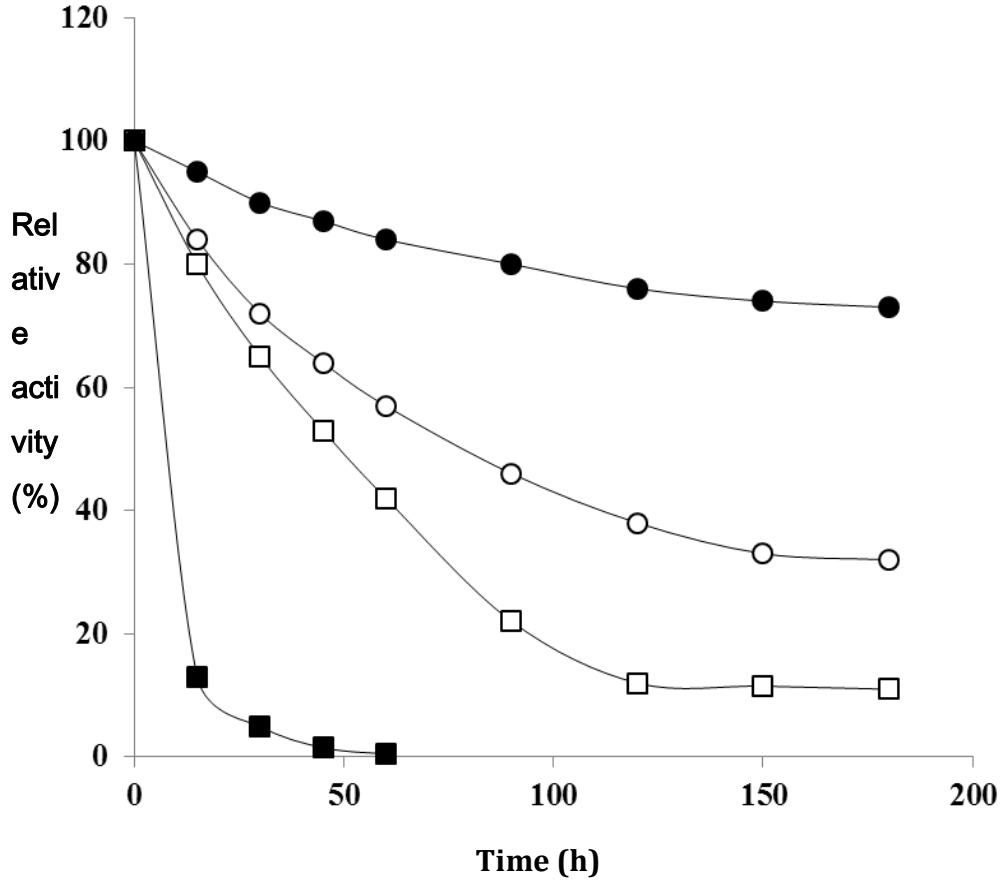

Figure 1: Stability of (●) dialysed and (▄) non-dialysed α-amylase produced by *Bacillus* RB_4 in presence (closed symbol) and absence (open symbol) of calcium ions (150ppm)

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