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Full Length Research Paper

Amylase activity of a starch degrading bacteria isolated from soil receiving kitchen wastes

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Starch degrading bacteria are most important for industries such as food, fermentation, textile and paper. Thus isolating and manipulating pure culture from various waste materials has manifold importance for various biotechnology industries. In the present investigation a bacterial strain was isolated from soil sample receiving kitchen waste and growth pattern as well as optimum growth condition was determined. Characteristic feature of the strain indicates that it belongs to the genus *Bacillus*. The optimum temperature for this strain was 37°C, whereas maximum growth was observed at 2% starch concentration. The pH range was found to be 6.8 - 7.2 for optimum growth. Amylase activity was maximum in the temperature range of 50 - 70°C, whereas this temperature range was deleterious for this bacterial strain. Also maximum enzyme activity was observed at 2% of starch concentration.

Key words: Amylase, *Bacillus*, kitchen waste, starch degrading.

INTRODUCTION

Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper (Pandey et al., 2000). Amylases can be obtained from several sources such as plant, animal and microbes (Kathiresan and Manivannan, 2006). The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. Microbial amylase has almost surpassed the synthetic sources in different industries (Pandey et al., 2000). Amylolytic enzymes are widely distributed in bacteria and fungi. They are categorized in to exo-acting, endo-acting and debranching enzymes. Among the amylases, β-amylase is exo-acting whereas α-amylase is endo-acting enzyme. Unusual bacterial amylases are found in acidophilic, alkalophilic and thermoacidophilic bacteria (Boyer and Ingle, 1972). Nowadays amylase from these sources is vastly used in amylase production under extreme conditions of pH and temperature.

There are various reports on starch degrading microorganisms from different sources and respective amylase activity (Aiba et al., 1983; Tonkova et al., 1993; Kathiresan and Manivannan, 2006). Soil receiving the kitchen wastes is one of the rich sources of starch degrading microorganism as it contains mostly starchy substrate. The present investigation dealt with isolation of a bacterial strain, from soil samples collected from different disposal sites of kitchen waste and physiological and biochemical features were determined. Its amylolytic activity under different physiological conditions were correlated with the growth kinetics.

MATERIALS AND METHODS

Microorganism

During the present investigation *Bacillus* strain was isolated from soil samples collected from different sites receiving kitchen wastes. One gram of soil samples was added to 99 ml of 2% starch broth. After 24 h of shake flask culture fresh inoculum was taken for batch culture at three different temperatures; 25±2 (RT), 37 and 50°C,

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which was followed by plating on starch agar medium after 7 days.

Media

Starch broth and starch agar supplemented with the following components peptone 0.05%, KCl 0.01%(w/v), $MgSO_4 .7H_2O$ 0.05% (w/v), $(NH_4)_2SO_4$ 0.01% (w/v), NaH_2PO_4 0.01% (Srivastava and Baruah, 1986) and 2% starch. The starch agar medium was composed of all these components along with 0.8% agar. Colonies of the isolated strain were transferred by replica plating on to starch agar plates and incubated at 25±2°C, 37, 40 and 50°C for 48 h.

Plates with bacterial colonies were then flooded with Gram's iodine reagent (0.01 M I_2-KI solution). If a strain was amylolytic then it started hydrolyzing the starch present in the surrounding and in the zone degradation there was no blue colour formation. Selection was done as per colonies with and without clear and transparent zone as amylase producing (Amy^+) and amylase non-producing (Amy^-) strain, respectively.

Identification of the isolated strain

Gram staining was performed to know whether the isolate was Gram positive or negative. Morphology was observed under light microscope. The result obtained from various morphological and physiological analysis were used to identify the isolates using Bergey's Manual of Determinative Bacteriology.

Culture condition growth kinetics

The starch nutrient medium was inoculated with a single isolated colony and culture for 48 h at 37°C with continuous shaking on a rotary shaker (Environmental Incubator shaker, NJ, USA) at 200 rpm. From this 1 ml of inoculum (approximately 1% culture) was transferred to 100 ml Erlenmeyer flask with sterile nutrient medium incubated at 37°C at 200 rpm for different time intervals (24 – 96 h). Growth kinetics was obtained by measuring the cell density at 600 nm in different time intervals.

To select the optimum concentration of starch the isolate was subjected to starch medium devoid of agar with 5 different concentration of starch (0.15, 0.5, 1, 1.5 and 2%). Similarly, optimum temperature range was selected by culturing the inoculums at different temperatures (RT, 37, 40, 45 and 50°C). Optimum pH range was selected taking different pH (5.2, 5.6, 6, 6.2, 6.4, 6.8, 7, 7.2, 7.4, 7.6 and 8). Growth pattern under these conditions were observed by measuring absorbance of cells at different durations at 600 nm.

Crude enzyme preparation and enzyme assay

To obtain crude enzyme 48 h old cultures were transferred to microcentrifuge tubes and centrifuged at 4000 rpm for 15 min. Cells were discarded and resultant supernatant was used as the crude enzyme for various enzyme assay.

Amylase assay was done by using a reaction mixture consisting 1 ml of substrate solution (1.1% soluble starch in 50 mM phosphate buffer pH 7.2) and 100 µl of the enzyme solution. The reaction mixture was incubated for 10 min at 30°C. Reaction was stopped by adding 2 ml of dinitrosalicyclic acid (DNSA) reagent (Miller, 1959). The reaction mixture was heated to 100°C for 10 min and cooled. Optical density of each sample with reaction mixture was taken at 575 nm in a spectrophotometer (Shimadzu, Japan). Enzyme activity was expressed in units (1 unit/ml = amount of enzyme which releases 1 µ mole glucose under the assay condition).

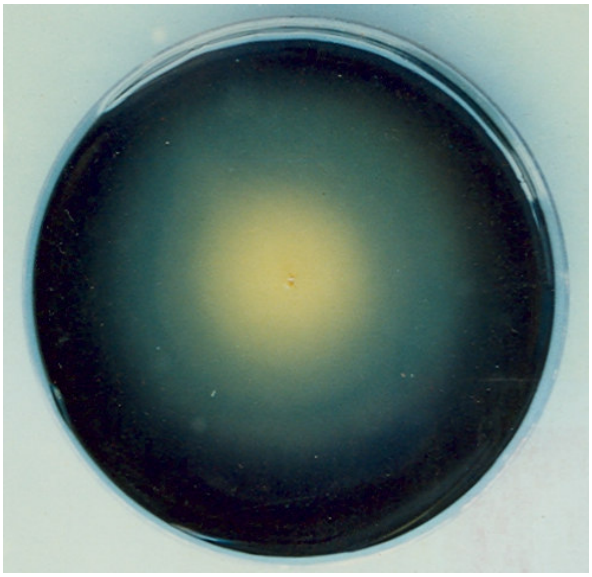

Figure 1. A starch agar plate showing amylase activity with clear white zone at the center surrounding the single colony of *Bacillus* isolated from soil sample collected from kitchen waste.

Entrapped cells and amylase activity

From an overnight grown culture of *Bacillus* isolate, 10 ml of culture was transferred to a sterile falcon tube and centrifuged to pellet down the cells. Then these cells were re-suspended in 4 ml of fresh sterile nutrient medium and used for entrapped cell culture in the dialysis sac incubated at 37°C. The dialysis chamber was maintained in a cold chamber with constant stirring and supplemented with fresh buffer in every 12 h interval. Aliquots were collected at different time interval of 24 h and various studies was done.

In order to see the effect of temperature on amylase activity 1 ml each of dialyzed solution was exposed to 40, 50 and 70°C for 5 min and 10 min, in a water bath. Amylase activity was determined in this range under standard assay condition (Miller, 1959) as mentioned above. Enzyme activity was expressed in units (1 unit/ml = amount of enzyme which releases 1 µ mole glucose).

Similarly to observe the effect of different substrate concentration on amylase activity, the dialysed aliquot was added to different starch concentrations (0.15, 5, 1, 1.5, and 2%) and the activity was observed following the method of Miller (1959).

RESULTS

Out of three different ranges of temperature, maximum colonies were recorded at 37°C and among them an average of 59±6.4 were found to be amylase positive (Amy^+). The Amy^+ colonies exhibited clear and wide transparent zone due to the production of amylase (Figure 1). Also we observed that colonies were translucent and cream whitish in color. All colonies were with entire regular margin (Table 1). The strain closely resembled to *Bacillus* species. For the present investigation, one of these colony was selected and taken for all physiological and biochemical analysis.

Table 1. Morphological and biochemical characteristics of isolated strain.

Test	Response of the strain
Gram's staining	+
Shape	Bacilli
Motility	+
Growth at temperature (^{0}C)	
R.T.	+
37	+++
50	++
Growth at pH	
5.2	+
5.6	+
6.0	+
6.8	++
7.0	+++
7.2	+++
7.4	++
8.0	+
Growth on starch agar	+
Starch hydrolysis	+
Utilization of carbohydrates	
Glucose	+
Fructose	-
Maltose	+

= None; + = Poor, ++ = good, +++ = very good.

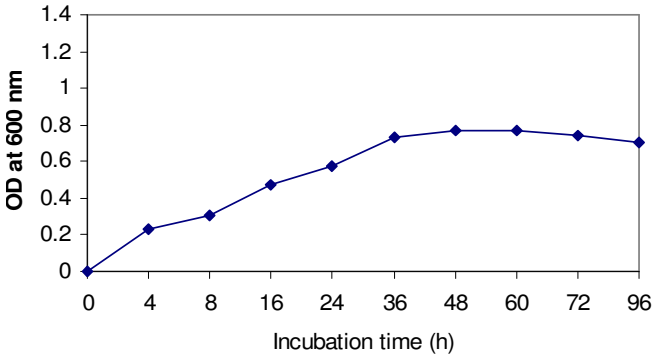

Figure 2. Growth kinetic of the isolated *Bacillus* strain from kitchen wastes. Readings were taken initially at 4, 8, 16 and then in every 12 h intervals till 48 h. For 72 and 96 h old culture, readings were recorded at the respective time.

Growth kinetics of the presently isolated *Bacillus* strain started lag phase right after inoculation. Stationary phase started from late 48 h which continued till 72 h and after that growth declined at 92 h (Figure 2). Growth pattern utilization of carbon sources varied with the supplement of starch. Low starch concentration (0.15, 0.5 and 1%) favored a 22 h lag phase for this *Bacillus* strain. However, culture grown at 1.5 and 2% starch concentration exhibited a very narrow lag phase with prolonged exponential phase. Particularly at 2% concentration of starch the exponential phase was observed till 55 h (Figure 3). Thus we selected 2% as the suitable concentration of carbohydrate and substrate for our investigation. Similarly, 37^{o}C was the optimum growth temperature for the presently isolated *Bacillus* strain. Higher temperature did support an early stationary phase. Interestingly this strain of *Bacillus* revealed a wide pH range of 6.8 to 7.2 (Figure 4).

Amylolytic activity from the sample taken from crude enzyme varied with the increase in incubation period. In the present study, increase in incubation period enhanced the amylase activity and reached maximum at 72 h (Figure 5).

The most interesting observation we found with the presently reported *Bacillus* strain was, the enzyme activity by entrapped cells at different temperature regimes. There was enhanced in amylase activity with the enhancement of temperature. There was 2 fold increases in activity at 70^{o}C both at 5 and 10 min exposure (Table 2). Also a positive correlation between the carbon source concentration and temperature was observed. Increase in substrate concentration, resulted more enzyme activity at each range of temperature. The entrapped cells showed maximum amount of 75.7 U/ml enzyme activity at 2% starch concentration. This amount was almost 4 times higher than activity at 1% (Figure 6).

DISCUSSION

In the present investigation a pure strain of *Bacillus* was isolated from kitchen waste. The kitchen wastes, mostly consists up starchy materials and we found bacteria isolated from such places may have better potential to produce enzyme under adverse condition. On the other hand, conversion of organic waste through microbial processes decreases the amount of waste disposed by land-filling (Sakai et al., 2001). Optimization of growth condition is a prime step in using microorganisms in fermentation technology (Kathiresan and Manivannan, 2006). In the present study we observed 37^{o}C as the optimum growth temperature for the presently reported *Bacillus* strain and higher temperature (50^{o}C) supported less number of colonies. This could be due to the mesophilic nature of the species. As per earlier report of Aiba et al. (1983) high temperature may inactivate the expression of gene responsible for the starch degrading enzyme. Among the physiological parameters, optimum temperature, substrate concentration and pH range are the most important for enzyme production by microbes (Bose and Das, 1996; Gupta et al., 2003). Most of the starch degrading bacterial strain revealed a pH range

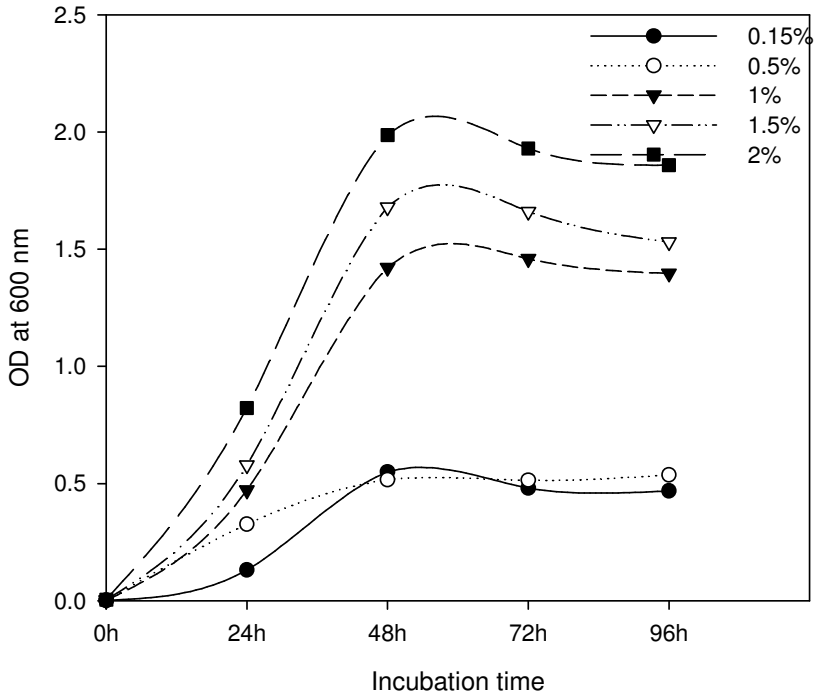

Figure 3. Effect of different concentration of starch on growth and activity of the isolated *Bacillus* strain from kitchen waste soil samples. Samples were collected in every 24 h intervals.

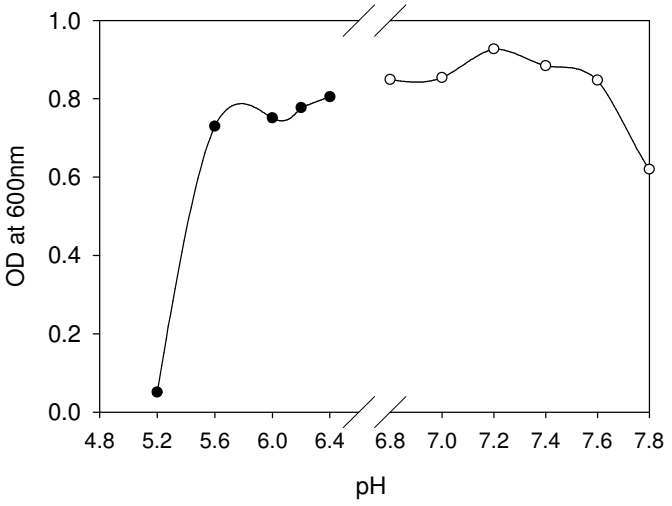

Figure 4. Effect of different pH, on the growth of the isolated *Bacillus* strain.

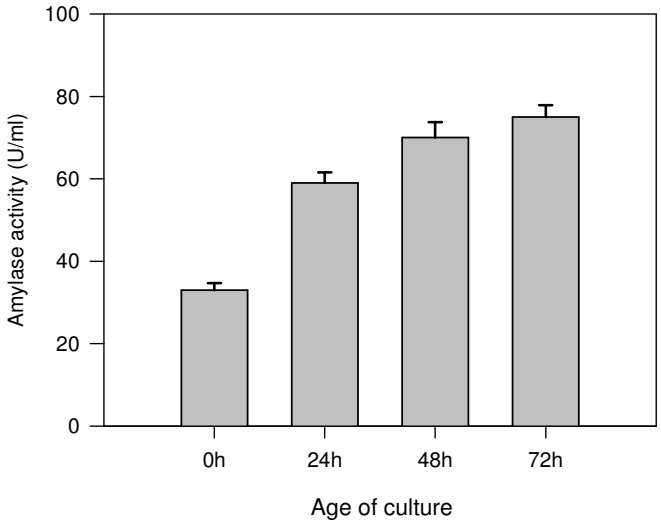

Figure 5. Amylolytic activity of the isolated *Bacillus* strain taken from crude enzyme in different incubation period.

between 6.0 and 7.0 for normal growth and enzyme activity (Gupta et al., 2003). The presently isolated *Bacillus* strain also showed optimum growth in this range. The composition and concentration of media greatly affect the growth and production of extracellular amylase production in bacteria (Chandra et al., 1980; Srivastava and Baruah, 1986). Starch is ubiquitous and is an easily accessible source of energy (Ryan et al., 2006). In past studies, a number of carbon and nitrogen sources have been examined for amylase production in several *Bacillus* species (Bose and Das, 1996; Srivastava and Baruah,

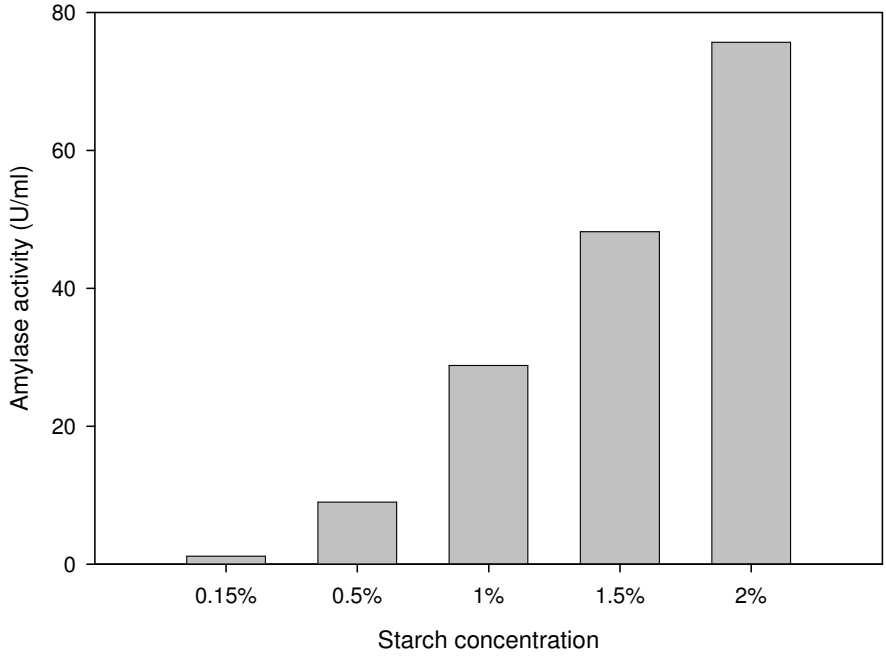

Figure 6. Effect of different substrate concentration on amylolytic activity of *Bacillus* strain isolated from kitchen waste. Cells were kept for dialysis and collected after 48h for enzyme assay.

Table 2. Effect of temperature on amylase activity in entrapped cells exposed at different time intervals. Isolated strain was grown at the optimum growth condition on 2% starch nutrient broth.

Temperature (°C)	Exposure time	
	5 min	10 min
40	10±1.67	10.44±1.25
50	15.55±0.87	24.44±3.65
70	35.54±4.52	36.44±2.98

1986; Ryan et al., 2006). Similar to these past reports, the present study also observed increasing the starch concentration increase both growth kinetics and amylase production.

Enzyme production from microorganism is directly correlated to the time period of incubation (Smitt et al., 1996). The present study observed enhanced enzyme activity with the increase in incubation time. This result is different from some of the recent observation in some *Bacillus* species (Aiyer, 2004), where increase in incubation time decrease enzyme activity. It was also observed that amylase activity by entrapped cells in a dialysis chamber, increased with the increase in temperature. The influence of temperature on amylase production is related to the growth of microbes (Kathiresan and Manivannan, 2006). Saito and Yamamoto (1975) observed similar result in *Bacillus licheniformis*. There might be catabolite repression in the early stages of culture grown at high temperature, but as growth proceeds the inducers may become active and induce more enzyme secretion (Saito and Yamamoto, 1975). Bose and Das (1996) observed that amylase activity in starch degrading bacteria is non-growth related. This could be a possible explanation for optimum growth of the presently reported strain at 37°C whereas maximum enzyme activity was observed at 70°C and making the isolated *Bacillus* species more suitable for future use in various industries.

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