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Original Article

Comparative Studies on the Production of Extracellular α-Amylase by Three Mesophilic *Bacillus* Isolates

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In the present study three mesophilic *Bacillus* isolates were analyzed for their α-amylase activity in shake-flask cultures. The organisms were capable to produce hydrolysis zone around their colonies on starch agar medium. The effect of various fermentation conditions on α-amylase production was investigated, and in every case it was found that *B. subtilis* was the best producer of the enzyme, which was followed by the newly isolated *Bacillus* sp. and *B. amyloliquefaciens*. The synthesis of extracellular α-amylase by the bacteria was repressed by the presence of readily metabolizable carbon source like glucose in the culture medium. Maximum α-amylase activity by the *Bacillus* isolates was obtained at 37°C with an initial medium pH 7.0 under agitation at 160-180 rpm for 72 h of growth.

Keywords: *Bacillus* species, α-Amylase, Enzyme production, Shake-flask culture

Introduction

Enzymes involved in starch conversion technology are of major importance and considerable interest exists in obtaining new enzymes having improved properties or new applications[1-3]. Enzymatic processes now produce over 75% of syrup and solid dextrose in the USA. New developments have taken place in the area of starch-degrading enzymes. Enzymes have several advantages. First, the specificity of enzymes allows the production of sugar syrups with well-defined physical and chemical properties. Second, the milder enzymatic hydrolysis results in few side reactions and less browning[4].

α-Amylases are group of enzymes classified as hydrolases that catalyze the hydrolysis of ortho-glycosyl compounds of starch and glycogen. They catalyze the hydrolysis of starchy materials into smaller glucose subunits that in turn are acted upon by other amylases to produce glucose[5]. Amylases, starch-degrading enzymes, have numerous biotechnical applications. These enzymes are used in textile and garments, paper industries, starch liquefaction, food, adhesive and sugar production and pharmaceuticals[6].

Carbohydrates in the form of sugar and starch represent a major part of total caloric intake for humans and for most animal life as well as for many microorganisms. The vast amount of starch and other carbohydrates made by photosynthesis become the ultimate energy and carbon sources for non-photosynthetic cells of the animal, plant and microbial worlds[7]. Most agricultural biomass containing starch can be used as a potential substrate for the production of gaseous or liquid fuels, feed proteins and chemicals by microbial processes. These substrates include corn (maize), wheat, oats, rice, potato, cassava etc. α-Amylases are produced by fermentation of such starchy substrates. There are several sources of α-amylases, which include bacteria, fungi, animals and high starch containing plants. However, industrial α-amylases is produced through fermentation using bacteria and fungi[4].

Because of the commercial and industrial uses, α-amylases from many sources has been studied in great detail. The genus *Bacillus* is the single most important bacterial source of this enzyme. Due to the thermostability of the enzyme produced by genus *Bacillus*, they have commercial significance[4]. This paper reports on the α-amylase from *Bacillus subtilis*, *Bacillus amyloliquefaciens* and a newly isolated *Bacillus* sp. under various conditions.

Materials and Methods

Microorganisms

Bacillus subtilis and *Bacillus amyloliquefaciens* were obtained from Department of Microbiology, University of Dhaka, Bangladesh. In addition, a soil organism identified as the member of *Bacillus* was also included. The organisms were maintained on nutrient agar (NA) medium.

Plate assay method

The *Bacillus* isolates were tested for amylase activity by employing zone clearing technique[8-9] using starch agar medium. The inoculated plates were incubated at 37°C for three days. After incubation, the zone of hydrolysis of starch was detected by flooding the plates with iodine solution. The development of blue

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colour indicated the presence of starch, while the areas around the hydrolytic bacteria appeared clear.

Enzyme production in shake-flask cultures

The basal medium[10] for α-amylase production contained 1.0% starch, 0.5% peptone, 0.5% corn steep liquor, 0.8% $(NH_4)_2SO_4$, 0.2% $MgSO_4.7H_2O$), 0.05% $CaCl_2.2H_2O$, 1.4% K_2HPO_4 and 0.6% KH_2PO_4. The organisms were cultivated in 250-ml Erlenmeyer flasks containing 50 ml of medium with an initial pH 7.0. The cultures were shaken at 150 rpm in a Gallenkamp orbital shaker incubator at 37°C for at least 72 h unless otherwise stated. After incubation, the cells were removed by centrifugation and the enzyme activity measured in the cell-free supernatant.

Enzyme assay and analytical methods

α-Amylase was determined by using soluble starch, 1% (w/v), as substrate in 0.05 *M* phosphate buffer (pH 6.5) essentially according to Gomes *et al*.[11]. The reaction mixture containing 1.8 ml substrate solution and 0.2 ml suitably diluted enzyme solution was incubated at 50°C for 10 min. The reaction was stopped by adding 3 ml dinitrosalicylic acid (DNS). The reducing sugar released was determined by the method of Miller[12]. One unit (U) of enzyme activity is defined in all cases as the amount of enzyme releasing 1 μmol of glucose or glucose equivalents from the substrate per min under the assay conditions.

Results and Discussion

α-Amylase production by three mesophilic *Bacillus* isolates was compared. Table 1 shows the extent of hydrolysis of starch by the organisms using the plate assay. All three isolates were able to hydrolyze starch and the diameter of zone of hydrolysis was more or less similar in all cases. The organisms were used for extracellular α-amylase production in shake-flask culture using a medium containing 1% soluble starch, 0.5% peptone, 0.5% corn steep liquor and 0.8% $(NH_4)_2SO_4$ in addition to various salt as described by Sarikaya[10]. In shake-flask cultures, the highest α-amylase activity (4.5 U/ml) was obtained from *Bacillus subtilis*, followed by a newly isolated *Bacillus* sp. (4.1 U/ml) and *Bacillus amyloliquefaciens* (3.8 U/ml).

Table 1. *Demonstration of starch hydrolysis ability by three Bacillus isolates using plate assay method*

Organism	Incubation time (h)	Diameter of zone of hydrolysis (cm)	Diameter of colony (cm)	Ratio
Bacillus subtilis	24	2.7	1.2	2.25
	48	4.0	1.8	2.22
	72	5.3	2.5	2.12
Bacillus amyloliquefaciens	24	2.2	1.0	2.20
	48	3.2	1.5	2.13
	72	4.7	2.2	2.14
Bacillus sp. (Soil isolate)	24	2.5	1.1	2.27
	48	3.8	1.7	2.23
	72	5.0	2.4	2.08

Although the medium contains substantial amount of protein (0.5% peptone and 0.5% corn steep liquor), we thought that the concentration of essential nutrients was not well-balanced. Therefore, various complex nitrogen sources were added separately to the original medium at 0.5% concentration to assess their effect on α-amylase production by the organism. It was observed that the supplementation of the medium with additional nitrogen sources led to the reduced production by all the isolates (Figure 1). It is likely that in a medium containing 1% organic nitrogen source is sufficient for the growth and α-amylase production by the organisms. A 1% organic nitrogen source is optimum for maximum α-amylase production by *Bacillus licheniformis*[6]. It was surprising to note that in a medium supplemented with 0.5% peptone, the activity was found to be much lower that that containing 0.5% yeast extract, beef extract, or casein. This indicates that a combination of nitrogen sources, *e.g.*, peptone plus yeast extract, is a better option in the culture medium for extracellular α-amylase production by the *Bacillus* isolates. Other investigators have also reported that the maximum α-amylase production by *Bacillus licheniformis* is achieved with a combination of peptone and tryptone[6], or peptone and yeast extract[13] as complex nitrogen sources.

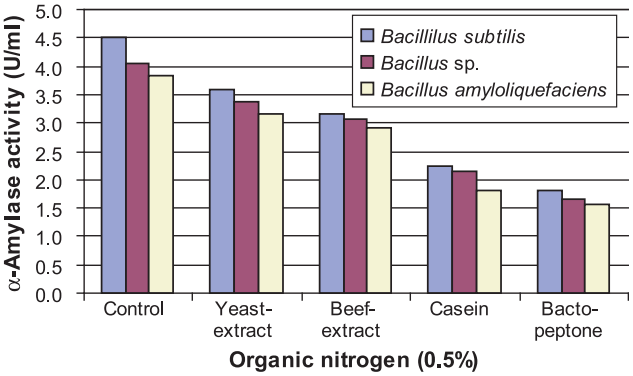

Figure 1. *Effect of supplementation of the culture medium (initial pH 7.0) with 0.5% organic nitrogen sources on extracellular α-amylase production by Bacillus isolates in shake-flask culture. The cultivations were carried out at 37°C under continuous agitation (150 rpm) on an orbital shaker for 72 h.*

Although glucose, a readily metabolizable carbon source, is a common catabolite inhibitor but it supports growth of the bacteria. It was expected that the α-amylase production by the bacteria would be enhanced by growing the organisms on the medium with the addition of glucose. However, the synthesis of α-amylase by the *Bacillus* isolates was seriously suppressed when the bacteria was grown on glucose although the medium contained 1% starch (Figure 2). The growth of the bacteria in glucose-containing media was found to be very good. The inhibitory effect of glucose on α-amylase synthesis increased with the increase of glucose concentration in the medium. It has been reported earlier that an inverse ratio existed between the rate of growth and the

total amount of α-amylase produced[6,14-15]. Utilization of carbon sources such as glucose gave rise to good growth with concomitant reduction in α-amylase production[6,16].

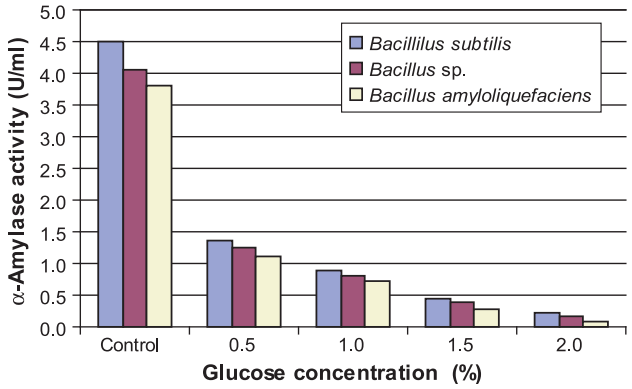

Figure 2. *Effect of addition of various concentrations of glucose in the medium (initial pH 7.0) on extracellular α-amylase production in shake-flask culture. The cultivations were carried out at 37°C under continuous agitation (150 rpm) on an orbital shaker for 72 h.*

Various parameters associated with the production of α-amylase by three *Bacillus* isolates were studied in the medium used for the enzyme production. The level of α-amylase increased linearly from 24 h to 72 h, and thereafter decreased more rapidly as the fermentation approached its end point (Figure 3). The finding was in accordance to that reported by Bajpai *et al*.[17] who obtained maximum α-amylase production after 72 h incubation of *Bacillus* sp. on cheese whey as carbon source in shake-flask cultures. A possible reason for α-amylase inactivation after 72 h might be due to release of high levels of intracellular proteases in the culture medium at the end of exponential phase. Abate *et al*.[18] reported that the production of α-amylase by *Bacillus amyloliquefaciens* starts at the beginning of the exponential growth phase reaching the maximum level after 24 h, and after that α-amylase level decreased drastically probably due to the accumulation of high level of protease activities concomitant with the sporulation process at the end of the exponential growth phase.

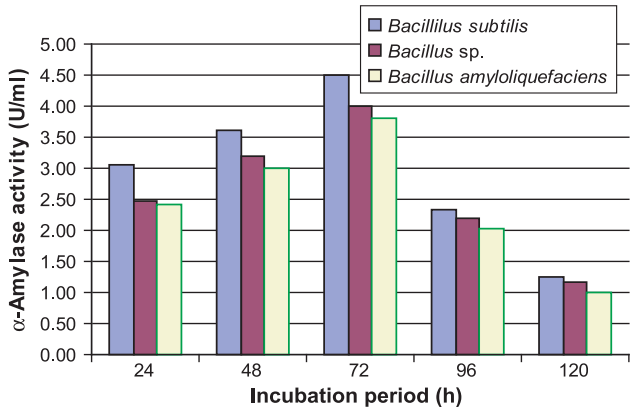

Figure 3. *Time-course of extracellular α-amylase production by the Bacillus isolates on starch-containing medium (initial pH 7.0) in shake-flask culture. The cultivations were carried out at 37°C under continuous agitation (150 rpm) on an orbital shaker for 120 h.*

The bacteria were found to grow at pH 5.0-8.5, with growth resulting in an increase of the pH of the medium (Figure 4). Maximum production of enzyme occurred at pH 7.0 in case of *B. subtilis* and *B. amyloliquefaciens*, and at pH 6.5 in case of *Bacillus* sp. It is evident from the results that α-amylase production by the *Bacillus* isolates is better at neutral to alkaline range of pH. These results are in agreement to that reported for *B. licheniformis*[6].

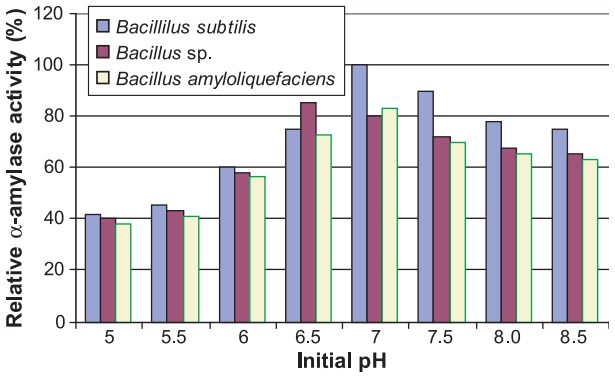

Figure 4. *Effect of initial pH of the culture medium on extracellular α-amylase production by three Bacillus isolates in shake-flask culture. The cultivations were carried out at 37°C under continuous agitation (150 rpm) on an orbital shaker for 72 h.*

Bacillus isolates was found to grow and produce α-amylase at temperatures from 30 to 50°C. Maximum enzyme production was observed at 37°C (Figure 5). Growth and enzyme production both decreased drastically above 40°C. Several investigators reported maximum α-amylase production from *Bacillus* spp. at 35-37°C[6,10].

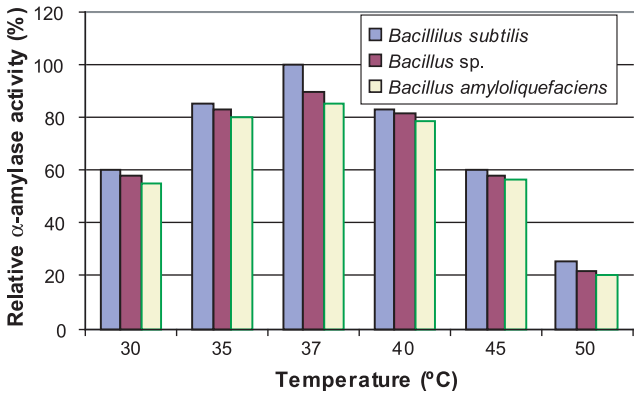

Figure 5. *Effect of incubation temperature on extracellular α-amylase production by three Bacillus isolates in shake-flask culture. The cultivations were carried out in the medium (initial pH 7.0) at various temperatures under continuous agitation (150 rpm) on an orbital shaker for 72 h.*

Agitation speed has important impact on extracellular α-amylase production[10]. In this study, it was observed that the α-amylase production from all three *Bacillus* isolates increased steadily with the increase of agitation up to 160-180 rpm. The best agitation speed that supported maximum enzyme production by *B. amyloliquefaciens*, *Bacillus* sp. and *B. subtilis* was at 160, 170

and 180 rpm respectively (Figure 6). Almost similar observations were evident in other studies where increase of α-amylase yield by some *Bacillus* strains was observed[10].

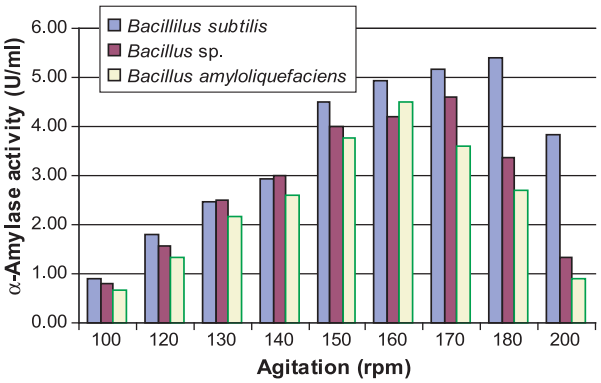

Figure 6. *Effect of shaking speed on extracellular α-amylase production by the Bacillus isolates in shake-flask culture. The cultivations were carried out at 37°C under continuous agitation (150 rpm) on an orbital shaker for 72 h.*

The *Bacillus* isolates merit further attention as potential sources of α-amylase. Among the three isolates compared *B. subtilis* seems more potent than the other isolates. The newly isolated *Bacillus* sp. showed α-amylase activity higher than that produced by the well-known starch hydrolyzing bacterium *B. amyloliquefaciens*.

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