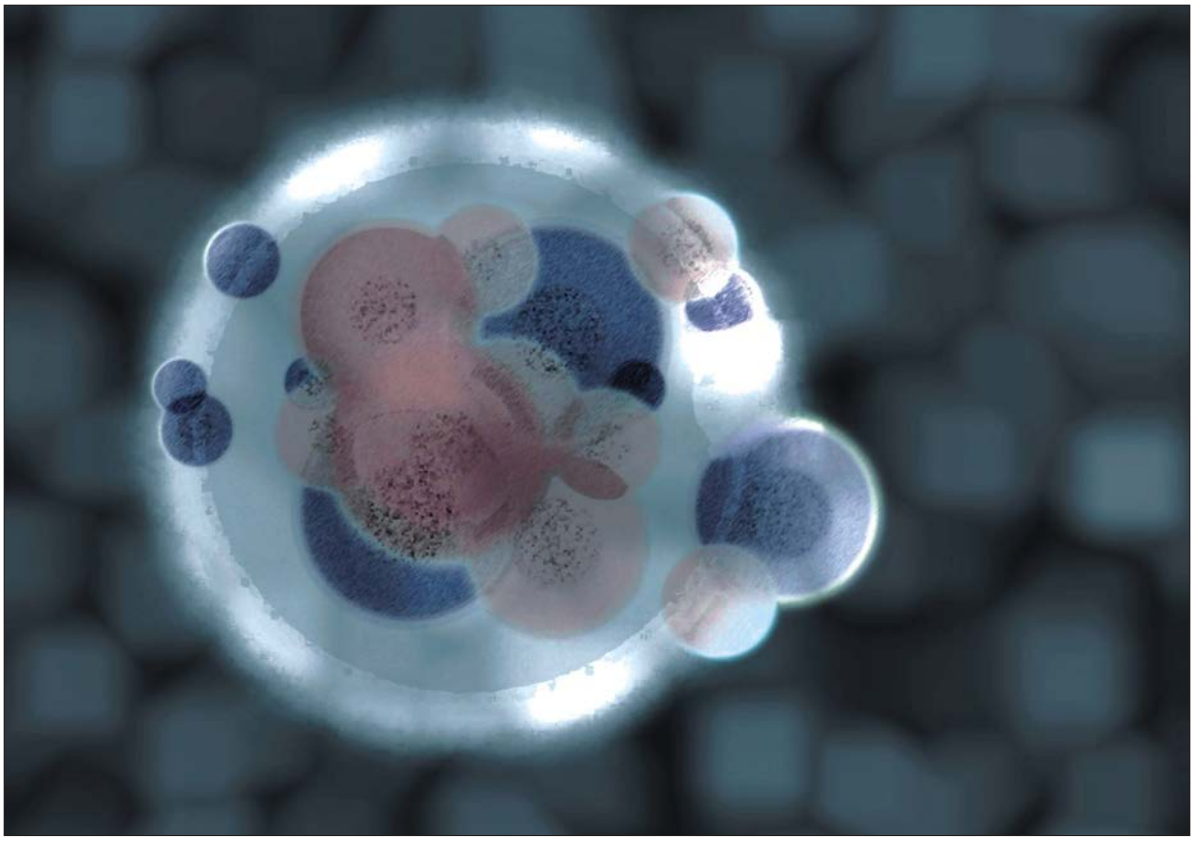

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Effect of Various Growth Conditions on Production of Extracellular Amylase from Thermotolerant *Bacillus* Species Isolated from Hot Springs in Jordan

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Abstract: All tested *Bacillus* species in this study had the ability to produce extracellular amylase when grown at pH 5-10 and at incubation temperature between 43-73°C. *Bacillus laterosporus* and *Bacillus schlegelii* were able to produce extracellular amylase at incubation temperature of 28°C. *Bacillus sphaericus* was the only species that produced extracellular amylase at medium pH of 4. *Bacillus circulans* showed extracellular amylase activity even in the presence of EDTA in its culture media. The presence of NaCl in the culture media variably promoted extracellular amylase abundance with *Bacillus schlegelii* was able to secrete amylase even in presence of 5% NaCl. The presence of fructose, maltose, lactose and glucose diminished extracellular amylase in all studied species. Presence of detergents Tween-20 or Tween-80 in the culture medium variably affected secretion of amylase.

Key words: *Bacillus circulans, Bacillus laterosporus, Bacillus marinus, Bacillus schlegelii, Bacillus sphaericus,* amylase activity, environmental conditions

INTRODUCTION

Enzymes are well known biological catalyst, produced by living cells to accelerate and coordinate the multitude of chemical reactions necessary to develop and sustain life; they possess a high level of selectivity, work under mild reaction conditions, easy to dispose off and are environmentally friendly. The majority of enzymes used to date have been obtained from mesophilic organisms. The applications of these enzymes are restricted because of their limited stability to extreme temperature, pH and ionic strength (Hough and Danson, 1999). Thermophiles which are known to prefer to live at 60-110°C (Brown and Doolittle, 1997; Blochl *et al*., 1997) are preferable sources of thermostable enzymes which are optimally active at temperatures above the host organism's optimum growth temperature (Brown and Kelly, 1993); due to their enzyme stability, thermophiles have been exploited commercially in food and other industries (Rao Mala *et al*., 1998).

Amylases that hydrolyze starch molecules (EC 2.4.1.25) (Gupta *et al*., 2003) may be classified based on location of bond hydrolyzed into α-amylases, β-amylases, amylo-glucosidases, pullulanases and cyclomalto-dextrin glycosyltransferases (Ashok *et al*., 2000; Vieille and Zeikus, 2001). Starch bioprocessing involves liquefaction and saccharification; both processes are run at high temperatures (Crabb and Mitchinson, 1997). Calcium-independent thermostable amylase is preferred in starch industries, although metals have long been known to affect enzymes activity and stability (Boel *et al*., 1990; Chung *et al*., 1995).

Bacillus species are considered as an ideal host for the industrial production of bulk extracellular enzymes (Van Wely *et al*., 1998), hence it lacks an outer membrane and proteins secreted are therefore immediately released into the external medium.

The aim of this study was to evaluate extracellular amylase abundance by *Bacillus* species isolated from Jordanian hot springs under various cultural conditions (Temperatures, pH-values, NaCl concentrations, EDTA, Tween, sugars and metal ions) in an effort to locate an industrially suitable *Bacillus* species as an amylase source and optimize its extracellular amylase secretion capabilities.

MATERIALS AND METHODS

Bacteria: *Bacillus circulans*, *Bacillus laterosporus*, *Bacillus marinus*, *Bacillus schlegelii* and *B. sphaericus* were originally supplied by Dr. Amjad Khalil (Department of Bioagriculture, Al-Balq'a University, Salt, Jordan) who had isolated them from Jordanian thermal ponds (Khalil, 2002).

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Bacterial growth: Vials containing 900 μL of isolation medium were inoculated using 100 μL (10^6 cells) of 24 h old bacterial cultures grown in isolation medium broth. Vials were then incubated at preferred temperature for 24 h.

Crude enzyme preparation: A the end of the incubation period, vials were centrifuged at 5000 rpm for 30 min at room temperature and the supernatant was used for amylase activity measurement.

Amylase activity measurement: Amylase Activity were measured according to the modified method of Al-Baker *et al.* (2000); activity were measured using peptone yeast extract agar plates prepared in the presence of 1% starch as substrate; then 6 mm diameter holes were created in agar plates by sterile Pasteur pipette; then 20 μL of supernatant of each species were introduced into each hole. The plates were incubated for 24 h at 43°C; at the end of incubation period, the diameter (mm) of clear halo zone was measured after Lugol's iodine solution (1% w/v iodine and 2% w/v potassium iodide) addition; the diameter indicates the hydrolysis of starch (Lim *et al.*, 1987) and represents amylase activity.

Residual activity of extracellular amylase: Thermostability of the crude enzyme was investigated by assaying residual activity after heat treatment of the supernatant at 73°C for 0, 1/2, 1, 2, 4 and 8 h. The heat-treated extracellular crude enzyme of each species was prepared and assayed as mentioned.

Effect of growth temperatures on production of extracellular amylase: Vials containing 900 μL of isolation medium were inoculated using 100 μL (10^6 cells) of 24 h old bacterial cultures grown in isolation medium broth. Vials were then incubated at 28, 37, 43, 53, 63 and 73°C; then, extracellular amylase activities present were examined as mentioned earlier.

Effect of growth medium pH on production of extracellular amylase: To evaluated effects of growth medium pH on the production of extracellular amylase activities, vials containing 900 μL of isolation medium adjusted at pH-values (4-11) were inoculated using 100 μL or 10^6 cells of 24 h old bacterial cultures grown in isolation medium broth. Vials were then incubated at 43°C for 24 h; extracellular amylase activities present were examined as mentioned above.

Effect of EDTA, divalent cations, tween, NaCl and sugars addition to the growth medium on production of extracellular amylase: EDTA final concentration of 4.0 mM, or one of the following divalent cations (Co^{++}, Mg^{++}, Zn^{++}, Mn^{++}, Hg^{++}, Ca^{++} or Fe^{++}) at 1 mM, or Tween-20 or Tween-80 at 1%, or sodium chloride to a final concentration of 0.5, 1, 3, 5, 7 and 10%, or one of the following sugars, fructose, glucose, lactose, maltose, sucrose, galactose, xylose, cellulose and starch at final concentration of 1% were individually added to the culture media and the extracellular amylase activity was evaluated as above.

RESULTS

Effect of incubation temperature on growth and abundance of extracellular amylase activity: Our results indicated that the optimum growth temperature for studied species was 43°C; very poor growth was obtained upon incubation at temperatures below 37°C or higher than 63°C.

At incubation temperature of 28°C, *B. laterosporus* and *B. schlegelii* were able to produce extracellular amylase activities; while, *B. circulans*, *B. marinus* and *B. sphaericus* produced no extracellular amylase activity at this incubation temperature (Table 1).

At growth temperatures of 37°C and 43°C, the five *Bacillus* species were able to produce extracellular amylase. At 53°C, *B. circulans*, *B. marinus*, *B. schlegelii* and *B. sphaericus* were able to produce extracellular amylase at this temperature. In contrast, *B. laterosporus* was unable to produce any extracellular amylase at this temperature. At 63°C, secretion of amylase was limited to *B. marinus*, *B. circulans* and *B. sphaericus*. At 73°C all studied species were unable to produce any extracellular amylase (Table 1).

Effect of culture medium pH on abundance of extracellular amylase: *B. sphaericus* was able to produce extracellular amylase if grown at pH range between 4-11; in fact among the five *Bacillus* species studied,

Table 1: Effect of growth temperature range, culture pH range,% NaCl range and EDTA on extracellular amylase abundance (B = *Bacillus*)

Bacillus species	Growth temperature ranges that allows amylase production	Culture pH range that allows amylase production	Range of NaCl% in culture media that allows amylase production	Effect of EDTA (0.4 mM) presence in culture media on amylase production
B. circulans	37-63°C	6-9	0.5-1	+
B. laterosporus	28-43°C	5-10	0.5-1	Nil
B. marinus	37-63°C	5-11	0.5-3	Nil
B. schlegelii	28-53°C	5-10	0.5-5	Nil
B. sphaericus	37-63°C	4-11	0.5-1	Nil

B. sphaericus was able to produce extracellular amylase at acidic growth medium pH 4; *B. laterosporus* was able to produce extracellular amylase activity if grown at pH between 5-10; on the other hand , *B. circulans* was able to produce extracellular amylase activity if grown at pH between 6-9 (Table 1).

The ability of each of the five studied species to produce extracellular amylase upon growth in cultures at pH-value between 4- 11 were different.

Effect of EDTA and divalent ions: In the presence of 0.4 mM EDTA in the growth media, *B. circulans* was the only species that showed extracellular amylase activity; on the other hand, no extracellular amylase activity was observed for *B. marinus*, *B. laterosporus*, *B. schlegelii* as well as *B. sphaericus*.

In the presence of Co^{++}, Zn^{++} and Hg^{++} at 1 mM in culture media, no extracellular amylase was found in the culture media of all studied species. The presence 1 mM of Fe^{++}, Mg^{++}, Mn^{++} and Ca^{++} in the culture media had no or little effect on the amylase abundance produced by *B. circulans, B. laterosporus, B. sphaericus* and *B. schlegelii*. Whereas, *B. marinus* showed variable response to the presence of such divalent ions; the abundance of extracellular amylase in the presence of metal ions may be arranged in the following decreasing order: $Fe^{++}>Mg^{++}>Mn^{++}>Ca^{++}$.

Effect of NaCl: All studied *Bacillus* species were able to produce extracellular amylase in the presence of 0.5 or 1% NaCl in the culture medium; at higher NaCl concentrations variable effects were noticed; for example, in the presence (in the culture medium) of 3.0% NaCl, only *B. schlegelii* and *B. marinus* showed amylase secretion; moreover, in the presence of 5.0% NaCl only *B. schlegelii* was able to secreted amylase. No extracellular amylase activity was observed in the culture media upon inclusion of 7% and 10% NaCl in the culture media for all species studied (Table 1)

Effect of the presence of different sugars: No amylolytic activity was detected in the presence of 1% fructose, 1% maltose, 1% lactose or 1% glucose for all tested species. On the other hand, the presence of 1% xylose, 1% galactose, 1% sucrose or 1% cellulose in the culture media of *B. circulans* and *B. marinus* showed extracellular amylase activity (Table 2).

Effect of the presence of Tween-20 and Tween-80: The presence of 1% Tween-20 or Tween-80 caused complete absence of extracellular amylase activity in the culture media of both *B. laterosporus* and *B. schlegelii;* while detergents presence had variable effect on amylase secretion from *B. circulans, B. marinus* and *B. sphaericus*. (Table 3)

Table 2: The effects of inclusion of 1% of Xylose, Galactose, Sucrose and cellulose in culture media prepared at pH 7.4 and incubated at 43°C on extracellular amylase abundance. (Note: 1% of each of Fructose, maltose, lactose and glucose produce no extracellular amylase activity). Amylase Activity Measurements were performed as indicated in the Method section. (*B = Bacillus*)

	Amylase activity (Diameter in mm)			
Bacillus species	Xylose	Galactose	Sucrose	Cellulose
B. circulans	8.9±2.1	17±3.7	10.5±2.3	12.5±2.8
B. laterosporus	Nil	Nil	Nil	Nil
B. marinus	11.2±2.6	17.6± 3.3	13±2.4	15.3±2.9
B. schlegelii	Nil	Nil	Nil	Nil
B. sphaericus	Nil	Nil	Nil	Nil

Table 3: The effects of the presence of 1% Tween-20 or Tween-80 in the culture medium at pH 7.4 and growth incubation temperature of 43°C on the abundance of extracellular amylase for all studied species. Amylase Activity Measurements were performed as indicated in the Method section. (*B = Bacillus*)

	Amylase activity (Diameter in mm)	
Bacillus species	Tween-20	Tween-80
B. circulans	16.7± 3.1	14.2±2.9
B. laterosporus	Nil	Nil
B. marinus	14.4± 3.1	10.5± 2.2
B. schlegelii	Nil	Nil
B. sphaericus	11.5± 3.0	9.7±2.1

Residual activity of amylase enzymes after incubation at 73°C: After 30 min incubation at 73 °C, activity was detected for amylase produced by all five *Bacillus* species; Amylase abundance after the heat treatment may be arranged in the following decreasing order: *B. schlegelii* > *B. marinus* > *B. sphaericus* > *B. circulans*. No amylase activity was detected after the incubation for 60, 120, 240 min and 8 h in all studied species.

DISCUSSION

Bacillus species are considered as an ideal host for the industrial production of bulk extracellular enzymes (Van Wely *et al*., 1998). Vieille *et al.* (1996) reported that thermophiles produced enzymes optimally at temperatures close to the host organism's optimal growth temperature; on the other hand, Fujiwara *et al.* (1996) reported that enzymes may be optimally produced at 10-20°C below the organism's optimum growth temperature. In our study, temperatures of maximum extracellular amylase production were not the organism's optimal growth temperature with the exception of that for *B. laterosporus*.

Ashok *et al.* (2000) found relationship between pH of the growth medium and the optimum pH of amylase

activity. Variation in extracellular amylase production in this study may reflect the differences in pH optimum for amylase of each strain.

Four of the *Bacillus* species studied required the presence of Ca^{++} or Mg^{++} ion in the culture media to produce extracellular amylase as required for synthesis of metalloenzymes as part of holoenzyme (Marg and Clark, 1990; Dey *et al*., 2002; Hagihara *et al*., 2001; Najafi *et al*., 2005). *Bacillus circulans* on the other hand, as our study indicated, continued the production of extracellular amylase activity even in the presence of EDTA in the culture media, which suggested that amylase enzyme produced was not a metalloenzyme. Calcium-independent thermostable amylase is preferred in starch industries.

The complete absence of amylase activity in presence of Co^{++}, Zn^{++} and Hg^{++} in the culture media may indicate decreased functional metalloenzyme production (Lin *et al*., 1998).

The inclusion of NaCl, Tween-20 and Tween-80 detergents in the culture media may affect stability and/or solubilization of membrane enzyme (Carlos *et al*., 2002); similar results were reported on *Geobacillus thermoleovorans* (Uma and Satyanarayana, 2003).

Amylase secretion in presence of different sugars in the culture media, was poorly produced in almost all strains studied ; this may be related to amylase gene repression modulated by sugars (Lin *et al*., 1998)

In conclusion, enhancement of extracellular amylase abundance in the culture media was achieved under various cultivation conditions and some *Bacillus* species can produce calcium-independent and thermostable amylase, which can find use in starch industries.

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