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Production and Immobilization of α-amylase from *Bacillus subtilis*

[1]Tarek E. El-Banna, [1]Ahmed A. Abd-Aziz, [2]Mohamed I. Abou-Dobara and [1]Reham I. Ibrahim
[1]Department of Microbiology, Faculty of Pharmacy, Tanta University, Egypt
[2]Department of Botany (Microbiology), Damietta, Faculty of Science,
New Damietta, Egypt

Abstract: α-amylase production by *Bacillus subtilis* was studied under different cultivation conditions. The maximum α-amylase production occurred after an incubation period of 48 h, temperature 40°C and pH 7.5. Among the defined carbohydrates, starch (1%) was the best carbon source. The organism grew better and produced high levels of α-amylase using peptone as nitrogen source. The produced α-amylase was immobilized on various carriers by different methods and the properties of the enzyme were compared before and after immobilization. The optimum pH of the immobilized enzyme was changed to acidic range. The optimum reaction temperature of immobilized enzyme was shifted slightly to 70-80°C. Both of K_m values and V_{max} and thermal stability of immobilized enzyme were found to be higher than that of free one. Among the tested metals $CaCl_2$ exerted a stimulating effect on the activity of α-amylase.

Key words: Production, immobilization, α-amylase, *Bacillus subtilis*

INTRODUCTION

α-amylase (EC3.2.1.1, 1, 4-α-D-glucan glucanohydrolase, endoamylase) hydrolyzes starch, glycogen and related polysaccharides by randomly cleaving internal α-1, 4-glucosidic linkages. It is widely distributed in various bacteria, fungi, plants and animals and has a major role in the utilization of polysaccharides (Kelly *et al.*, 1997). α-amylases are used commercially for the production of sugar syrups from starch that consist of glucose, maltose and higher oligosaccharides (Guilbot and Mercier, 1985).

Immobilized enzymes are becoming increasingly popular as reusable, selective analytical chemical reagents in solid phase flow-through reactors, as membranes in sensors and as films in dry reagent kits. For industrial application, the immobilization of enzymes on solid support can offer several advantages, including repeated usage of enzyme, ease of product separation, improvement of enzyme stability and continuous operation in packed-bead reactors (Abdel-Naby, 1993). A wide variety of carriers have been used for immobilization of amylase (Emne'us and Gordon, 1990; Kurakake *et al.*, 1997; Tien and Chiang, 1999; Dey *et al.*, 2003).

In our laboratory we isolated and identified a bacterial species as *Bacillus subtilis* that able to produce

α-amylase. Optimization of different cultural conditions is expected to improve the α-amylase production. The aim of this research is to investigate the effect of some factors on α-amylase production by *Bacillus subtilis* and immobilize the produced α-amylase onto various carriers to compare the effectiveness of the different methods for immobilization. The properties of the immobilized enzymes were also compared to those of free enzyme.

MATERIALS AND METHODS

Present study was conducted throughout the years 2005 and 2006 and carried out in the laboratories of Botany Department, Faculty of Science, New Damietta, Mansoura University, Egypt.

Organism: *Bacillus subtilis* was isolated from a fertile soil sample collected from Egypt and identified according to Bergey's manual of systematic bacteriology (Sneath, 1986).

Cultivation: The organism was grown in 250 mL Erlenmeyer flasks, each containing 50 mL of starch nutrient broth medium that has the following composition (g/L): beef extract, 3.0; peptone, 10.0; NaCl, 5.0; starch, 10.0; distilled water, 1000 mL. The pH was adjusted to 7.0. The flasks were inoculated with 1 mL of the spore

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Corresponding Author: Dr. Mohamed I. Abou-Dobara, Department of Botany (Microbiology), Damietta, Faculty of Science,
New Damietta, Egypt

suspension obtained from nutrient agar cultures and incubated at 40°C with shaking at 150 rev min^{-1} for 48 h. The filtrate was then separated by centrifugation at 10,000 for 10 min.

Estimation of α-amylase: α-amylase was assayed by measuring the amount of reducing sugar released from 1% starch. The amount of reducing sugar was estimated according to Miller (1959). The reaction mixture contained 0.4 mL of (1%) (w/v) soluble starch plus 0.5 mL each of diluted culture supernatant or partial purified enzyme preparation and 1.1 phosphate buffer (0.2 M, pH 7). The mixture was incubated at 50°C for 20 min. centrifuged at 10,000 rpm for 10 min and aliquots of the supernatants were assayed for reducing sugars. One unit of the enzyme activity was defined as the amount of enzyme that released 1 μmole of reducing sugar (expressed as glucose equivalents) per milliliter per minute under assay conditions. Enzyme and substrate controls were included routinely.

Growth estimation: Growth was determined spectrophotometrically by measuring optical density of the culture at 600 nm. The final pH was also recorded at the end of each experiment.

Factors affecting α-amylase production by *Bacillus subtilis*

Incubation periods: The basal medium containing 1% soluble starch was used; 25 mL medium in 250 mL conical flasks was inoculated with 1 mL of an overnight culture in triplicate manner. The culture was incubated in an incubator shaker at a shake rate of 150 rpm. Samples were taken at 12 h intervals (12, 24, 36, 48, 60 and 72 h).

Effect of pH: Different flasks of basal amylase production media of different initial pH values (4, 5, 6, 7, 8 and 9). The pH was adjusted by using 0.1 N NaOH and 0.1 N HCl. The inoculated flasks were incubated at 40°C for 48 h.

Effect of different temperatures: Temperature effect was carried out by inoculating the flasks containing α-amylase production medium at pH 7 with the tested strain and incubated at different temperatures, notably, 30, 35, 40, 45 and 50°C for 48 h.

Effect of different carbon sources: Different carbon sources including; D-glucose, fructose, lactose, xylose, galactose, mannitol, sucrose, potato starch, rice starch, flower starch) were incorporated into flasks containing the production medium to the final concentration of 1% (w/v). The flasks were inoculated and incubated at 40°C for 48 h.

Effect of different concentration of soluble starch: Different concentrations of soluble starch solution (0.5, 1.0, 1.5 and 2.0%) in 25 mL basal medium and the flasks were inoculated with the bacterial culture and after incubation at 40°C for 48 h.

Effect of different nitrogen sources: Different nitrogen sources were added to the medium according to the nitrogen content of peptone (16.16% nitrogen) and beef extract (12% nitrogen). These nitrogen sources included peptone, tryptone, beef extract, yeast extract, NH_4Cl, $(NH_4)_2SO_4$, NH_4NO_3 and KNO_3 were inoculated and incubated at 40°C for 48 h.

Physicochemical properties of the α-amylase
The pH: To study the effect of pH on the activity of α-amylase, citrate phosphate buffer with pH's (from 3 to 5), phosphate buffer with different pH's (from 6 to 8) and carbonate buffer with pH's (from 9.5 to 10.5) were used in the standard assays. Aliquots of the enzyme preparations were mixed with the different buffers at each pH and the enzyme activity was assayed as previously described. Activities were expressed as a percentage of the maximal activity.

Temperature: Reaction mixtures at different temperature (30, 40, 50, 60, 70, 80°C), while the assay was carried out under the standard assay methods. Activity was expressed as % of the maximal activity. The effect of temperature on the stability of α-amylase was determined by incubating the enzyme solution in the absence of substrate for different time at 60°C at pH. The residual activity was assayed as described before.

Effect of metals: 0.5 mL of the dialyzed precipitated α-amylase was mixed with 0.5 mL of calcium chloride, sodium chloride, cobalt chloride, nickel chloride and EDTA solutions, 1, 5 and 10 mmol of each metal. The α-amylase activity was determined as previously.

K_m and V_{max}: The K_m and V_{max} of α-amylase were determined at pH 7 and 40°C by varying the substrate concentration from 1.0 to 15 mg of starch per mL. Data plotted by the method of Lineweaver-Burk plot.

Partial purification of the amylase enzyme: The crude enzyme was precipitated with ammonium sulphate. The precipitation was carried out at 4°C under constant stirring and the precipitated proteins were centrifuged at 10,000 rpm for 10 min. The fraction was dialyzed against 0.1 M phosphate buffer (pH 7) to remove the remaining salt. Specific activity of the enzyme was estimated in all precipitates. This partially purified enzyme was used for the preparation of the immobilized enzyme.

Protein estimation: The quantitative estimation of protein was carried out according to Bradford (1976). Protein concentrations were determined from a standard curve with Bovine Serum Albumin (BSA).

Immobilization methods
Immobilization on sodium-alginate (Entrapment): An equal volume of tested enzyme solution and sodium alginate solution was mixed to give a 4% (w/v) final concentration of sodium alginate solution. The mixture obtained was extruded drop wise through a Pasteur pipette (1 mm diameter) into a gently stirred 2% (w/v) $CaCl_2.2H_2O$ solution 2 h to give bead size of 3 mm. The calcium alginate beads containing the enzyme were thoroughly washed with distilled water and used for further studies (Roy *et al.*, 2000).

Immobilization on chitin (physical adsorption): Chitin beads were prepared as follows; 0.5 g of chitin was shacked in 10 mL phosphate buffer containing 2.5% glutaraldehyde for 2 h at room temperature. The solid material was filtered and washed to remove excess of glutaraldehyde. The treated chitin was mixed with 30 mL of the enzyme solution for 24 h at 4°C. The mixture was centrifuge at 10,000 rpm for 5 min and the supernatant was used for further study. The precipitated was washed by phosphate buffer containing small amount of 0.1 M sodium chloride then subjected to the immobilized enzyme activity (Ohtakara *et al.*, 1988).

Immobilization on sephadex: One gram of Sephadex was added to 30 mL of the partial purified enzyme solution in phosphate buffer (pH 7) and kept at 4°C for 24 h. The unbound enzymes were removed by washing three times with the same buffer (Kusunoki *et al.*, 1982).

Immobilization on dowex (ionic binding): One gram Dowex was incubated with 30 mL of the partial purified enzyme solution in phosphate buffer (pH 7) and kept at 4°C for 24 h. The unbound enzymes were removed by washing three times with the same buffer to remove unbounded protein.

Physical properties of the immobilized α-amylase: Effect of pH, temperature, thermal stability and kinetic constant K_m and V_{max} and some metal on physical properties of the immobilized α-amylase were studied as mentioned before.

Statistical analysis and data presentation: The experiments were carried out in three replicates with standard deviation and standard error presented the statistical procedure used where appropriate was the t-test.

RESULTS

The time course for the production of α-amylase by *Bacillus subtilis* in the basal liquid medium containing 1% soluble starch as a substrate is shown in the Fig. 1. The α-amylase activity of the *Bacillus subtilis* increased during the growth phase of the culture and the optimum period for the production was reached after 48 h. After 60 h, α-amylase production declined significantly (p<0.05, t-test). α-amylase production reached the minimum level after 72 h. The maximum growth measured after 24 h and the growth declined significantly to reach the minimum level after 48 to 60 h.

As shown in the Table 1 *Bacillus subtilis* was able to grow in basal liquid medium supplied with the different carbon sources which were glucose, xylose, galactose, mannitol, rice, flower, potato, sucrose, fructose, lactose and starch. The best carbon source for production of α-amylase for *Bacillus subtilis* was starch with activity $(3.19 \mu mL^{-1})$ and the maximum growth measured with galactose. Glucose, xylose, galactose, sucrose, mannitol, fructose and lactose were found to be good sources for the production of α-amylase. The growth was recorded when using flower powder xylose, galactose, rice powder, potato powder, starch and lactose. Little growth was observed with glucose, mannitol, sucrose and fructose.

The optimum concentration of starch for α-amylase production was 0.25 mg mL^{-1} with activity of 3.6 μmL^{-1} (Fig. 2). At the concentration of 0.375 mg mL^{-1} the activity could be decreased or still constant, no change in the α-amylase production. Also at 0.125 mg mL^{-1} the activity well be increased until reached to 0.25 mg mL^{-1}.

The optimum pH for α-amylase production was at pH 7 with activity 1.95 μmL^{-1} (Fig. 3.). The activity of α-amylase was decreased significantly at pH 9.0 (p<0.05, t-test). The range of maximum growth was found from pH 5 to 8, respectively.

An optimum temperature for α-amylase production from *Bacillus subtilis* was 45°C (Fig. 4.). At 50°C the activity was declined significantly (p<0.05, t-test). The range of maximum growth from 40 to 45°C, respectively.

Table 1: Effect of different carbon sources on α-amylase production and growth of *Bacillus subtilis*

Carbon source	α-amylase $(\mu mL^{-1}) \pm SE$	Growth (Optical density at 600 nm) $\pm SE$
Glucose	1.89±0.214	0.134±0.0001
Xylose	2.543±0.222	0.39±0.0003
Galactose	1.426±0.117	0.168±0.0001
Mannitol	0.244±0.020	0.022±1.74
Sucrose	1.58±0.020	0.161±0.0001
Fructose	1.94±0.052	0.138±0.0001
Lactose	1.601±0.230	0.358±0.0002
Starch	3.19±0.033	0.33±0.0002
Rice	2.243±0.106	0.365±0.0002
Flower	2.33±0.309	0.385±0.0003
Potato	2.238±0.153	0.259±0.0002

Each results is the means of three replicates±standard error (SE)

Table 2: Effect of different nitrogen sources on α-amylase production and growth of *Bacillus subtilis*

Nitrogen source	α-amylase (μ mL^{-1}) ±SE	Growth (Optical density at 600 nm) ±SE	Final pH
Ammonium sulphate	0.021±0.0006	0.01±0.0007	5.30
Ammonium chloride	0.043±0.003	0.04±0.003	5.00
Ammonium nitrate	1.34±0.0287	0.29±0.0217	5.28
Potassium nitrate	1.52±0.047	0.31±0.023	6.50
Tryptone	0.787±0.0169	0.181±0.013	8.53
Peptone	2.71±0.0344	0.587±0.044	8.06
Beef extract	2.4±0.0877	0.485±0.036	8.76
Yeast extract	1.72±0.025	0.378±0.0284	8.67
Peptone±Beef extract	1.89±0.0488	0.423±0.0317	7.14

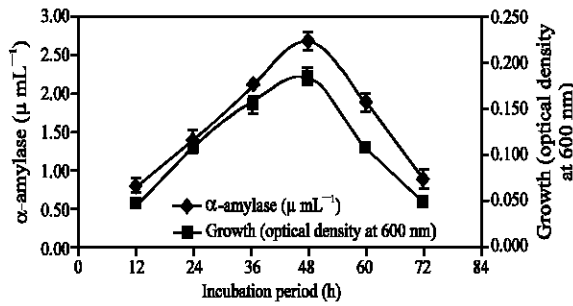Each results is the means of three replicates±standard error (SE)

Fig. 1: Effect of different incubation periods on α-amylase production and growth of *Bacillus subtilis*. Values are shown as means of triplicates±standard error
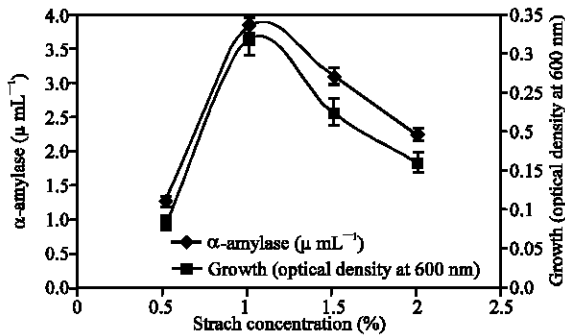

Fig. 2: Effect of different starch concentration on α-amylase production and growth of *Bacillus subtilis*. Values are shown as means of triplicates±standard error

Fig. 3: Effect of different pH values on α-amylase production and growth of *Bacillus subtilis*. Values are shown as means of triplicates±standard error

Fig. 4: Effect of different temperatures on α-amylase production and growth of *Bacillus subtilis*. Values are shown as means of triplicates±standard error

The best nitrogen source for production of α-amylase from *Bacillus subtilis* was peptone with activity (2.71 μ mL^{-1}) and the maximum growth measured with beef extract as shown in Table 2.

Physicochemical properties of free and immobilized α-amylase: Free α-amylase activity increased with increasing pH within the range pH (3-7) till reached to the optimum point of pH 7 (Fig. 5). Generally, the optimum pH of the immobilized α-amylase was shifted to acidic range in comparison to the free enzyme (Fig. 5). After the optima pH of the free and immobilized α-amylase, the relative
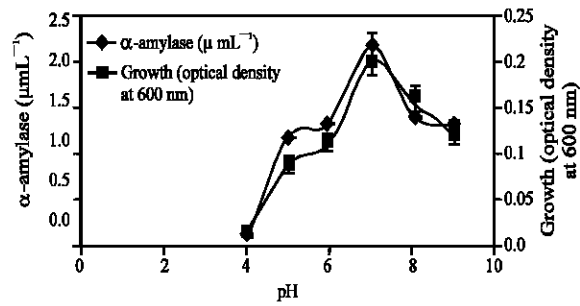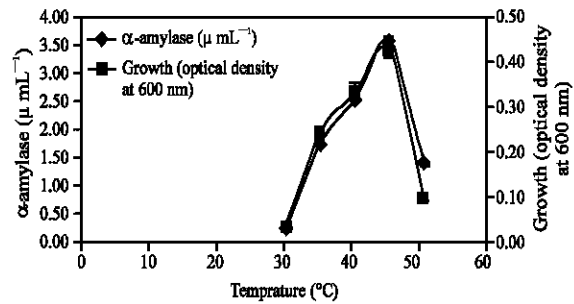
activity decreased and reached to minimum level at pH 8 and 9.5 as shown in Fig. 5.

The activity of free α-amylase increased with increasing temperature starting from 40°C till reached to the optimum activity at 50°C (Fig. 6). The optimal reaction temperature of the free α-amylase shifted from 50°C to 70°C for the enzyme immobilized on Dowex and Ca alginate and to 80°C for immobilization on sephadex and chitin (Fig. 6). The minimum level of the free and immobilized α-amylase activity occurred at 90°C as shown in Fig. 6.

Generally, some metals increased the activity of free and immobilized α-amylase such as CaCl$_2$ and NaCl with the three used concentrations (1, 5 and 10 mM). The other

Table 3: Effect of metal ions on relative activity (%) of free and immobilized α-amylase of *Bacillus subtilis*. Values are shown as means of triplicates

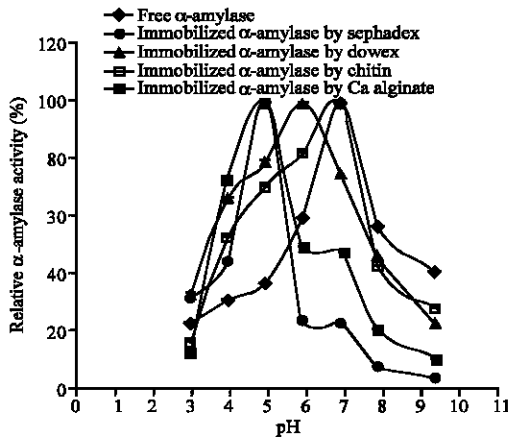|				Immobilized α-amylase			
Chemical	Concentration (mM)	None	Free α-amylase	Sephadex	Dowex	Chitin	Ca alginate
CaCl₂	1	100	105	109	106	106	103
	5	100	109	118	109	111	104
	10	100	116	121	112	114	107
NaCl	1	100	91	103	103	103	102
	5	100	94	107	104	105	103
	10	100	97	111	106	107	105
CoCl₂	1	100	92	93	78	81	87
	5	100	79	84	48	65	72
	10	100	65	71	39	43	38
NiCl₂	1	100	52	64	71	79	65
	5	100	41	46	42	54	28
	10	100	29	32	28	37	23
EDTA	1	100	24	35	37	19	25
	5	100	12	19	7	6	5
	10	100	2	5	5	2	2

Fig. 5: Effect of pH on activity of α-amylase of *Bacillus subtilis* before and after immobilization. Values are shown as means of triplicates±standard error (SE)
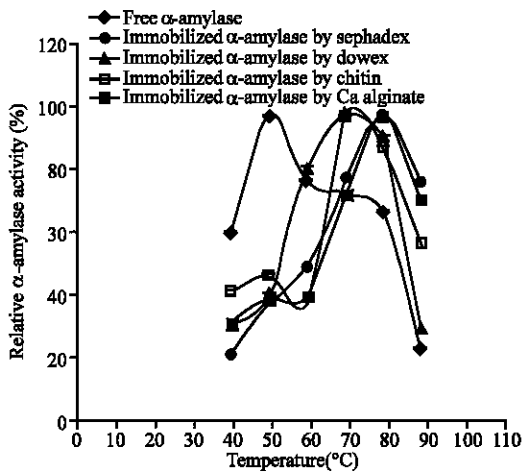

Fig. 6: Effect of temperature on activity of α-amylase of *Bacillus subtilis* before and after immobilization. Values are shown as means of triplicates±standard error (SE)
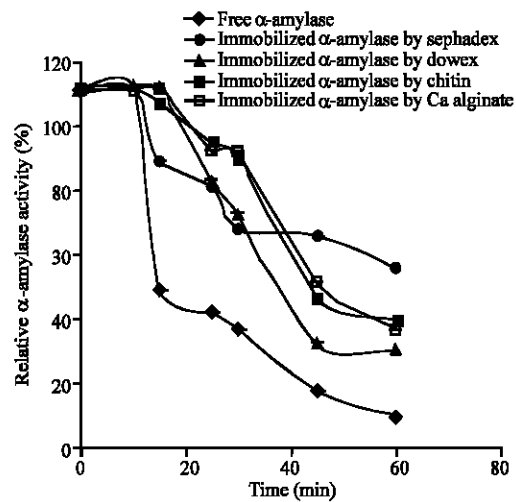

Fig. 7: Thermal stability of α-amylase of *Bacillus subtilis* before and after immobilization. Values are shown as means of triplicates±standard error (SE)

metals, notably $CoCl_2$, $NiCl_2$ and EDTA inhibited the activity of free and immobilized α-amylase. The minimum level of the activity was reported when using EDTA in all concentrations (Table 3).

In general, the calculated K_m and V_{max} values of the immobilized enzymes are higher than that of the free enzyme (Table 4).

Thermal stability of free and immobilized α-amylase was completely stable at 60°C for 10 min (Fig. 7). The free enzyme lost about 50% of the relative activity after 15 min while the immobilized ones retain 60% in case of utilization of sephadex and dowex; to 80% of the activity after 30 min when chitin and Ca-alginate were used for immobilization, respectively (Fig. 7). After 60 min, the free and immobilized enzyme lost more than 50% of the activity.

Table 4: K_m and V_{max} values of free and immobilized α-amylase of *Bacillus subtilis*. Values are shown as means of triplicates

Value	Free α-amylase	Immobilized α-amylase			
		Sephadex	Dowex	Chitin	Ca alginate
K_v (mg mL^{-1})	8.3	16.66	16.66	9.09	14.28
V_{max} (μ mole mL^{-1} min^{-1})	10	30.30	25	11.11	30.30

DISCUSSION

Extracellular α-amylase production and growth of *Bacillus subtilis* was maximal at pH 7 and this result is in accordance with the work of Bajpai and Bajpai (1989) and Lin *et al.* (1998). The composition of cell wall and plasma membrane of microorganisms is known to be affected by the culture pH (Ellwood and Tempest, 1972a, b). The change of the initial pH of the medium may be leads to change of the nature of the cell membrane and/or cell wall and hence affecting the α-amylase production and the growth of *Bacillus subtilis*. On the other hand, Mamo and Gessesse (1999) reported that high level of α-amylase at pHs 5.0 and 6.0 and when the pH was increased low level of α-amylase was obtained.

The optimal temperature for production of α-amylase and growth of *B. subtilis* occurred at 45°C. Similar observations were recorded by Bajpai and Bajpai (1989), Lin *et al.* (1998) and Anyangwa *et al.* (1993). Bacterial cells have various mechanisms that allow them strictly to control excretion (Mamo amd Gessesse, 1999). Change in the nature of cell envelope can affect the release of extracellular enzymes to the culture medium (Antranikian, 1990). Temperature is one of the factors that induces such changes on cell membranes and cell walls (de Vrij *et al.*, 1990; Nordstrom, 1993). It is also reported that in Bacillaceae, the surface protein layer (S-layer) in involved in the control of exoenzyme release (Egelseer *et al.*, 1996a, b).

The best carbon source for α-amylase production from *B. subtilis* was found to be starch. Starch is known to induce amylase production in different bacterial strains (Saito and Yamamoto, 1975; Fogarty and Bourke, 1983; Wind *et al.*, 1994; Aiyer, 2004; Ryan *et al.*, 2006). Low levels of α-amylase were detected when the organism was grown with mannitol. Similar observations were recorded by Wind *et al.* (1994), Chandra *et al.* (1980) and Meers (1972). Glucose and fructose showed repression effects on enzyme production. It has been reported that the synthesis of carbohydrate degrading enzymes in most species is subjected to catabolite repression by readily metabolize substance such as glucose and fructose. Among the undefined carbon source that led to high levels of amylase were flower, potato and rice. Undefined carbon sources are known to induce a high level of

amylase production in many bacterial strains (Fogarty *et al.*, 1974; Mamo and Gessesse, 1989; Tonkova *et al.*, 1993; Ryan *et al.*, 2006).

α-amylase production and growth of *Bacillus subtilis* increased after 24 to 48 h. After this period, the production of the enzyme decreased. The observed peaking and throughing of the production of extracellular enzymes can be attributed to (1) The products of action of one component inducing the synthesis of another, (2) Differential inhibition by products of substrate hydrolysis, (3) A decrease in growth was observed after 48 h of growth of *B. subtilis*. This probably resulted from cellular lysis, an observation previously reported. These results indicate that the production of extracellular α-amylase by *B. subtilis* was growing associated and this is agreement with other investigators (Bajpai and Bajpai, 1989; Stephenson *et al.*, 1998; Riaz *et al.*, 2003).

The maximum starch concentration for the optimal α-amylase production from *Bacillus subtilis* was 1.0%. Similar results were reported by Bajpai and Bajpai (1989). *Bacillus subtilis* produced different levels of α-amylase with varying C: N ratio and this may be explained as different types of induction of enzymes. This may suggest different mechanisms of production of extracellular enzymes of *Bacillus subtilis* when grown with different C: N ratio.

The optimal production of α-amylase and growth of *B. subtilis* were obtained when peptone was used as nitrogen sources and this is in accordance similar with Aiyer (2004), Chandra *et al.* (1980) and Babu and Satyanarayana (1995). Peptone containing medium is complex and contains high nutritional amino acids and this may be lead to high α-amylase production and good growth of *Bacillus subtilis*. Proteose peptone was recorded as good nitrogen source for amylase production and growth of thermophilic *Bacillus* sp. WN11 (Bajpai and Bajpai, 1989).

The pH optimal for the free α-amylase of *B. subtilis* was found to occur at pH 7. These results are in accordance with those reported by Vihinen and Mäntsälä (1989), Hamitton *et al.* (1999), Saito (1973) and Khoo *et al.* (1994). The immobilized-amylase showed optimum pH in the acidic range in comparison to the free enzyme. These effects may be dependent on the ionic environment around the active site of the enzyme bound to the carrier (Abdel-Naby, 1993). Similar shifts of pH optima were reported by Sadhukhan *et al.* (1993) and Yoshida *et al.* (1989).

The free α-amylase of *B. subtilis* showed temperature optima at 50°C and this result is in accordance with Mamo and Gessesse (1999) and Vihinen and Mäntsälä (1989). The immobilized α-amylase showed higher relative activity at the temperatures 70 and 80°C than that of free

enzyme. Increase in temperature optima had been found in immobilized enzymes (Sadhukhan *et al.*, 1993; Yoshida *et al.*, 1989). The increase in temperature optima for the activity of immobilized enzyme could be due to the fact that actual temperature in the micro-environment of the gel matrix incases of Ca-alginate as a carrier was lower than in the bulk solution (Kennedy, 1987).

Free α-amylase of *B. subtilis*, the relative activity was declined to 44% after 15 min, to 38% after 25 min at 60°C, these results are also in accordance with that to Gerhartz, (1990), Shaw and Chuang (1982) and Shaw and Ou-Lee (1989). The thermoinactivation at 60°C of free and immobilized enzyme showed remarkable achievement of thermostability by the immobilization with *Bacillus subtilis* with different carriers. The high thermostability of immobilized-amylase is consistent with the results obtained for other enzymes (Sheffield *et al.*, 1995). The thermal stability of the enzyme increased the tolerance to thermal denaturation, therefore, had been imparted by the gel entrapment covalent cross-linking of the enzyme protein.

The free α-amylase from *Bacillus subtilis* showed high affinity towards starch with a K_m and V_{max} values of 8.3 mg mL^{-1} and 10.0 µ mole min^{-1} mL^{-1}, respectively. The K_m and K_{max} of the immobilized α-amylase increased with the four carriers. This increase is most likely a consequence of either structural changes in the enzyme introduced by the applied immobilization procedure and/or lower accessibility of the substrate to the active site of the immobilized enzyme (Abdel-Naby, 1993). The latter, as explained above, may result either from diffusional resistance of the matrix or steric hinderance in the immediate vicinity of the enzyme molecules (Abdel-Naby, 1993).

The results obtained in the our study were similar to those that have been reported for immobilized glucoamylase (Koji *et al.*, 1999). For practical application, an immobilized system with lower K_m value and faster rate of reaction is preferred.

Among the tested metals, $CaCl_2$ was the most activator metals. The relative activity of free and immobilized α-amylase from *B. subtilis* increased in the presence of all concentration of calcium. These results are in accordance with the work of other investigators (Lin *et al.*, 1998). Ca^{2+} ions has a positive effect on the activity of the α-amylase enzyme. The binding of Ca^{2+} ions has been shown to increase the-helical structure of the α-amylase enzyme leading to increased stability. Na^+ ions similar to Ca^{2+} increased the activity of the α-amylase. The ions likes Co^{2+}, Ni^{2+} slightly inhibited amylase activity. Some amylases are metalloenzymes,

containing a metal ion with a role in catalytic activity by Ni^{++} and Co^{++} ions could be due to competition between the exogenous cations and the protein-associated cation-resulting in decreased metalloenzyme activity. The inhibitory effect of the chelating agent EDTA demonstrated the ion requirement of this amylase similarly. EDTA has been found to be a potent inhibitor of amylases from other bacteria (Sadhukhan *et al.*, 1993; Yoshida *et al.*, 1989; Shaw *et al.*, 1995).

REFERENCES

Abdel-Naby, M.A., 1993. Immobilization of *Aspergillus niger* NRC 107 xylanase and xylosidase and properties of the immobilized enzymes. Applied Biochem. Biotechnol., 38: 69-81.

Aiyer, P.V.D., 2004. Effect of C: N ratio on alpha-amylase production by *Bacillus licheniformis* SPT 27. Afr. J. Biotechnol., 3: 519-522.

Antranikian, G., 1990. Physiology and enzymology of thermophilic anaerobic bacteria degrading starch. FEMS Microbiol. Rev., 75: 201-218.

Anyangwa, E.M., C. Mapsev, P. Musanage and M. Elemva, 1993. The effect and removal of starch in the sugar refining industry. J. Int. Sugar, 95: 210-213.

Babu, K.R. and T. Satyanarayana, 1995. Extracellular calcium in habited α-amylase production in batch and fell batch cultures of *Bacillus subtilis*. Folia Microbiol., 29: 359-364.

Bajpai, P. and P. Bajpai, 1989. High temperature alkaline α-amylase from *Bacillus licheniformis* TCRFC-BI3. Biotechnol. Bioeng., 33: 72-78.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem., 72: 248-254.

Chandra, A.K., S. Medda and A.K. Bhadra, 1980. Production of extracellular thermostable-amylase by *Bacillus licheniformis*. J. Ferment. Technol., 58: 1-10.

De Vrij, W., G. Speelmans and R.I.R. Heyne, 1990. Energy transduction and amino acid transport in thermophilic aerobic and fermentative bacteria. FEMS Microbiol. Rev., 75: 183-200.

Dey, G., B. Singh and R. Banerjee, 2003. Immobilization of α-amylase produced by *Bacillus circulans* GRS 313. Brazilian Arch. Biol. Technol., 46: 167-176.

Egelseer, E., I. Schocher and M. Sara, 1996a. The S-layer from *Bacillus stearothermophilus* DSM 2358 functions as an adhesion site for a high-molecular-weight amylase. J. Bacteriol., 177: 1444-1451.

Egelseer, E., I. Schocher and U. Sleytr, 1996b. Evidence that an N-terminal S-layer protein fragment triggers the release of a cell-associated high-molecular-weight amylase in *Bacillus stearothermophilus* ATCC 12980. J. Bacteriol., 178: 5602-5609.

Ellwood, L.L. and D.W. Tempest, 1972a. Effects of environment on bacterial wall content and composition. Adv. Microb. Physiol., 7: 83-117.

Ellwood, L.L. and D.W. Tempest, 1972b. Influence of culture pH on the content and composition of teichoic acid in the walls of *Bacillus subtilis*. J. Gen. Microbiol., 73: 395-397.

Emne'us, J. and L. Gordon, 1990. Effects on the hydrolysis of native starch and glycogen by a thermostable α-amylase after immobilization on solid supports. Anal. Chim. Acta, 234: 97-106.

Fogarty, M.M., P.J. Griffin and A.M. Joyce, 1974. Enzymes of *Bacillus* species. Part 1. Process Biochem. July/August, pp: 11-24.

Fogarty, M.M. and E.J. Bourke, 1983. Prodcution and purification of maltose-producing amylase from *Bacillus subtilis* IMD 198. J. Chem. Technol. Biotechnol., 33B: 145-154.

Hamitton, L.M., C.T. Kelly and W.M. Fogarty, 1999. Purification and properties of the raw starch degrading α-amylase of *Bacillus* sp. IMD 434. Biotechol. Lett., 21: 111-5.

Gerhartz, W., 1990. Enzymes in Industry. VCH, Wienheim, pp: 78-79.

Guilbot, A. and C. Mercier, 1985. Starch. In: The Polysaccharides, 3rd Edn. Aspinall, G.O. (Ed.), New York: Academic Press, pp: 209-282

Kelly, C.T., D.J. Botton and W.M. Forgaty, 1997. B1-phasic production of α-amylase of *Bacillus* flavor thermus in batch fermentation. Biotechnol. Lett., 19: 675-677.

Kennedy, J.F., 1987. Enzyme technology. In: Biotechnol. Kenda, J.F. and D.M.S Cabral (Eds.), Vol. 7A. Weinhuin, Germany, VCH publisher-verlagsgeslls Chaft Mbtl, pp: 398-399.

Khoo, S.L., A.A. Amirul, M. Kamaruzaman, N. Nazalan and M.N. Azizan, 1994. Purification and characterization of α-amylase from *Aspergillus flavus*. Folia Microbiol., 39: 392-398.

Koji, N., T. Koji, S. Fumio and K. Youichi, 1999. Entrap-immobilization of urease on composite gel fiber of cellulose acetate and Zirconia. J. Soc. Fiber Sci. Technol., 55: 563-568.

Kurakake, M., M. Ueki, S. Hashimoto and T. Komaki, 1997. Adsorption of α-amylase on dextrin immobilized on kieselguhr or chitin. Carbohyd. Polym., 34: 54-59.

Kusunoki, K., K. Kawakami, F. Shiriashi and M.A. Katok-Kai, 1982. Kinetic expression for hydrolysis of soluble starch by glucoamylase. Biotechnol. Bioeng., 24: 347-354.

Lin, L.L., C.C. Chyau and W.H. Hsu, 1998. Production and properties of a raw starch degrading amylase from the thermophilic and alkaliphilic *Bacillus* sp. TS-23. Biotechnol. Applied Biochem., 28: 61-68.

Mamo, G. and A. Gessesse, 1999. Effect of cultivation conditions on growth and amylase production by a thermophilic *Bacillus* sp. Lett. Applied Microbiol., 29: 61-65.

Meers, J.L., 1972. The regulation of α-amylase production in *Bacillus licheniformis*. Antonie Van Leeuwenhok, 38: 585-590.

Miller, G.L., 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem., 31: 426-428.

Nordstrom, K.M., 1993. Effect of temperature on fatty acid composition of a white *Thermus* strain. Applied Environm. Microbiol., 59: 1975-1976.

Ohtakara, A., G. Mukerjee and M. Mitsutomi, 1988. In: Proceeding of 4th International Conference on Chitin and Chitosan. Skjak-Break, G., T. Anthonsen and P. Standford (Eds.), Trondheim, Norway, pp: 643.

Riaz, N., I. Ul-Haq and M.A. Qadeer, 2003. Characterization of amylase by *Bacillus subtilis*. Int. J. Agric. Biol., 5: 249-252.

Roy, I., M. Sardar and M.N. Gupta, 2000. Exploiting unusual affinity of usual poly-saccharides for separation of enzymes on fluidized beds. Enzyme Microb. Technol., 27: 53-65.

Ryan, S.M., G.F. Fitzerald and D. Sinderen, 2006. Screening and identification of starch-, amylopectin-and pullulan-degrading activities in Bifidobacterial strains. Applied Environ. Microbiol., 72: 5289-5296.

Sadhukhan, R., S.K. Roy and S.L. Chakrabarty, 1993. Immobilization of α-amylase from *Myceliophthora thermophila* D-14 (ATCC48104). Enzyme Microbiol. Technol., 15: 801-804.

Saito, N., 1973. Thermophilic extracellular α-amylase from *Bacillus licheniformis*. Arch. Biochem. Biophys., 15: 290-298.

Saito, N. and K. Yamamoto, 1975. Regulatory factors affecting α-amylase production in *Bacillus licheniformis*. J. Bacteriol., 121: 848-856.

Shaw, J.F. and L.Y. Chuang, 1982. Studies on the α-amylase from the germinated rice seeds. Bot. Bull. Acad. Sin., 23: 45-61.

Shaw, J.F. and T.M. Ou-Lee, 1989. Simultaneous purification of α-and β-amylase from germinated rice seeds and some factors affecting activities of the purified enzymes. Bot. Bull. Acad. Sin., 25: 197-204.

Shaw, J.F., F.P. Lin, S.C. Chen and H.C. Chen, 1995. Purification and properties of an extracellular α-amylase from *Thermus* sp. Bot. Bull. Acad. Sin., 36: 195-200.

Sheffield, D.J., T.R. Harry, A.J. Smith and L.J. Rogers, 1995. *Corallina officinalis* bromoperoxidase immobilized on agarose. Phytochemistry, 38: 1103-1107.

Sneath, P.H.A., 1986. Endospore-forming Gram-positive rods and cocci. In: Bergey's Manual of Systematic Bacteriology. Holt, J.G., N.R. Krieg, P.H.A. Sneath, J.T. Staley and S.T. Williams (Eds.), Williams and Wilkins, Baltimore., Vol: 2, Section 13.

Stephenson, K., N. Mo. Carter, C.R. Harwood, M.F. Petitglatron and R. Chambert, 1998. The influence of protein folding on late stages of the secretion of α-amylase from *Bacillus subtilis*. FEBS Lett., 430: 385-389.

Tien, C.J. and B.H. Chiang, 1999. Immobilization of α-amylase on a zirconium dynamic membrane. Proc. Biochem., 35: 377-383.

Tonkova, A., R. Monolov and E. Dobreva, 1993. Thermostable α-amylase from depressed *Bacillus licheniformis* produced in high yields from glucose. Process. Biochem., 28: 539-542.

Vihinen, M. and P. Mäntsälä, 1989. Microbial amylolytic enzymes. Crit. Rev. Biocheme. Mol., 24: 329-418.

Wind, R.D., R.M. Buitelaar and G. Eggink, 1994. Characterization of a new *Bacillus stearothermophilus* isolate: A highly thermostable α-amylase producing strain. Applied Microbiol. Biotechnol., 41: 155-162.

Yoshida, M., K. Oishi, T. Kimura, M. Ogata and I. Nakakuki, 1989. Continuous production of maltose using a dual immobilized enzyme system enzyme System. Agric. Biol. Chem., 35: 3139-3142.