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Biosynthesis and Properties of Extracellular Amylase by Encapsulation *Bifidobatrium bifidum* in Batch Culture

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Abstract: Cell encapsulation is likely to play a major role in cell and transplantation therapies in the next decade. The capsules provide a special microenvironment in which cells always have different behaviors compared with free non-encapsulated culture. In this work, the encapsulated cells of the probiotic bacterium *bifidobatrium bifidum* No.1, 791 cultures on different carriers were investigated for the production of amylase. Amylase production by encapsulation *bifidobatrium bifidum* No.1, 791 cultivated in liquid media containing soluble starch reached a maximum at 48h. The entrapped cells of *bifidobatrium bifidum* No.1, 791 in calcium alginate showed the highest enzyme activity (90 U mL^{-1}). Encapsulation matrix was optimized. In repeated batch fermentation process, the encapsulated cells preserve their ability to produce consistently over 21 cycles and the activity remain between (90 and 95 U/ mL^{-1}) throughout the cycles. Some properties of the crude enzyme produced by entrapped cells of *bifidobatrium bifidum* No.1, 791 in repeated batch were studied. The enzyme properties revealed that the optimum temperature for activity was 75ºC. The enzyme was stable for 2h at 60ºC, while at 60ºC, 70ºC and 90ºC, 4%, 13% and 38% of the original activities were lost, respectively. The optimum pH of the enzyme was 7.5. After incubation of crude enzyme solution for 24h at pH 7.5, a decrease of about 5% of its original activity was observed. The enzyme was strongly inhibited by Cu^{2+} and Mg^{2+} but less affected by Ca^{2+}, Mn^{2+}. The enzyme in 1M and 5M NaCl solutions the enzyme retained 70% and 47% of the original activity after 24h of incubation at 4ºC, respectively.

Key word: probiotics bifidobacteria; amylase, cell encapsulating

INTRODUCTION

Bifidobacteria are important constituents of the intestinal microflora of humans and animals (Molder, 1994). These organisms are the predominant group of bacteria in the normal intestinal flora of healthy breast-fed newborns where they constitute more than 95% of the total population(Yildirim *et al.*, 1999). Recently it has been reported that bifidobacteria the natural inhabitants of the human large intestinal tract, especially healthy breast-fed infants, and exert many beneficial effects on human health(Benno and Mitsuoka, 1992). High numbers of *Bifidobacterium* are reported to form a barrier pathogens by prohibiting colonization or by controlling the intestinal pH through the release of acetic and lactic acids and stimulate the immune response of the host, for these reasons they have been incorporated into various commercial products such as yogurts, fermented milk, baby milk powder, pharmaceutical tablets and feed additives (Hughes and Hoover, 1991). The human colon is described as a complex ecosystem, containing over 400 species of microbes, the majority of which are anaerobic microorganisms. The distribution of amylolytic microorganisms in the human large intestinal tract was investigated in various individuals of differing ages using anaerobic culture techniques. A large percentage of the amylolytic microorganisms present belonged to the genus Bifidobacteria. The number of Bifidobacteria increased significantly at 2 yr of age. Adults and children above 2 yr old carried ~ 0.8×10^9 - 2.0×10^{10} colony forming units (CFU/g) of amylolytic Bifidobacteria (Ji *et al.*, 1992).

Amylase production could be induced by starch in a stable form. When cells were grown on maltose or glucose, amylase production was much lower than on starch and amylase activity disappeared after 24 h growth on these media (Ji *et al.*, 1992). Encapsulation has been effective in protecting sensitive bacteria from harsh

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environments where they may not normally survive. Modification of biotechnology and processes, using immobilized biocatalysts, has recently gained the attention of many biotechnologists. Application of immobilized enzymes or whole cells is advantageous, because such biocatalysts display better operational stability (Fortin and Vuillemard, 1990) and higher efficiency of catalysis (Ramakrishna *et al*., 1992) and they are reusable.Microbial products are usually produced either by free or encapsulated cells. The use of capsulated cells as industrial catalysts can be advantageous compared to batch fermentation process (Venkatasubramanian, 1979). Whole cell immobilization (Capsulation or entrapment form) has been a better choice over enzyme immobilization(Kennedy *et al*., 1990; Ramkrishna and Prakasham, 1999) . Wholecell encapsulation is a widely used and simple technique. This paper reports the experimental results for extra-cellular amylase activities production by immobilized *Bifidobacterium Bifidum* No., 1 791, also evaluated the using of the immobilized cells of *Bifidobacterium Bifidum* No.1, 791 for extra-cellular amylase production in repeated batch according to the productivity, and long-time operational stability. To the best of our knowledge, this is the first report dealing with the capsulation of human intestinal tract *Bifidobatrium bifidum* No.1, 791 for biosynthesis of amylases enzymes.

MATERIALS AND METHODS

Microorganisms:

The bacterial strain *Bifidobacterium Bifidum* No.1, 791 used in the present work from department of microbiology, Kazakh National University, Almaty, Kazakhstan.

Culture Media and Growth Conditions:

The medium used for the cell biomass production for the cell encapsulation was Thioglycolate Broth with Resazurine (Fluid Thioglycolate Medium, Thioglyolate Medium 90404, Fluka). The medium was inoculated from 24 h old culture and was grown at 37ºC.

Extracellular Enzyme Amylase Production:

The organism was found to produce amylase on culture medium composed of 1% (w/v) soluble starch. The composition of Basal Medium (BM) contained (per liter) 30 g tryptone Pancreatic digest of casine (BD), 7 g yeast extract, 1 g $MgSO_4 \cdot 7H_2O$. After autoclaving BM was supplemented with 0.05% (wt/v) filter-sterilized cysteine-HCl, and strains were grown at 37°C under anaerobic conditions. The pH was adjusted to 6.0-6.5 with 1.0 M NaOH and this basal medium was sterilized by autoclaving at 121ºC for 15min. Yeast extract, Bacto-tryptone and Soluble starch were sterilized separately and aseptically added to the flasks containing the liquid medium, after cooling. The above medium (50 mL in 250 mL Erlenmeyer flasks) was inoculated with 1 mL of an overnight culture and incubated at 37ºC, under anaerobic conditions. The turbidity of the cultures was determined by measuring the optical density at 550 nm. Before assay, the cells were separated by centrifugation at 10.000 rpm for 20 min and the clear supernatant was used as crude enzyme preparation.

Amylase Activity Assay:

The activity of amylase was assayed by incubating 0.3 mL enzyme with 0.5 mL Soluble starch (1%, w/v) prepared in 0.05M Phosphate buffer, pH 6.5. After incubation at 90ºC for 10 min the reaction was stopped and the reducing sugars released were assayed calorimetrically by the addition of 1 mL of 3-5- dinitrosalicylic acid reagent (Miller 1959). An enzyme unit is defined as the amount of enzyme releasing 1 mmole of glucose from the substrate in 1 min at 90ºC.

Encapsulation Procedures:

All the immobilization processes were performed under aseptic conditions. In separate experiments, the cell pellets obtained from each culture (in the logarithmic phase of growth) were collected by centrifugation (10.000 rpm, 20 min) in a refrigerated centrifuge (4 ºC). Then, the wet cell pellets were suspended in 0.85% sterile saline and used for the cell immobilization experiments.

Encapsulation in Ca-alginate:

Unless otherwise stated, the wet cell pellets obtained from 100 ml culture of each organism were mixed with 10 ml of sodium alginate solution (Loba Chemie, Mumbai, India) in separate experiments. The alginate entrapment of cells was performed according method of (Johnsen and Flink, 1986). Sodium alginate solution was prepared by dissolving sodium alginate in 100 mL boiling water and autoclaved at 121°C for 15 minutes.

Encapsulation in Agar:

A definite quantity of agar–agar (Hi-media, Mumbai, India) was dissolved in 18 mL of 0.9% sodium chloride solution to get final concentration of 3 % and sterilized by autoclaving. The cell suspension (2 mL equivalent to 0.03 g wCW) was added to the molten agar-agar maintained at 40°C, shaken well for few seconds (without forming foam), poured into sterile flat bottom 4-inch-diameter petri plates and allowed to solidify. The solidified agar block was cut into equal size cubes (4 mm^3), added to sterile 0.1 M phosphate buffer (pH 7.0), and kept in the refrigerator (1 hour) for curing. After curing, phosphate buffer was decanted and the cubes were washed with sterile distilled water 3 to 4 times (Veelken and Pape, 1982).

Encapsulation in Polyacrylamide:

A cell suspension was prepared by adding 0.03 g cells to 10 mL chilled sterile distilled water. To another 10 mL of 0.2 M sterile phosphate buffer (pH 7.0), the following chemicals were added: 2.85 g acrylamide (Fluka, Buchs, Switzerland), 0.15 g bisacrylamide (Fluka), 10 mg ammonium persulphate, and 1 mL TEMED (NNN^1N^1 tetra methyl ethylene diamine). The cell suspension and the above phosphate buffer mixture was mixed well and poured into sterile flat bottom 10 cm-diameter petriplates. After polymerization (solidification), the acrylamide gel was cut into equal size cubes (4 mm^3), transferred to 0.2 M phosphate buffer (pH 7.0), and kept in the refrigerator for 1 hour for curing. The cubes were washed 3 to 4 times with sterile distilled water and stored in sterile distilled water at 4°C until use.

***Batch Process with Encapsulated Cells*:**

The batch experiments were performed in 250 ml Erlenmeyer flasks each containing 100 ml of BM. The flasks were inoculated with the beads obtained from 10 ml gel with the calculated amounts of the immobilized cells. Parallel experiments were carried out with equal amounts of free cells. The cultures were incubated for 48 h at 37 °C. All the experiments were carried out in triplicates.

Repeated Batch Process with Encapsulated Cells:

One of the advantages of using immobilized biocatalysts is that they can be used repeatedly and continuously. Therefore, the reusability of *Bifidobacterium Bifidum* No.1, 791 cell immobilized in matrix was examined. This was done in 250 ml Erlenmeyer flasks each containing 100 ml of BM. Each flask was inoculated with the beads obtained from 10 ml alginate gel comprising a cell loading of 1.08 g wet cells 100 ml / culture. After attaining the maximum production of amylase enzyme. the spent medium was replaced with fresh production medium BM (100 mL) and at the end of each run, the gel particles were filtered and washed with 25 ml of 0.05 M $CaCl_2$ and distilled water and the process was repeated for several batches until the beads started disintegrating. The enzyme titers and cell leakage of each cycle were determined.

Cell Growth and Cell Leakage:

Both cell growth in freely suspended cultures and cells leaked from the gel matrix were determined as cell wet weight by measuring the optical density at 600 nm. One absorbance unit was equivalent to 0.16 g/L (cell wet / weight).

Effect of pH on Activity and Stability of Amylase:

Effect of pH on the activity of amylase was measured by incubating 100mL of enzyme and 500mL of buffers, adjusted to pH of 5.5 to 8.5, containing Soluble starch (0.5%). The buffers used were: sodium acetate pH 5.5; phosphate pH 6.0 – 8.0; Tris-HCl pH 8.5. Stability of the enzyme at different pH values was also studied by incubating the enzyme at various pH values ranging from 5.5 – 8.5 for 24h and then estimating the residual activity.

Effect of Temperature on Activity and Stability of Amylase:

The effect of temperature on the enzyme activity was determined by performing the standard assay procedure as mentioned earlier for 10 min at pH 6.5 within a temperature range of 40 – 110ºC. Thermostability was determined by incubation of crude enzyme at temperatures ranging from 40-110ºC for 2h in a constant-temperature water bath. After treatment the residual enzyme activities were assayed.

Effect of Various Metal Ions:

The effect of different metal ions on amylase activity was determined by the addition of the corresponding ion at a final concentration of 1mM to the reaction mixture, and assayed under standard conditions. The enzyme assay was carried out in the presence of $Ca\ cl_2$, $Cu\ SO_4$. $Fe\ Cl_3$, $MnSo_4$, nacl and $ZnSo_4$.

RESULTS AND DISCUSSIONS

Secretion of Extracellular Amylase with Encapsulated Cells in Various Matrices Techniques:

The suitability for Biosynthesis of amylase activity by the encapsulation entrapped cells of *Bifidobacterium Bifidum* No.1, 791 in different encapsulated matrices was investigated in submerged culture. In another set of experiments, the same amount of free cells from *Bifidobacterium Bifidum* No.1, 791 culture was inoculated along with the same amount of the encapsulated cells. The results (Table 1) indicated that, in all cases the activity of the encapsulat cells was lower than the corresponding amount of free cells. The effectiveness factor of the encapsulating cells, which is the ratio of the enzyme activity of the encapsulating cells to that of the same amount of free cells under identical conditions, was in the order of 0.5±0.8. These values are similar to those reported elsewhere for other encapsulated bacterial amylases (Yong *et al.*, 1990; Jamuna and Ramakrishna, 1992). The effectiveness factor of the encapsulated cells would always be less than one because the encapsulated cells represent a heterogeneous catalysis fermentation in which the activity, or rather synthesis, of primary or secondary metabolites is dependent upon the external and internal mass transport and adequate oxygen supply (Jamuna and Ramakrishna, 1992), of all preparations, the cells of *Bifidobacterium Bifidum* No.1, 791 encapsulated in Ca-alginate showed the highest amylase activity (90.0 U ml $^{-1}$) and the highest specific productivity (60.0 U g wet cells h $^{-1}$).

Table 1: Production of amylase enzymes by free and encapsulated bacterial cultures.

Carrier for encapsulation	Cell Biomass loaded g wet cell 100 ml culture^{-1}	Protein content (mg ml $^{-1}$)	amylase enzyme productivity (U ml $^{-1}$)	Specific productivity (U g wet cells^{-1} h^{-1})	Effectiveness factor
Free cells	1.48	2.72	110.50	74.66	------
Agar	1.48	0.62	53.64	36.24	0.60
Ca- alginate	1.48	0.93	90.0	60.81	0.81
Polyacrylamide	1.48	0.72	50.24	33.94	0.56

Optimum Conditions for Encapsulation of Bifidobacterium Bifidum No.1, 791 in Ca-alginate:

Different concentrations of sodium alginate (2%, w/v) were investigated for the immobilization process. In all cases, a constant amount of cells was used (equivalent to 1.48 g wet cell pellets per 10 ml gel). Inoculation of 100 ml BM was performed with the beads resulted from 10 ml Na alginate solution. The results recorded in (Table 2) indicated that beads prepared from 2 % (w/v) alginate concentration were much softer and showed the highest number of leaked cells (1.65 mg/ml) and therefore, they were excluded. On the other hand the enzyme yield of the beads made of 6 % (w/v) was 70.8 less than that recorded for the beads prepared from 4 % (w/v) alginate concentration. This is most likely because the resulting beads had lower diffusion efficiency. Maximal enzyme yield (90.0 U) was obtained at 4% (w/v) alginate concentration. This concentration was recommended for the production of amylase (Jamuna and Ramakrishna, 1992) by encapsulated bacterial cells. Using the same conditions from the previous experiment.

Table 2: Effect of alginate concentration on the production of amylase by the encapsulated cells of *Bifidobacterium Bifidum* No.1, 791.

Alginate concentration (%)	Cell Biomass loaded g wet cell 100 ml culture^{-1}	Protein content (mg ml $^{-1}$)	amylase enzyme productivity (U ml $^{-1}$)	Specific productivity (U g wet cells^{-1} h^{-1})	Leak cell (mg/ml)
2	1.48	1.95	79.8	74.66	1.65
4	1.48	0.62	90.0	36.24	0.45
6	1.48	0.20	70.8	60.81	0.3

The Effect of Different Concentration of Starch on Productivity of Amylases Enzyme:

Amylase is an inducible enzyme and is generally induced in the presence of starch. Using the optimum conditions reached from the previous experiments (4 % w/v, alginate concentration, cell loading of 1.48 g wet cell pellets per 10 ml gel), the effect of different concentration of starch of productivity of amylases enzyme (1.0 -6.0) was investigated for the production of amylase. Maximal enzyme activity (95 U / ml) was attained at 5.0 % of starch concentration Fig. (1). The concentration of starch as substrate had influences on the production of the enzyme. It was also observed that increase in the concentration of starch in the culture medium enhanced *Bifidobacterium Bifidum* No.1, 791 growth and enzyme production. Similarly strong amylase induction by starch in the case of *A. oryzae* DSM 63303 has been reported by

Lachmund *et al.,* 1993. *Bacillus thermooleovorans* is reported to prefer starch, glucose, non-conventional substrates lactose, maltose and maltodextrins as carbon sources for a- amylase secretion(Narang and Satyanarayana, 2001). In contrast, carbon sources such as glucose, maltose and starch did not enhance a-amylase production by thermophilic *B. coagulans* in solid-state fermentation using wheat bran(Babu and Satyanarayana, 1995). The carbon sources as glucose and maltose have been utilized for the production of amylase. However, the use of starch remains promising and ubiquitous.

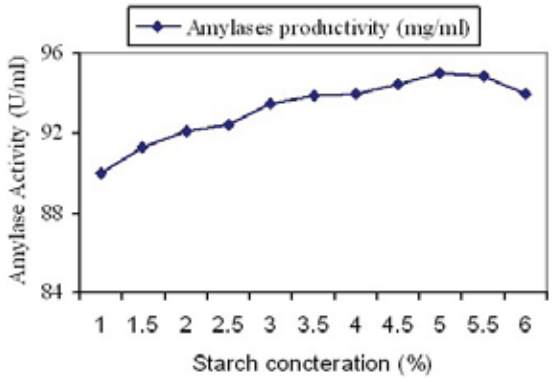

Fig. 1: Different concentration of starch (%).

Repeated Batch Operation with Immobilized Cells:

The activity of encapsulated *Bifidobacterium Bifidum* No.1, 791 in cells for the production of amylase continuously was investigated by using the cell- encapsulated beads, respectively, for several batches. The medium was replaced every 48 h and the beads were washed thoroughly with 0.05 M $CaCl_2$, and distilled water at the end of each cycle before reuse. The results (Fig. 2) show the activity of 11 cycles (21 Days). The activity of the encapsulated cells remain between 90 and 95.5 U / ml throughout the cycles. These results are similar to those reported by Jamuna and Ramakrishna (Jamuna and Ramakrishna, 1992) for the production of extracellular a- amylase by the immobilized cells of *Bacillus sp*. in repeated batch fermentation.

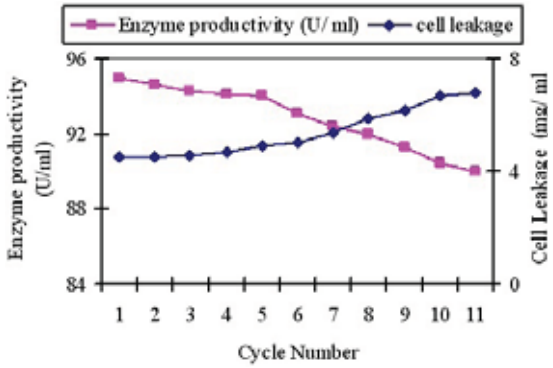

Fig. 2: Repeated batch fermentation used encapsulated cells of *Bifidobacterium Bifidum* No.1, 791 for amylase production.

Effect of Ph on Activity and Stability of Amylase:

The effect of pH on amylase activity is shown in Fig.3. Optimum pH was found to be 7.5. The enzyme activity at pH 5.5 and 10.0 were 73% and 55% of that at pH 7.5, respectively. After incubation of crude enzyme solution for 24h at pH 5.0–10, a decrease of about 5% of its original activity at pH 7.5 was observed. At pH 10.0, the decrease was of 44%. Thus, amylase of *Bifidobacterium bifidum* No.1, 791 strain seems to be active in very broad pH range.

Effect of Temperature on Activity and Stability of Amylase:

The supernatant amylase activity was assayed at different temperatures ranging from 40ºC - 110ºC at a constant pH of 7.5 and a substrate concentration of 0.5% as shown in (Fig. 4). Enzyme activity increased with temperature within the range of 40ºC to 70ºC. A reduction in enzyme activity was observed at values

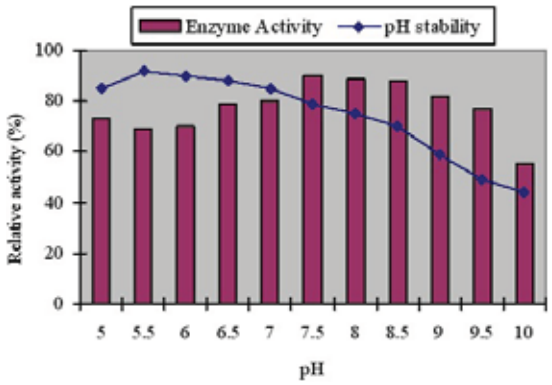

Fig. 3: Effect of pH on activity and stability of amylase.

above 70ºC. The optimum temperature of this amylase was 70ºC, which was higher or similar to that described for other *Bacillus* amylases(Ara *et al.*, 1993)**.** The residual activity of crude amylase incubated at different temperatures for a period of 2h and 24h was estimated at optimum temperature. The enzyme was stable for 2h at temperatures ranging from 40-60ºC while at 70ºC and 90ºC, 13% and 38% of the original activities were lost respectively.

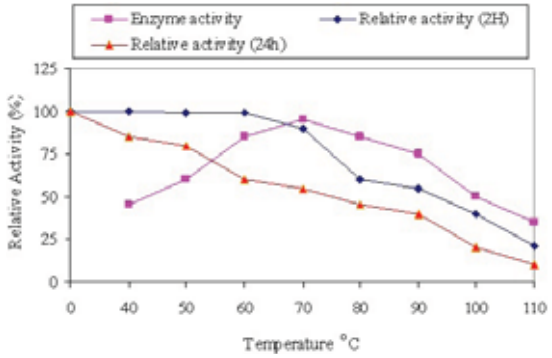

Fig. 4: Effect of temperature on activity and stability of amylase.

Effect of Metal Ions on the Activity of Amylase Enzyme:

The effect of some metal ions at the concentration of 1 mM in the activity of amylase was investigated. As can be observed in Fig. (5) , the amylase did not require any specific ion for catalytic activity. A slight activity inhibition was produced in the activity by Ca Cl_2, and $MnSO_4$, and a stronger inhibitory effect was observed in the presence of Cu^{2}+ some amylases are metallo enzymes, containing a metal ion for catalytic activity. The inhibition of *Bacillus subtilis* JS-2004 a - amylase by Co_2+, Cu_2+, and

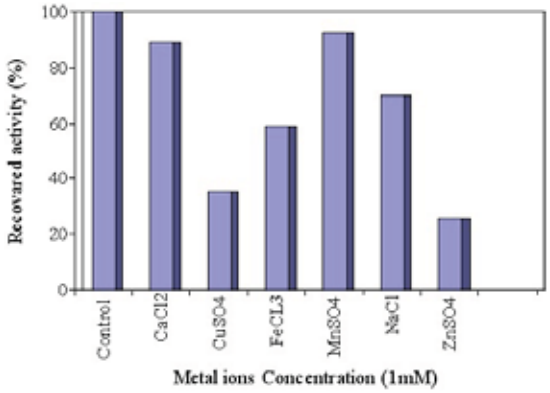

Fig. 5: Effect of metal ions on the activity of amylase enzyme.

Hg_2+ ions but less affected by Mg_2+, Zn_2+, Ni_2+, Fe_2+, and Mn_2 could be due to competition between the exogenous cations and the protein-associated cation, resulting in decreased metalloenzyme activity(Laderman *et al.*, 1993). There is also report where Ca^{+2} did not have any effect on the enzyme (Asgher *et al.*, 2007). Kundu *et al.,* (1973) reported that Ca^{2+} was inhibitory to amylase production by *A. oryzae* EI 212. The enzyme activity in NaCl 1m M solution retained 70% of the original activity. Dutta *et al.,*(2006) had earlier reported that amylase produced by *Heliodiaptomus viduus* was retained of its activity at 60% in 2 M NaCl after 24 h incubation, while full activity was found in 0.5 M NaCl for the same duration of incubation.

Conclusion:

It is known that starches are a major carbohydrate source in the human colon. Some starches escape complete digestion due to their size and molecular conformation during passage through the human small intestine; these compounds arrive in the colon as fermentable carbohydrate sources for intestinal bacteria . The approach used in this study was to use encapsulation technology as a means to protect a recognized probiotic organism from the harsh environment during fermentation . A variety of wall materials including alginate has been used for the successful encapsulation of probiotics (Doleyres and Lacroix, 2005). Alginate wall materials were chosen in the present study as they were found to provide the best protection for *Bifidobacterium bifidum* No.1, 791 against external condition (Muthukumarasamy *et al.*, 2006). Increased bacterial stability following encapsulation in alginate is reported in the literature and has met with varying levels of success (Iyer and Kailasapathy, 2005). The alginate matrix was found to be superior to the other matrices studied in this work . In addition, the alginate carrier is not expensive, nontoxic, and preparation of biocatalyst involves mild conditions, which is an added advantage. The results show that calcium alginate shows potential method for microencapsulating of *Bifidobacterium bifidum* No.1, 791 for amylases production. amylases production by biomicroencapsulating is superior to that of free cells because it leads to enzyme productivity within the same time of fermentation. Specific advantages of this technique such as long life-term stability 21 days (11 cycles), reusablility, and possibility of regeneration to be adaptable also to scale-up the obtained data. In addition, the experiments with repeated batches of alginate encapsulated bacterial growth by introducing fresh nutrients every 48 hours leads to activity of the encapsulated cells continue between 90 and 95.5 U / ml, throughout the cycles. To our knowledge the present work is the first study investigating the potential use of microencapsulation technology to produce amylases from bifidobacteria. The results showed that microencapsulated *Bifidobacterium bifidum* No.1, 791 potential strain for amylase production using fermentation with soluble starch as substrate. Interesting observation was that it showed and retained 95 % enzyme activity at 70 °C. Furthermore, the enzyme was found to show active in very broad pH range.

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