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Microencapsulation and Fermentation of *Lactobacillus acidophilus* LA-5 and *Bifidobacterium* BB-12

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Abstract

Because of poor survival of probiotic bacteria, microencapsulation evolved from the immobilized cell culture technology is used in the biotechnological industry. *Bifidobacterium* (BB-12) and *Lactobacillus acidophilus* (LA-5) were immobilized in calcium alginate by extrusion method. Encapsulation parameters and efficacy were evaluated. Growth factors of the above two bacteria were also measured by culturing in fermenter. Growth trend was obtained with respect to optical density and dry biomass weight. Encapsulation yield was over 60% in each experiment. Scanning electron microscopy of entrapment of cells in alginate matrix and cross-sections of dried bead were obtained and illustrated. According to fermentation results, *Bifidobacterium* BB-12 shows better biotechnological properties.

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1. Introduction

According to FAO/WHO, probiotics are living microorganisms that, when administered in adequate amounts, confer a health benefit on the host [1]. Probiotic bacteria have been reported to suppress the growth of pathogens [2,3], prevention of diarrhea and constipation diseases [4-6], improvement of lactose utilization by producing β-galactosidase [6,7], and prevention of cancer and mutation activities in the human gut [8,9].

Due to the particular nature of many probiotic bacteria, survival during the processing and storage of functional foods and during passage through the human gastrointestinal tract remains major challenges for effective delivery of these beneficial bacteria [10]. Providing probiotic living cells with a physical barrier against adverse environmental conditions is among the approaches currently receiving considerable interest. The technology of microencapsulation of probiotics has evolved from the immobilized cell culture technology used in the biotechnological industry. Immobilization in polymer provides a physical retention of cells. It facilitates the purification process and separation of cells from metabolites. Encapsulation is used to enhance viability, stability, and protection of lactic cultures [11]. The majority of materials used for formation of microcapsules are natural or synthetic polymers. Several characteristics and qualities should be taken into account for choosing polymers for this purpose [12]. Among the various carriers such as chitosan, carrageenan and gelatin, microencapsulation in calcium alginate network has been widely used for the immobilization of lactic acid bacteria due to its ease of handling, nontoxic nature, and low cost [13,14]. Alginates are natural unbranched binary copolymers of 1-4 linked s-D-mannuronic acid (M) and a-L-guluronic acid (G) [12].

On the other hand, many strains of intestinal origin are difficult to propagate. The most frequently used bacteria in probiotic products include the *Lactobacillus* (L.) and *Bifidobacterium* (B.) species [15]. Lactobacilli have complex growth requirements. Bifidobacteria are anaerobic micro-organisms [16]. Thus, producing these bacteria on large scale is in much demand in probiotic industry, and most commercial strains are selected on the basis of their technological properties. Industrial processes for food culture production, including probiotics, almost exclusively use conventional batch fermentation with suspended cells [17].

In this study, encapsulating of two commercial probiotic strains was performed, and the yields of the products were measured. Furthermore, to evaluate the growth in large scale based on substrate utilization and biomass production, fermentation of these strains was performed separately using 10-l fermenter.

2. Materials and Methods

2.1. Materials

Sodium alginate was obtained from Sigma Aldrich (USA). Calcium chloride, L-cysteine-HCl, MRS medium (broth and agar), glucose, yeast extract and other materials were supplied by Merck Germany. *B. animalis* subsp. lactis strain BB-12 and *L. acidophilus* strain LA-5 were obtained from Christian Hansen (Denmark).

2.2. Microencapsulation of microorganisms

To achieve biomass for encapsulation, freeze-dried cells were suspended in sterile physiological serum, and after inoculating on MRS agar, incubated at 37°C for 2 days. The cells were sub-cultured twice in 50 ml and 150 ml MRS broth under the same conditions. After 20 h, the cells were harvested by centrifuging at 3800×g (4°C and 20 min), washed with sterile physiological serum, and then collected by centrifugation. *L. acidophilus* LA-5 was incubated under aerobic conditions while *Bifidobacterium* BB-12 was cultured in a medium supplemented with 0.5 gl^{-1} L-cysteine hydrochloride and incubated anaer-obically using the Gas Pack system.

The extrusion technique of microencapsulation was used [18]. For each strain, encapsulation was performed with freeze-dried bacteria (used intact as supplied by the manufacturer) and also with fresh biomass, which was obtained as described in the previous section .

The alginate mixture was prepared by adding sodium alginate in distilled water and 5% freeze-dried cells in physiological serum; then they were mixed together. The cell pellet was re-suspended in saline solution and mixed with sodium alginate solution. In both procedures, a cell suspension with the final concentration of 2% (ww^{-1}) alginate was achieved .

Microbial suspension was introduced in a stainless steel device designed particularly for this study (Dissertation of Jamshid Fooladi, Braunschweig, Germany 1990). This devise has three main parts; a column, a cap with an inlet for air pressure system and lower part with one inlet for air (use of this inlet is optional), and one outlet, which can fit on syringe needle. Its outlet was fitted on a 27.5 G needle. The suspension was dropped aseptically into 250 ml of 0.1 M calcium chloride (using sterile air pressure), which caused the droplets to harden in sphere shapes with gentle agitation. After 30 min, the beads were separated by decantation and rinsed with 0.1 M calcium chloride [19]. The distance between the syringe and $CaCl_2$ solution was 20 cm [20].

2.3. Calculation of encapsulation parameters

For dissolution of alginate beads, citrate buffer (pH=6.8) was used since citrate chelate calcium ions weaken alginate network for effective release of cells [21]. The samples were diluted to appropriate concentrations, and pour plated in MRS agar. The plates were incubated for three days at 37˚C, and the encapsulated bacteria were enumerated as CFU ml^{-1} (colony forming unit per milliliter). The encapsulation yield (EY), which is a combination measurement of the efficacy of entrapment and survival of viable cells during the microencapsulation procedure, was calculated as $EY= N/N_0 \times 100$; where, N is the number of viable entrapped cells released from the beads, and N_0 is the number of free cells added to the biopolymer mix during the production of beads [22].

Cell load of the beads (number of the cells in each bead) was calculated by dividing the amount of cells to the number of beads in each sample.

Survival of encapsulated *L. acidophilus* LA-5 in $CaCl_2$ (0.1 M) and distilled water during two weeks at 4°C was compared.

2.4. Electron microscopy

A sample of beads containing biomass of *Bifidobacterium* BB-12 was air dried at 4˚C. After gold coating, entrapment of cells in alginate matrix and cross-sections of the dried beads were analyzed by scanning electron microscopy (SEM) (TESCAN, VEGA), and the photos were taken at different magnifications .

2.5. Cell growth of strains

Fermentations were done without aeration in a 10-l laboratory bioreactor (Bench Top Fermentation System – Major Science) with pH, temperature and agitation control. Consumption of sugar in anaerobic conditions resulted to produce acids and reduce pH; this was, consequently, compensated by adding NaOH automatically. Inoculation was done at 8% (*L. acidophilus* LA-5) and 6% (*Bifidobacterium* BB-12) using the same medium as fermentation for each strain.

2.5.1. *L. acidophilus* LA-5

Fermentation was carried out in MRS medium at a constant pH of 6±0.5. The pH was kept constant by the automatic addition of 10 N NaOH solution. The fermentation temperature was adjusted at 37˚C, and the agitation rate was fixed at 100 rpm [23].

2.5.2. *Bifidobacterium* BB-12

The cultivation medium contained yeast extract 30 gl^{-1}, glucose 20 gl^{-1}, phosphates (K_2HPO_4 and NaH_2PO_4) 4 gl^{-1}, Tween 80 1 gl^{-1}, L-cysteine-HCl 1 gl^{-1}, and $MgSO_4$ 0.2 gl^{-1} [24]. The pH was kept constant using 4 N NaOH .The fermentation temperature was kept at 37°C and the agitation rate was fixed at 60 rpm.

During the fermentation, the hourly samples were withdrawn aseptically for analysis of cell growth and sugar consumption. Cell growth was measured by optical density at 650 nm (OD650), and cell dry mass (CDM, expressed in g ml^{-1}) was measured after 72 h incubation at 105°C. In the samples having OD values above 0.9, dilutions were carried out before the final OD determination. Sugar level was determined by phenol sulfuric acid method [25, 26].

3. Statistical analysis

The data were analyzed using the SPSS software (ver.14). All means±SEM (in triplicate) were compared with One-Way ANOVA. The differences were considered significant at $p<0.05$.

4. Results and Discussion

In this study, two challenges of probiotic industry, microencapsulation and fermentation, were evaluated for two probiotic strains. Numerous encapsulation strategies have been evaluated for their ability to protect probiotics from environmental stresses; most of them using alginate, which presents the benefits of being non-toxic, biodegradable and biocompatible. Encapsulation process resulted to the formation of spherical beads containing bacteria. The initial number of viable *L. acidophilus* LA-5 in aqueous suspension used to prepare the beads was $2.76±0.35×10^{10}$ CFU. After encapsulation, the number of viable cells recovered in pH 6.8 citrate buffer was $1.06±0.47×10^{9}$ CFU g^{-1} of beads. The encapsulation yield was calculated as 77±9%, and the cell load was $5.45±0.35×10^{6}$ CFU. About *Bifidobacterium* BB-12, the number of viable cells in sodium alginate suspension was $1.38±0.04×10^{11}$ CFU. The number of viable bacteria per gram of bead was $7.3±3.4×10^{9}$ CFU. The yield of encapsulation for this strain was calculated as 82±9%, and the cell load was $3.81±1.81×10^{7}$ CFU. Although encapsulation technology was developed to protect cells against harsh conditions, the process itself can cause missing some cells. Extrusion is the simplest and most common technique used to produce probiotic capsules with hydrocolloids [27]. The mild formulation conditions used to produce alginate beads by cross-linking alginate with Ca^{2+} divalent cations allowed the encapsulation of both probiotic bacteria with minimum effect on their viability and good entrapment efficiency. Encapsulation yield was over 60% in all experiments without any significant difference between the two species. Since, after ion exchange of sodium alginate with $CaCl_2$ and formation of beads a portion of the initial water maintains in the calcium alginate network, N and N_0 were calculated with respect to the weight of initial suspension and the weight of final beads.

Like this study, Graff et al. used alginate for encapsulation of yeast cells by extrusion method, and the obtained yield was reported to be 87% [28]. Extrusion method is usually done by syringe in laboratory scale [20, 29]. To make a model for industrializing the process, stainless steel device with higher suspension capacity was used.

The number of encapsulated *L. acidophilus* LA-5 cells in $CaCl_2$ and distilled water at 4˚C is shown in Figure 1. After 15 days, the number of survived encapsulated cells kept in $CaCl_2$ was about 2.8 logs more than beads in distilled water. More survival of encapsulated cells kept in $CaCl_2$ solution can be due to more stability of the alginate network in this condition.

Alginate beads were spherical with wrinkled surface. The wrinkle surface was probably due to the drying process and loss of water content (Figure 2).

4.1. Fermentation process

In the fermentation process, *Bifidobacterium* BB-12 grows better than *L. acidophilus* LA-5, and produces more biomass. *L. acidophilus* LA-5 reached a maximal biomass of about 1.2 g CDM l^{-1}, while the amount of biomass for *Bifidobacterium* BB-12 strain was 3.9 g CDM l^{-1} (Figure 3).

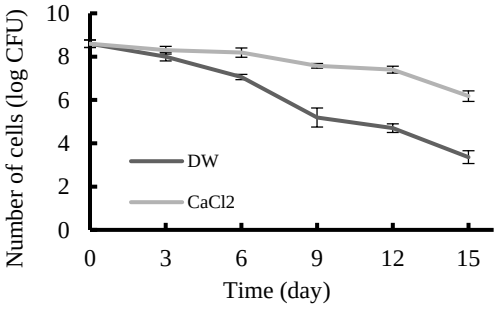

Figure 1. Survival curves of encapsulated *L. acidophilus* LA-5 during 15 days in $CaCl_2$ and distilled water ($p<0.05$).

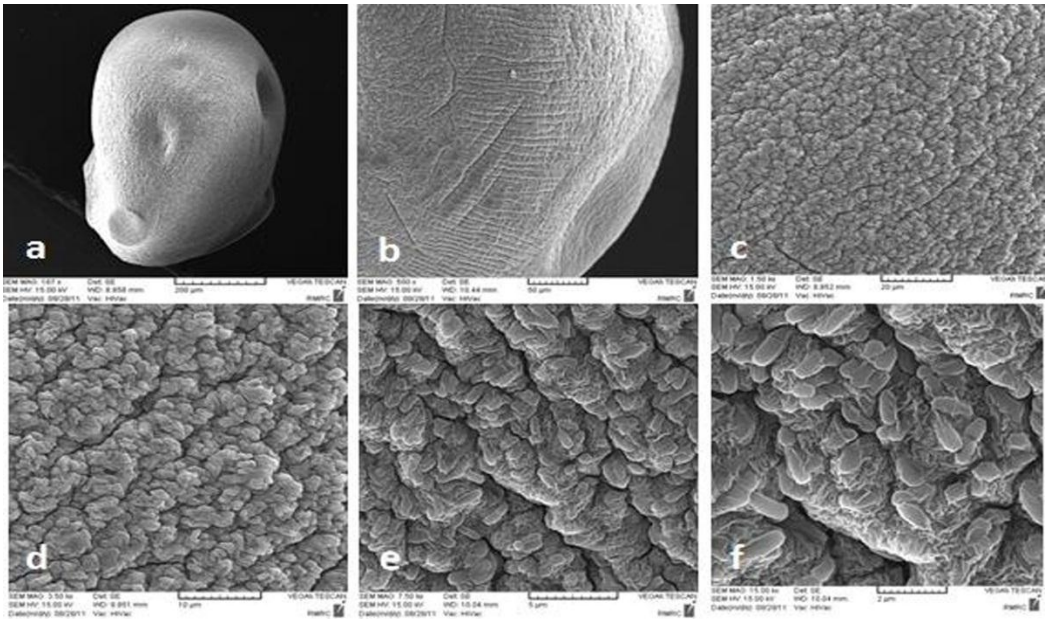

Figure 2. Micrograph of the beads containing *Bifidobacterium* BB-12 encapsulated in alginate (Magnification: a: ×187, b: ×500, c: ×1500, d: ×3500, e: ×7500, f: ×15000).

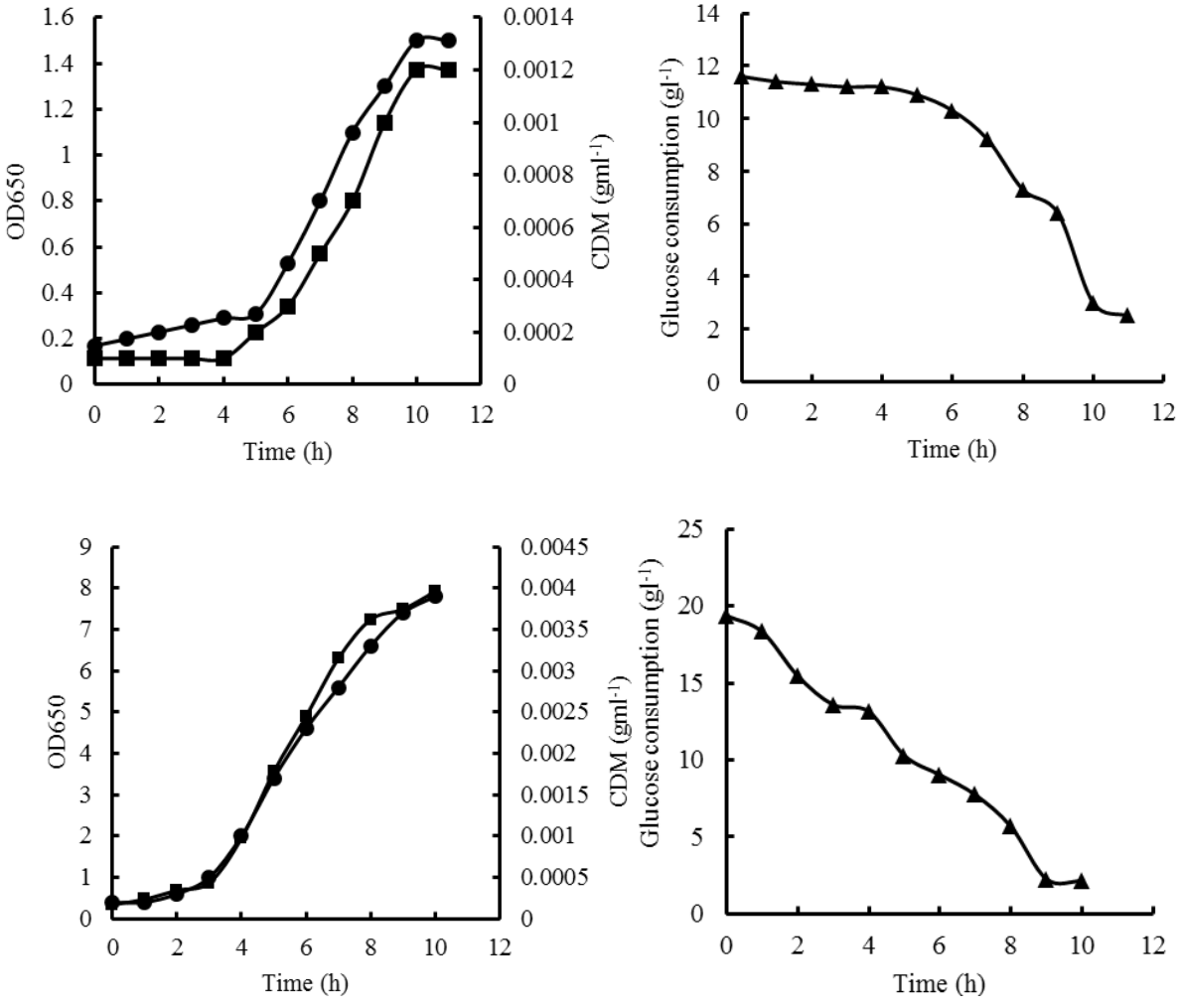

Figure 3. Growth curves of *L. acidophilus* LA-5 (top) and *Bifidobacterium* BB-12 (down) base on optical density (●), cell mass (■) and glucose consumption (▲).

In the primary experiment, *L. acidophilus* LA-5 did not grow well in the prepared media like *Bifidobacterium* BB-12. Thus, the procedure of Avonts et al. was performed in this study using MRS medium, preferably for 10 L fermentation of this strain. Most strains of bifidobacteria are unable to grow in a totally synthetic medium and require complex nitrogenous substrates. Thus, the Kiviharju et al. procedure was applied in the case of *Bifidobacterium* BB-12 propagation [24].

Although *Bifidobacterium* BB-12 is an anaerobic strain, CO_2 was not used. L-cysteine acts as oxygen scavenger, and maintains low redox potential. Bifidobacteria are considered anaerobic; however, the degree of tolerance to oxygen depends on the species and the culture medium [30]. In a study, growth of *B. infantis* in aerated fermentation was evaluated by Gonzalez et al. Their results indicated that these bacteria are able to grow in the presence of up to 80% DO (dissolved oxygen), showing a high aerotolerance under the conditions used in that work (starting fermentations in the absence of oxygen) [31].

Nowadays, most commercial strains are selected on the basis of their technological properties, and can be produced on a large scale. However, still there are a lot of differences among these strains. Thus choosing the best strain for each process or optimizing the process for each specific strain should be considered, and are much still in demands.

4. Conclusion

Two probiotic strains, *Bifidobacterium* BB-12 and *L. acidophilus* LA-5, were immobilized in calcium alginate by extrusion method. Stainless steel device with higher capacity for suspension was a model instrument for industrializing of the process. The mild formulation conditions used to produce alginate beads allowed the encapsulation of both probiotic bacteria with minimum effect on their viability and good entrapment efficiency.

Although both strains are industrial probiotic strains, with conventional culturing media and method, *Bifidobacterium* BB-12 grew better and produced more biomass. This result indicates that *L. acidophilus* LA-5 needs more optimization than *Bifidobacterium* BB-12 for large-scale production.

5. Acknowledgement

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6. Conflict of interest

The authors declare that there is no conflict of interest.

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