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Optimization of Screw Pumping System (SPS) for Mass Production of Entrapped Bifidus

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Abstract Process of screw-pumping system (SPS) was optimized for mass production of encapsulated bifidus. SPS entrapment device was composed of feeding component, with optimized nozzle size and length of 18G (0.91 cm) and 4 mm, respectively, screw pump, and 37-multi-nozzle. Screw component had five wing turns [radius (r) =26 to 15 mm] from top to bottom of axis at 78-degree angle from middle of the screw, and two wings were positioned at screw edge to push materials toward nozzle. For nozzle component, 37 nozzles were attached to 20-mm round plate. Air compressor was attached to SPS to increase productivity of encapsulated bifidus. This system could be operated with highly viscous (more than 300 cp) materials, and productivity was higher than 1128 ± 30 beads/min. Viability of encapsulated bifidus was 5.45×10^8 cfu/bead, which is superior to that of encapsulated bifidus produced by other methods (2.51×10^8 cfu/bead). Average diameter of produced beads was 2.048 ± 0.003 mm. Survival rate of SPS-produced encapsulated bifidus was 90% for Simulator of the Human Intestinal Microbial Ecosystem test and 88% in fermented milk (for 14 days). These results show SPS is effective for use in development of economical system for mass production of viable encapsulated bifidus.

Keywords: encapsulation technique, screw pumping system, bifidobacterium species, alginate beads, multi-nozzle

Introduction

A high percentage of bifidobacteria lose their viability during processing and, after ingestion, passage though the gastrointestinal track due to the presence of free hydrochloric acid in the stomach and bile salts. Various methods have been employed to increase the viability of bifidobacteria, including immobilization of living cells by hydrogel entrapments (1-4) and microencapsulation of lactic acid bacteria (LAB) within the cross-linked chitosan (5) and cellulose acetate phthalate (6) membranes formed by emulsification/interfacial polymerization, as well as several biopolymers, such as gellan gum, carrageenan/locust bean gum, and alginate (7-10); alginate, in particular, is easy to handle, non-toxic in nature, highly viscous, and safe as a food additive (11). However, these encapsulation techniques still have limitations in that they require strict anaerobic strains and highly viscous fluids; at present, vibrating nozzle and air jet techniques are the most extensively used methods for mass production of entrapped cells (12, 13). However, as with most entrapment systems (14-19), these operations also make use of highly-viscous fluids (20).

A screw pumping system (SPS) is a very efficient tool for both mixing and pumping highly-viscous liquids (21, 22), and can provide a continuous pressure for the effective movement of materials without any negative side effects, which alleviates strain loss caused by the physical stresses involved in processing, thus allowing efficient entrapment of living cells. However, no current techniques allow simultaneous production of beads, maintenance of a satisfactory level of material utilization under non-stressful strain conditions, and easy scale up (23, 24). Therefore, a new entrapment method involving less physical stress during processing is needed for use with sensitive organisms.

Accordingly, the aim of the current study was to develop predictive tools for the continuous production of entrapped bifidobacteria and scale-up of the bead formation process, optimize the conditions of this continuous SPS entrapment device, and determine the effects of SPS device on bead productivity and the survival stability of the entrapped bifidobacteria.

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Materials and Methods

Bacterial strains and high cell density cultivation *B. longum* ATCC 15707 and *B. bifidum* ATCC 29521 were purchased in lyophilized form from the American Type Culture Collection (ATCC; Rockville, MD, USA). The strains were routinely prepared as inocula by incubation in an anaerobic system (Forma Scientific Inc., Marietta, GA, USA) filled with mixed gases, including N_2 (75%), H_2 (10%), and CO_2 (5%) for 20 hr at 37°C. Before cultivation, the strains were subcultured in a trypticase-proteose peptone-yeast extract (TPY) broth containing 0.5% glucose as the only carbohydrate source. The high cell density cultivation for bifidobacterial cell entrapment was conducted with a 20% (w/v) Na_2CO_3 buffer solution to control the pH at 5.5 under anaerobic conditions, while shaking at 100 rpm for 20 hr at 37°C in a 2.5-L fermenter containing 1 L TPY (2.5% glucose) broth and 2% (v/v) fresh inoculum. Subsequently, the bifidobacterial cells were centrifuged for 15 min at 3000 rpm, and the cell pellets resuspended in a 0.85% (w/v) NaCl saline solution. The cell suspension was then poured into a sterile disposable bag (Seward Co., Worthing, UK) containing a sterile AE solution (2.5% of commercial sodium alginate and 1.0% erythritol), and mixed in a laboratory blender (Stomacher 400, Seward Co., Worthing, UK) (4). Finally,

the viscosity of the alginate solution was determined using a DV-II+ model Brookfield Viscometer (Brookfield Eng Labs Inc., Middleboro, MA, USA) at 60 rpm using the appropriate spindle (11).

SPS entrapment device The SPS entrapment device was composed of a feeding component, a screw pump, and a multi-nozzle (1). The feeding component was a 1-L vessel with a connected tube (inner diameter, 25 mm; Length, 300 mm) for feeding the mixed sodium alginate into the screw (2). The screw pump included a single screw and close-fitting barrel manufactured with plastic for easy modification. The plan was drawn using the Solid Edge Version 12 (Unigraph Solution Inc., Plano, TX, USA) (3). A previously developed multi-nozzle was used (23), in which 37 nozzles were attached to a 20-mm round plate. Needles used for general injections were utilized for the multi-nozzle in the device. Various nozzle sizes and lengths were tested with fixed screw sizes, vessel sizes, motor speed, and motor power. To optimize the multi-nozzle, four different inside-diameters of the nozzles (18 G: 0.91 mm, 20 G: 0.58 mm, 22 G: 0.41 mm, and 24 G: 0.30 mm) were compared, and the device was tested with various nozzle lengths from 10 to 4 mm. A 37-multi-nozzle and an air compressor were also attached to the SPS to increase the productivity of the encapsulated bifidus.

Bead productivity AE solution mixtures containing bifidobacteria were transferred to the bead-forming device (SPS, Fig. 1), and dropped though a 18 G blunt-ended needle into a sterile 0.1 M $CaCl_2$ solution using compressed air filtered by a sequence of air filters located in the air compressor. The bead productivity of SPS was compared with those of other techniques using a syringe, peristaltic pump, and air gun. The nozzle size and length were identical for all the encapsulation equipment (nozzle size, 18 G; length, 4 mm).

Viable cell counts of bifidobacteria entrapped in beads

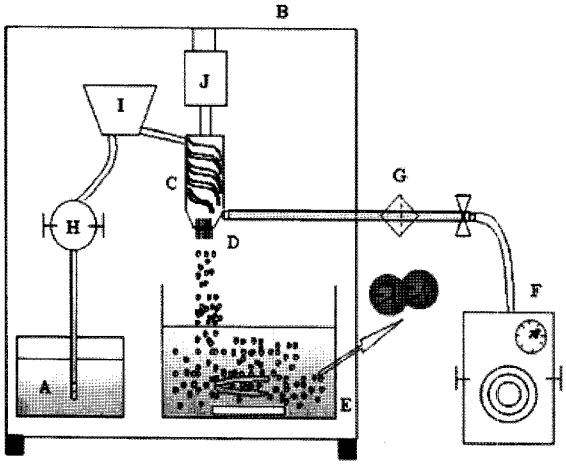


Fig. 1. Schematic diagram of SPS modeling. A: cell entrapment solution, B: clean box, C: extruding device (screw pump), D: muti-nozzle, E: bead collection tool, F: air compressor, G: air filter, H: pump, I: hopper, J: low speed motor.

Ten beads were dissolved in 5 mL sterile solution of 0.1 M sodium citrate and vortexed until the beads completely dissolved. The resulting solution was then used to measure the viable cell count. To enumerate the living cells, 0.1 mL of the solution in physiological saline was plated onto a TPY agar gel and incubated in an anaerobic system for 48 hr at 37. The cell colonies that grew in the medium were counted (25).

Bead size measurements The diameters of the calcium alginate beads were measured using an optical microscope at a magnification of 100×, and the mean diameter (Dm) was calculated. At least 20 randomly selected beads were washed with sterile saline to remove any excess calcium ions and untrapped cells, and were measured (25).

Structure of alginate lattices of beads The freshly produced beads were frozen with liquefied nitrogen for 5 s, lyophilized using a freeze-dryer (EYELA Co., Tokyo, Japan) for 16 hr, and coated with platinum for conductivity. The surfaces of the specimens were then examined using a scanning electron microscope (SEM; Hitachi S-700, Ibaraki, Japan) at 7 kV.

Simulator of human intestinal microbial ecosystem (SHIME) test SHIME was used to determine the survivability of the encapsulated bifidus when digested in the human gastro-intestinal system. The acid tolerance of the bifidobacteria immobilized in the beads was determined by measuring the viability of the bacteria after being exposed for 3 hr to stimulated gastric juices (pH 1.5), consisting of 0.08 M HCl and 0.2% NaCl without pepsin (6). Ten beads were exposed to 3 mL stimulated gastric juices at 37 and 100 rpm for 3 hr collected, washed, and dissolved in 0.1 M sodium citrated to enumerate the live bacteria. The bile salt solution was made from 0.6% Oxgall (Difco Co.), and the bead treatment similar to that used for the gastric juices reaction was used, except that the contact time was extended to 6 hr. The viable cell counts were determined as mentioned before.

Storage stability of encapsulated bifidus Skim milk (10%, w/v) was added to 200 mL fresh milk, and autoclaved at 85°C for 30 min. Subsequently, an ABT941 starter (YC-180, Hansen's Lab., Horsholm, Denmark) in lyophilized form was inoculated into the autoclaved milk and incubated at 37°C for 8 hr. The resulting yogurt, with a final pH of 4.4, was sterilized by autoclaving at 85°C for 30 min. The beads were then soaked in the pasteurized yogurt and stored at 4°C. The viable cell count was determined after 2, 4, 6, 8, 10, 12, and 14 days storage.

Results and Discussion

SPS device Figure 1 is a schematic diagram of the proposed SPS designed for the mass production of encapsulated bifidus. The SPS entrapment device consisted of a feeding component, a screw pump, and a 37 -multi-nozzle. An air compressor was selected as an additional tool to increase the productivity of the encapsulated bifidus. Special care was given to ensure that

Table 1. Comparison of numbers and sizes of beads produced from various nozzle sizes and lengths

Nozzle length (mm)	Productivity (beads/min)			D product[2)]
	18 G (0.91 mm)	20 G (0.58 mm)	22 G (0.41 mm)	
10	100 ± 2	-[1)]	-	
8	133 ± 4	-	-	
7	145 ± 1	-	-	
6	163 ± 5	-	-	
5	220 ± 4	-	-	
4	308 ± 3	63 ± 1	14 ± 1	
3	-	80 ± 4	30 ± 1	
2	-	144 ± 5	44 ± 3	
Bead size (mm)	2.20 ± 0.02	2.04 ± 0.04	2.00 ± 0.02	1.97 ± 0.07

The bead sizes and productivities were determined with various nozzle sizes and lengths. AE (2.5% alginate+1.0% erythritol) was used as the entrapment material. Results are presented as mean ± S.D. (n=3).
[1)](–) : no proper beads
[2)]D product : commercial yogurt

the entrapment device was powerful, but not stressful as to affect the viability of the sensitive anaerobic bacteria, and continuously operated. In particular, because the screw pumps required high-pressure for successful flow of the materials from the screw pump to the narrow droplet, the exit of the screw pump was located parallel to the axis of the screw core. The motor was designed to have low-speed and strong power, allowing the feeding of highly-viscous materials, while preventing stress on the strains when they moved though the system.

Bead size and productivity according to nozzle sizes and length The bead size and productivity values were determined using various nozzle sizes and lengths (Table 1) for the feeding components of SPS. Increasing the internal diameter of the nozzles resulted in higher bead production, with the difference in the bead sizes being about 0.2 mm. In the case of nozzles smaller than 18 G, however, the SPS pressure was insufficient for the materials to flow though the narrow nozzle. In addition, due to the short distance between the wing of the screw and the barrel, material back-blow also became possible. If the SPS device had been more accurately manufactured, this problem would not have occurred. The best productivity was 308 ± 10 ea/min when using an 18 G syringe needle with 4 mm nozzles.

Cell viability according to encapsulation technique Preliminary studies showed, among the four extrusion techniques, the productivity of alginate beads produced using a syringe needle injector was 20 ± 2 ea/min, the SPS productivity when using a 17-multi-nozzle was 308 ± 10 ea/min (Fig. 2). Thus, the alginate solution transport speed appeared to directly increase with the volume of extrusion by the screw and multi-nozzle. The viability of the encapsulated bifidus produced by SPS was 5.45×10^8 cfu/bead, which was slightly higher than the results of other trials, indicating that the SPS technique did not affect the bifidus activity during the course of production. Therefore, it was concluded that an SPS is an efficient and economic

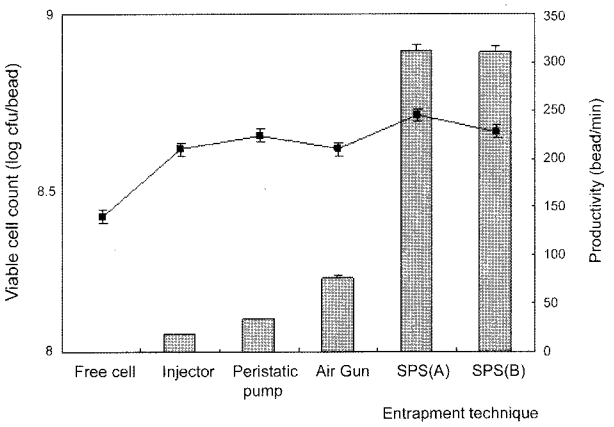


Fig. 2. Comparison of viable cell count and bead productivity among various encapsulation methods. SPS (A): *B. bifidum*, SPS (B): *B. longum*. Data are mean±S.D. (n = 3).

tool for the mass production of encapsulated bifidus.

Bead productivity in SPS devices In the present study, the screw pump of SPS had five turns of the wing from top to bottom on the axis, which, however, is changeable and can be used for a variety of purposes. The wing diameter was also decreased [Radius (r) =26 to 15 mm] at 78-degree angle from the middle of the screw. Two wings were positioned at the edge of screw (Fig. 3A) to push the materials toward the nozzles. The multi-nozzle was manufactured (Fig. 3B) and modified, as described previously (16). Finally, 18 G 4-mm nozzles were selected for the bead production, as determined by experimental results; a total of 37 nozzles were attached to the plate (thickness, 2 mm). The AE beads were then produced using the modified multi-nozzle (37 nozzles). The bead productivity values were 600 ± 20 and 540 ± 25 ea/min from 72.5 and 316.9 cp alginate solutions, respectively. However, considering the errors inherent in the mass

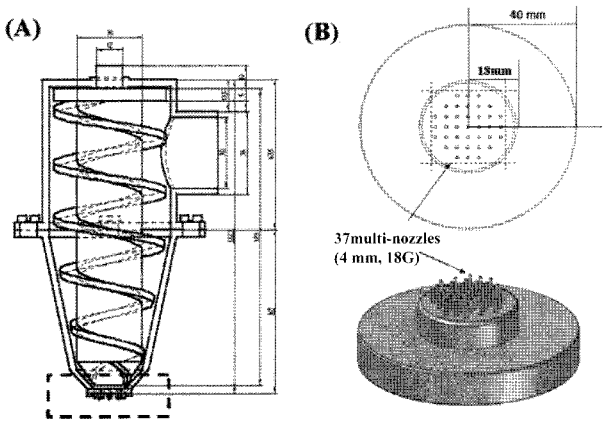


Fig. 3. Diagram of screw pump and multi-nozzles in SPS. The size of the SPS device was determined on an experimental scale. (A) Screw component with 5 wing-turns on axis from top to bottom. The wing diameter is decreased (r=26 to 15 mm) at 78-degree angle from the middle of the screw. Two wings are positioned at the edge of the screw to push the materials toward the nozzles. (B) Diagram of 37-multi-nozzle for mass production of encapsulated bifidus. Diameter of total plate: diameter: 80 mm, nozzle size: 18 G, nozzle length: 4 mm. Diameter of droplet plate is 36 mm, and projected from the center.

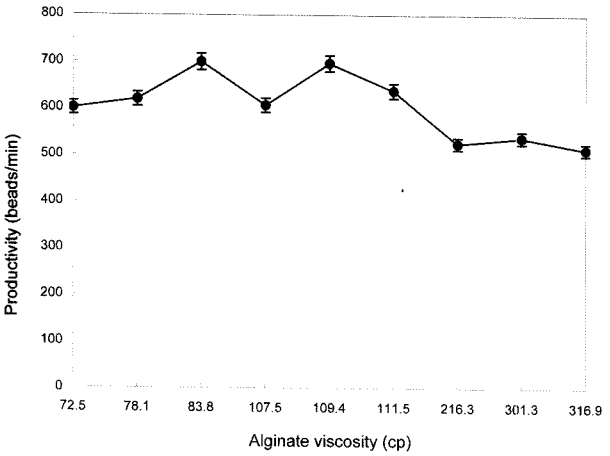


Fig. 4. Comparison of bead productivity according to concentration of AE solution. The bead productivity was calculated as the number of beads produced using the screw pumping system with 37-multi-nozzle. Results are presented as mean±S.D. (n = 3).

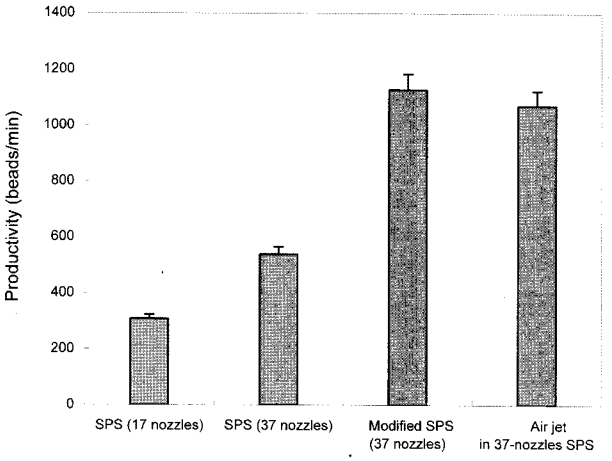
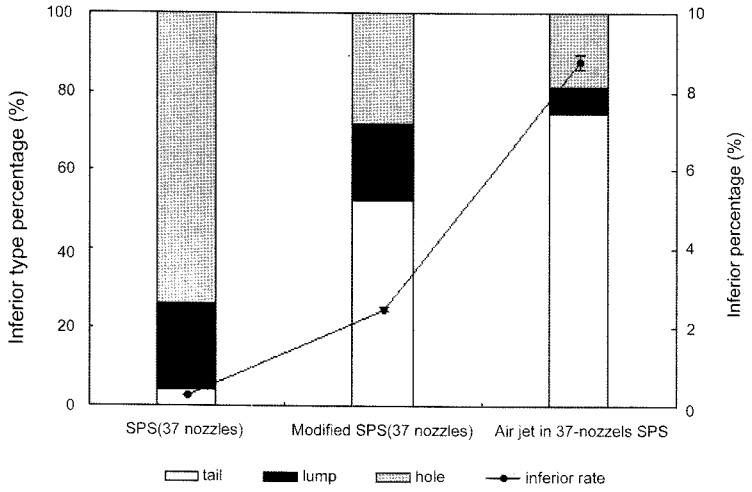


Fig. 5. Bead productivity of different SPS devices. The AE viscosity was 300 ± 10 cp, and bead productivity calculated as the number of beads produced by the screw pumping system. Data are expressed as mean±S.D. (n = 3).

production of alginate beads, the alginate bead productivity was unaffected by the viscosity of the alginate solution (Fig. 4).

Bead productivity with different SPS devices The productivities of alginate beads produced using 17- and 37-multi-nozzle SPSs, 37-multi-nozzle SPS attached to an air compressor (Modified SPS, M-SPS), and air jet modified-SPS with no solution transfer by screw (Air jet M-SPS) were 310 ± 5, 538 ± 20, 1128 ± 30, and 1071 ± 35 ea/min, respectively (Fig. 5); in general, M-SPS and A-SPS exhibited superior productivity (Fig. 6). These results suggested that M-SPS is the optimum type of SPS, showing a high overall rate of productivity and quality. Upon extrusion of the alginate beads using the M-SPS system, prompt recovery of the encapsulated bifidus was observed from each nozzle.



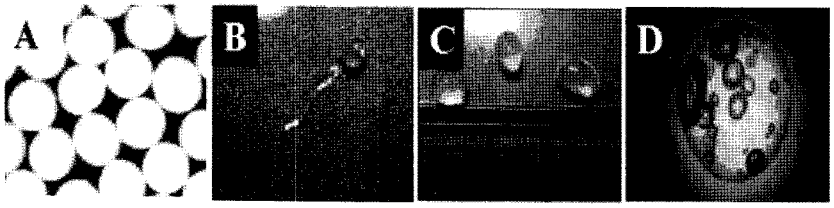


Fig. 6. Photogram and incidence analysis of inferior beads produced by each SPS device. The bead productivity was calculated as the number of AE-beads produced using the 37-multi-nozzle screw pumping system, and the inferior rates calculated as the number of inferior beads. Data are expressed as mean±S.D. (n = 3). A: uniform-shaped beads (bead size < 2mm), B: tail bead, C: lump bead (bead size > 4 mm), D: floating bead due to air inhalation (optical microscopy of bead, ×40).

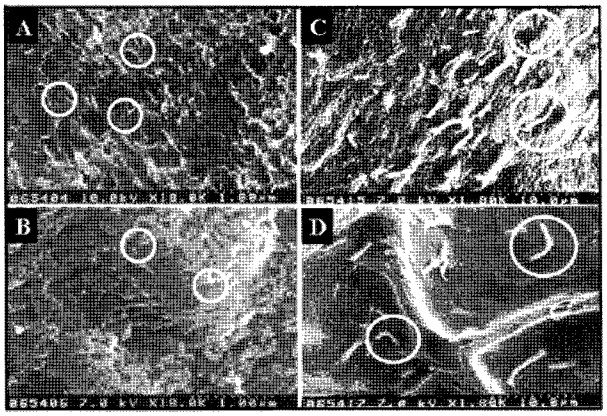


Fig. 7. SEM photograph of *B. longum* encapsulated in AE-beads produced using M-SPS system. A-C: Surface of bead encapsulated with 2.5% alginate, B-D: Surface of bead encapsulated with AE (2.5% alginate plus 1.0% erythritol).

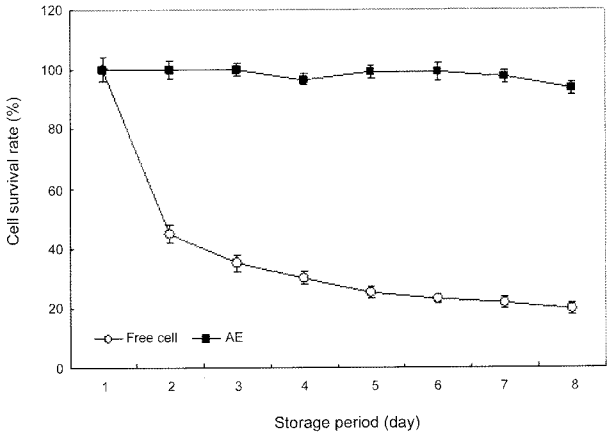


Fig. 9. Survivability of encapsulated bifidus produced using SPS in fermented milk for storage period. –○–: free cell (uncapsulated *B. bifidum*), –■–: *B. bifidum* encapsulated with AE. Data are expressed as mean±S.D. (n=3).

Structural analysis of bead surfaces produced using SPS Figure 7 shows that several pores were formed on the surfaces of the beads produced with only alginate, as observed in the SEM photographs (A, C), whereas, in beads produced with AE as the additive, the pores on the surfaces of the beads were filled (B, D). These results suggest that an additive is necessary to protect the beads against free hydrochloric acid in the stomach and bile salts produced by the gall bladder and secreted into the duodenum to increase the survivability of living cells during passage though the gastrointestinal tract.

Survivability of encapsulated bifidus in SHIME SHIME is used in many experiments involved with human food digestion. While the survivability of free cells passing though the stomach and intestine was nearly zero percent, the encapsulated bifidus produced using M-SPS exhibited a good survivability, in spite of the AE component of the encapsulated bifidus collapsing due to the effect of pH in the stomach and intestine model (26). However, the M-SPS technique generally led to a good survivability of the encapsulated bifidus in the gastro-intestinal system. The initial viable cell count of the AE-encapsulated bifidus was 2.20×10^8 cfu/bead, which, after being allowed to react for 22 hr in the SHIME system, increased to 3.72×10^7 cfu/bead; the cell counts of the viable bifidus and bifidus flowing out were 1.14×10^9 cfu/bead and 1.98×10^9 cfu/bead, respectively. Therefore, it was concluded that the transfer effect of AE was quite high in the intestine.

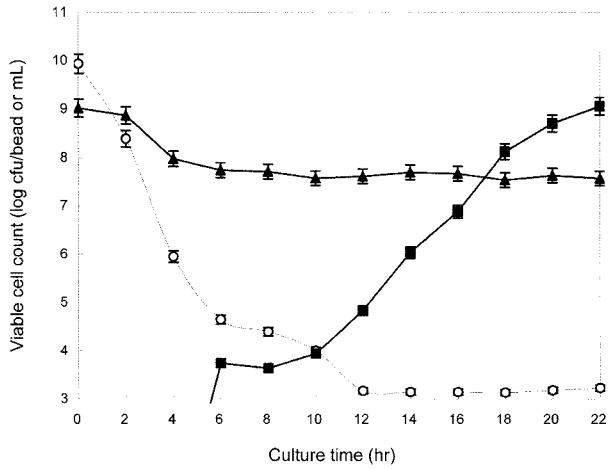


Fig. 8. Survivability of encapsulated bifidus produced using SPS in SHIME. –○–: Free cells (uncapsulated *B. bifidum*), –▲– : *B. bifidum* encapsulated with AE, –■–: outflow of encapsulated *B. bifidum* into broth. The reaction solution used HCl without pepsin and bile salt as a simulator of stomach and intestine conditions. The total SHIME retention time was 22 hr. Data are mean±S.D. (n=3).

Stability of encapsulated bifidus produced using SPS in fermented milk In the fermented milk, the initial viable cell count for the free cells and encapsulated bifidus produced using M-SPS was 2.4×10^9 cfu/mL and 5.4×10^8 cfu/bead, respectively. However, after 14 days, the viability of the free cells was rapidly reduced by the organic acid in the fermented milk (4.7×10^8 cfu/mL). In contrast, the viable AE cell count was 5.0×10^8 cfu/bead, representing only a slight reduction in the initial viable cell count (Fig. 9).

In conclusion, a new entrapment method on an experimental scale was developed using a screw pumping system, which is not only an efficient tool for mixing and pumping highly viscous liquids, but also a source of continuous pressure for feed materials, thereby producing beads without any negative side effects. The proposed method is also considered to be very valuable to the development of a novel entrapment device for use with live probiotic cells, including *Bifidobacterium* species. Further experiments on the pilot and commercial-scale uses of SPS device are currently in progress.

Acknowledgments

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