J. Microbiol. Biotechnol. (2006), **16**(3), 443–449

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Encapsulation of *Bacillus polyfermenticus* SCD with Alginate-Methylcellulose and Evaluation of Survival in Artificial Conditions of Large Intestine

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Received: July 6, 2005
Accepted: August 20, 2005

Abstract *Bacillus polyfermenticus* SCD was studied for its increasing stability by encapsulation, using 2, 3, and 4% sodium alginate. In these cases, 3% alginate resulted in the maximum survival of *B. polyfermenticus* SCD in artificial gastric juice for 3 h. Effects of several biopolymers on the encapsulated *B. polyfermenticus* SCD by 3% sodium alginate were investigated. Encapsulation with 0.5% methylcellulose showed the highest survival rate for 3 h in artificial gastric juice. Therefore, the optimized encapsulation material was 3% alginate with 0.5% methylcellulose. Furthermore, the survival of encapsulated *B. polyfermenticus* SCD was shown to be 122%, when 1% bile salt was added. Freeze-dried encapsulation resulted in lower survival than with non-dried encapsulation. Therefore, encapsulation was the most effective when 3% sodium alginate was used with 0.5% methylcellulose, but without freeze-drying.

Key words: Probiotics, encapsulation, alginate, *Bacillus polyfermenticus*

Probiotics are generally defined as viable microorganisms that, when applied to humans or animals, beneficially affect the health of the host by improving the indigenous microbial balance [7, 10, 17]. Most probiotics belong to the large group of bacteria empirically designated as lactic acid bacteria (LAB; *Lactobacillus*, *Bifidobacterium* [8], *Streptococcus*, and *Enterococcus*), which are important components of the human gastrointestinal microflora and exist as harmless commercial organisms. Probiotics require the following characteristics to be effective: (i) genera of human origin, (ii) stability and ability to adhere to the intestinal mucosa, (iii) colonization potential in the human gastrointestinal tract, (iv) production of antimicrobial substances, and (v) demonstrable efficacy and safety. In addition, it is important that the viability of the strain and stability of its desirable characteristics are maintained during commercial production as well as in the final product [9]. New probiotics also include other microbes, such as yeast (e.g., *Saccharomyces boulardii*) and other quite different types of bacteria (e.g., *Clostridium butyricum*, *B. subtilis*, *B. polyfermenticus*, and so on.). In particular, *B. polyfermenticus* SCD, which is commonly referred to as a "Bispan" strain, has been effectively used for the treatment of long-term intestinal disorders, since live strains in the form of active endospores can successfully reach the target intestine [9, 14, 18].

Capsule techniques have widely been utilized to protect microorganisms, cells, or tissues from environmental and physiological degradation and have been applied to increase the survival and delivery of bacterial cultures. Encapsulated *Bifidobacterium pseudolongum* was reported to have increased survival under artificial gastric acid conditions as compared with the non-encapsulated bacteria [1, 16, 20]. In order to increase viability, LAB were microencapsulated within cross-linked chitosan membranes formed by emulsification/interfacial polymerization [4, 11, 13, 21, 23, 29, 30]. In addition to these materials, several biopolymers such as gellan gum, carrageenan/locust bean gum, and alginate are commonly used for immobilizing bacteria [12, 23, 25, 28]. In particular, sodium alginate has been used most widely as an immobilizing vehicle, owing to its gentle and simple method of immobilization: It forms a gel when in contact with calcium and multivalent cations. Alginate capsules (or microparticles) are stable in low pH

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conditions, but swell in weak basic solutions, followed by disintegration and erosion [6, 12, 15].

The aim of this work was to determine the optimal conditions for encapsulation of *B. polyfermenticus* SCD and to investigate the effects of artificial gastric juices and bile salts on the survival of encapsulated *B. polyfermenticus* SCD in a gel matrix.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

B. polyfermenticus SCD was maintained at −70°C in Trypic Soy Broth (TSB; Difco Laboratories, MD, U.S.A.), to which 20% (v/v) glycerol was added. This strain was cultured in TSB with shaking at 30°C.

Encapsulation of *B. polyfermenticus* SCD

Large Capsule Preparation (Extrusion). *B. polyfermenticus* SCD was harvested in the late exponential phase and centrifuged for 15 min at 10,000 rpm. Then, the cells were washed twice with 20 ml of sterile normal saline and resuspended in 10 ml of sterile saline to approximately 10^8–10^9 cells/ml. Ten ml of cell suspension was added to 80 ml of 2, 3, and 4% sodium alginate solution in aseptic vinyl bags (Seward Co., London, U.K.). The solutions were thoroughly mixed with a stomacher 400 laboratory blender (Seward Co.). These mixtures were next transferred to the capsule-forming device and dropped through a Pasteur pipette and Tygon tube into a sterile 0.1 M $CaCl_2$ solution on a clean bench. The capsules were hardened in this solution for more than 1 h (Fig. 1).

Small Capsule Preparation (Emulsification). A modified method of Sheu and Marshall [22] was used. Five percent (w/v) $CaCO_3$ was mixed into culture containing 3% sodium alginate, and the mixture was dispersed with high-speed homogenization for 4 min. The mixture was dropped into oil. After the dropping was completed, the mixture was stirred vigorously until it was emulsified and appeared creamy. After the 5 min emulsification, glacial acetic acid was added and stirring continued for 5 min to solubilize the calcium carbonate. A solution of 0.1 M calcium chloride was then added quickly along the side of the beaker, and phase separation of the oil/water emulsion occurred. The mixture was left for 30 min to allow the calcium-alginate capsules to separate and settle at the bottom of the calcium chloride layer. The oil layer was drained, and capsules were collected by low-speed centrifugation, washed once with 0.85% saline, and stored at 4°C. Size of the capsules was adjusted using 1.18-mm steel sieves [21, 24].

Morphological Observation of Alginate Capsules

Microstructural properties of freeze-dried encapsulated cells were investigated by scanning electron microscopy (model S-4200; Hitachi Co., Japan). Encapsulated cells were refrigerated at 4°C and freeze-dried at −45°C for 12 h, using a vacuum tray freeze dryer (Ilshin Engineering Co., Korea). Specimens were loaded onto a specimen stub with two-sided adhesive tape, and were then coated with Pt-Pb in an ion sputter (ε-1030; Hitachi Co., Japan).

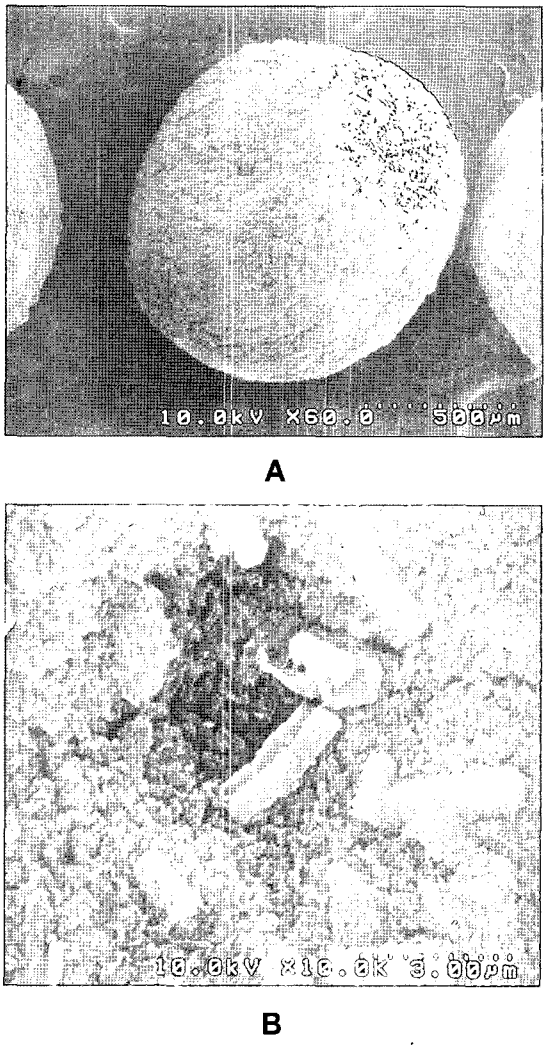

Fig. 1. Scanning electron microscopic (SEM) observation of **A** sodium alginate capsules, and **B** freeze-dried *B. polyfermenticus* SCD entrapped in the pore alginate lattice.

Determination of *B. polyfermenticus* SCD Viability in Alginate Capsules

To determine the cell viability, the encapsulated bacteria were released from the capsules according to the method of Sheu and Marshall [22]. Capsules (0.1 g) were washed with a sterile saline solution and dissolved in 1 ml of 0.1 M sodium citrate solution for 30 min, followed by vortex homogenization. Viable cells (cfu/g) were determined by plating on TSA plates and incubating at 37°C for 12 h.

Survival of Encapsulated *B. polyfermenticus* SCD in Artificial Gastric Juice Conditions

Artificial gastric juice was prepared using pepsin (0.1 M HCl containing 0.85% NaCl). The encapsulated *B. polyfermenticus* SCD was added to artificial gastric juice that had been adjusted to pH 1.5, 2.5, 4.0, and 7.0 (control). Samples were incubated at 37°C for 3 h. At 30-min intervals, encapsulated *B. polyfermenticus* SCD (0.1 g) was harvested, washed with physiological saline, and then dissolved in 1 ml of sterile 0.1 M sodium citrate solution with the aid of a vortex for 1 h. Total viable cell numbers were determined by the plate count method.

Survival of Encapsulated *B. polyfermenticus* SCD in Artificial Bile Salt Conditions

Filter sterilized oxgall was added to a final concentration of 0.6, 0.8, and 1%, and the samples were incubated at 37°C for 6 h. Total viable cell numbers were determined by the plate count method.

Selection of Capsule Material by Addition of Other Biopolymers

Three percent sodium alginate was combined with other materials such as 0.15% xanthan gum, 0.5% gellan gum, 0.5% methylcellulose, 0.5% corn starch, and 0.5% casein. Methylcellulose was also tested at different concentrations.

Comparison of *B. polyfermenticus* SCD Survival in Non-Dried and Freeze-Dried Sodium Alginate/Methylcellulose Capsules

Sodium alginate and methylcellulose capsules were washed twice with 0.85% saline. They were frozen at −75°C for 2 h, and then freeze-dried using a freeze dryer (Ilshin Engineering Co., Korea).

To compare the gastric resistance of *B. polyfermenticus* SCD, 0.1 g of non-dried and freeze-dried sodium alginate/methylcellulose capsules were added to 100 ml of artificial gastric juice (pH 1.5 within pepsin). After incubation at 37°C for 3 h, the survival of *B. polyfermenticus* SCD was determined in triplicate by using the plate counting method.

For comparison, samples of non-dried and freeze-dried sodium alginate/methylcellulose capsules were also incubated in artificial bile salt solution containing 0.6% oxgall at 37°C for 6 h. The survival of *B. polyfermenticus* SCD was determined by using the plate count method in triplicate.

RESULTS AND DISCUSSION

Morphological Observation of Alginate Capsules

Sodium alginate capsules exhibited a spherical shape (Fig. 1A), and the mean capsule size was 2.8 mm (diameter). The spatial distribution of cells within sodium alginate capsules is shown in Fig. 1B.

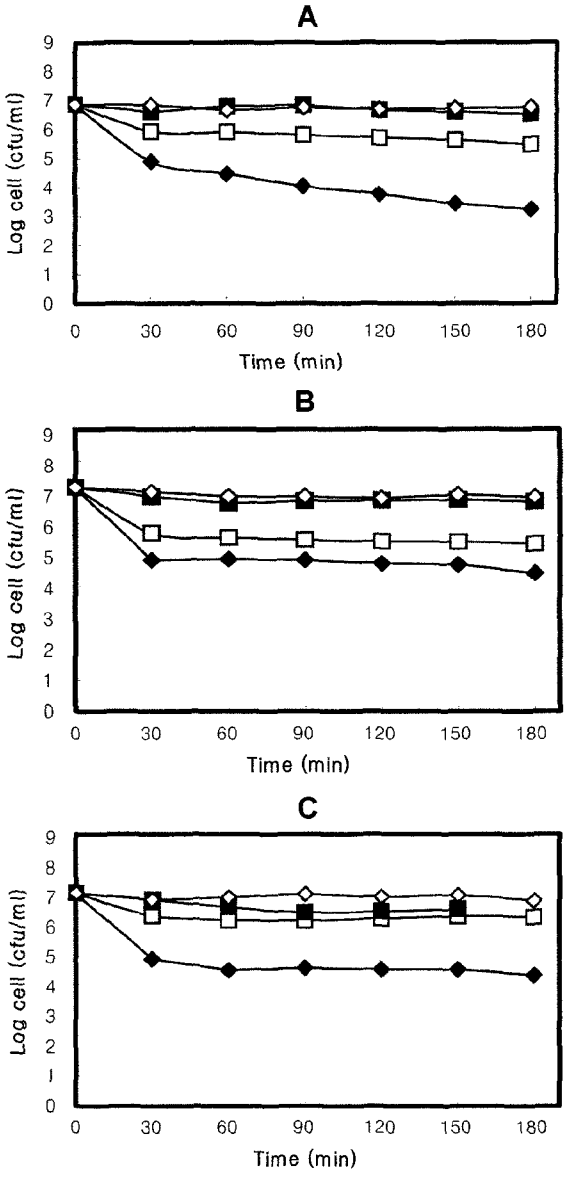

Fig. 2. Survival of encapsulated *B. polyfermenticus* SCD with different sodium alginate concentrations after the exposure to artificial gastric juice.
A, 2% sodium alginate capsule; **B**, 3% sodium alginate capsule; **C**, 4% sodium alginate capsule. ◆, pH 1.5; □, pH 2.5; ■, pH 4.0; ◇, pH 7.0.

Survival of Encapsulated *B. polyfermenticus* SCD in Artificial Gastric Juices

Capsules (about 2.8 mm diameter) were prepared separately using 2, 3, and 4% (w/v) sodium alginate. These capsules were then exposed to artificial gastric juice (pH 1.5, 2.5, 4.5, and 7.0) for 3 h. Figure 2 shows the survival of encapsulated *B. polyfermenticus* SCD in different concentrations of sodium alginate. It is known that *B. polyfermenticus* SCD is a halophilic bacterium that is very susceptible to pH change, and sodium alginate capsules are stable in gastric fluid, but erode and disintegrate in higher pH conditions

[19]. Even though *B. polyfermenticus* SCD can be protected by encapsulation in sodium alginate, the survival was also affected by the pH of the exposing medium: Survival was unchanged in neutral pH conditions, but rapidly decreased at lower pH. With 2% sodium alginate capsules, the initial cell viability of *B. polyfermenticus* SCD was 7.0×10^6 cfu/ml, whereas cell viability in artificial gastric acid was 1.7×10^3 cfu/ml. With 3% sodium alginate capsules, the cell viability of *B. polyfermenticus* SCD decreased from 1.4×10^7 cfu/ml to 2.5×10^4 cfu/ml after 3 h. With 4% sodium alginate capsules, the initial cell viability of *B. polyfermenticus* SCD was 1.1×10^7 cfu/ml, and 2.2×10^4 cfu/ml after 3 h. Therefore, the survival of encapsulated *B. polyfermenticus* SCD in 2, 3, and 4% sodium alginate capsules was 0.02, 0.18, and 0.19 %, respectively. The results on the survival of *B. polyfermenticus* SCD that was exposed to strong acid with free cells (data not shown) and the studies with encapsulated *B. polyfermenticus* SCD indicated that encapsulation of *B. polyfermenticus* SCD did not effectively protect this strain from the high acid conditions. However, the survival in 3 and 4% sodium alginate capsules at pH 1.5 was higher than with 2% sodium alginate capsules. The slower diffusion of these substances into capsules containing higher concentrations of sodium alginate was due to lower number and depth of micropores rather than their size [12]. The diffusion of a substance with a high molecular weight into sodium alginate capsules was limited by an increasing sodium alginate concentration in the capsule [27]. Therefore, encapsulation of *B. polyfermenticus* SCD in 3% sodium alginate mixture was considered to be optimal.

To test the effect of capsule size on the survival of encapsulated *B. polyfermenticus* SCD, capsules of different sizes were prepared using 3% sodium alginate mixture in the presence of artificial gastric juices (Fig. 3). The viability of *B. polyfermenticus* SCD in capsules with 850 μm diameter (small capsule) was 4.6×10^7 cfu/ml initially and 1.1×10^4 cfu/ml (0.02% survival rate) after 3 h. As expected, a lower death rate of encapsulated *B. polyfermenticus* SCD was observed with a larger capsule (2.8 mm). Therefore, the size of the capsule affected the physiological changes caused by artificial gastric juice: That is, the calcium alginate matrix increased the survival of *B. polyfermenticus* SCD in artificial gastric juice. Chandramouli *et al.* [3] reported that the survival of bacterial cells under *in vitro* gastric conditions increased proportionately to capsule size

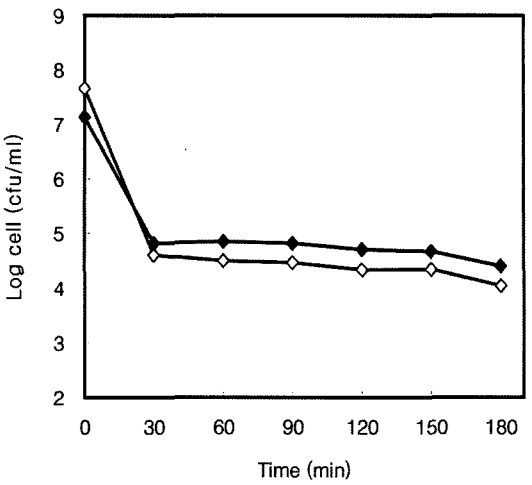

Fig. 3. Survival of *B. polyfermenticus* SCD in 3% alginate capsules with various capsule diameters after the exposure to artificial gastric juice (pH 1.5).
◇, Small capsule (850 μm); ◆, large capsule (2.8 mm).

Table 1. Survival of encapsulated *B. polyfermenticus* SCD in 3% sodium alginate after the exposure to artificial gastric juice with various additives.

		Exposure time (min)						
		0	30	60	90	120	150	180
Control	Log cfu/g	7.13	4.81	4.85	4.81	4.70	4.67	4.40
	Survival (%)	100	0.48	0.52	0.48	0.37	0.35	0.19
0.15% Xanthan gum	Log cfu/g	7.28	5.01	4.92	4.71	4.74	4.69	4.75
	Survival (%)	100	0.54	0.44	0.27	0.29	0.26	0.29
0.5% Gellan gum	Log cfu/g	7.19	4.94	4.87	4.66	4.45	4.41	4.54
	Survival (%)	100	0.57	0.48	0.30	0.18	0.17	0.23
0.5% Methylcellulose	Log cfu/g	7.07	6.88	6.88	6.93	6.94	6.92	6.83
	Survival (%)	100	64.6	64.6	72.4	74.1	70.8	57.5
0.5% Corn starch	Log cfu/g	6.56	4.79	4.71	4.75	4.7	4.64	4.53
	Survival (%)	100	1.70	1.41	1.55	1.38	1.20	0.93
0.5% Casein	Log cfu/g	6.89	4.76	4.58	4.47	4.82	4.5	4.79
	Survival (%)	100	0.74	0.49	0.38	0.85	0.41	0.79

Table 2. Effect of incorporating methylcellulose into capsules with sodium alginate on the survival of *B. polyfermenticus* SCD in artificial gastric juice.

Methylcellulose concentration (%)		Exposure time (min)						
		0	30	60	90	120	150	180
0.1	Log cfu/g	5.57	4.30	4.00	4.00	4.00	4.30	4.02
	Survival (%)	100	5.37	2.69	2.69	2.69	5.37	2.82
0.3	Log cfu/g	5.56	4.48	4.30	4.18	4.65	4.40	4.54
	Survival (%)	100	8.32	5.50	4.17	12.3	6.92	9.55
0.5	Log cfu/g	6.94	6.90	6.91	6.89	6.91	6.90	6.85
	Survival (%)	100	91.2	93.3	89.1	93.3	91.2	81.3
0.7	Log cfu/g	5.46	4.70	4.70	4.81	4.48	4.40	4.30
	Survival (%)	100	17.4	17.4	22.4	10.5	8.71	6.92

[2, 3, 22, 24, 26]. However, small beads of less than 100 mm in diameter did not significantly protect the probiotic bacteria in gastric juice, compared with free cells. Consequently, probiotic bacteria should be encapsulated within a limited range of bead size.

Determination of Encapsulating Material by Addition of Biopolymers

Table 1 indicates that the survival of *B. polyfermenticus* SCD entrapped in biopolymers containing calcium alginate capsules varied in artificial gastric juice according to biopolymer added. The survival of encapsulated *B. polyfermenticus* SCD prepared with all additives and sodium alginate in the presence of artificial gastric juice was higher than that of *B. polyfermenticus* SCD entrapped in capsules containing only 3% sodium alginate. Of all the capsules containing added biopolymers, the encapsulated *B. polyfermenticus* SCD in capsules containing 3% sodium alginate and 0.5% methylcellulose exhibited the highest survival in artificial gastric juice (Table 2): At pH 1.5, an 80–90% reduction in viability of *B. polyfermenticus* SCD encapsulated in sodium alginate was observed, whereas alginate/methylcellulose-encapsulated *B. polyfermenticus* SCD showed 15–20% reduction (80% survival rate). Therefore, the encapsulation of *B. polyfermenticus* SCD in sodium alginate capsules mixed with 0.5% methylcellulose is very effective in improving the survival of the bacteria in artificial gastric juice.

Determination of the Optimal Methylcellulose Concentration in Alginate Capsules

To determine the highest survival rate, we evaluated the viability of encapsulated *B. polyfermenticus* SCD exposed to artificial gastric juice after 2 h, using methylcellulose concentrations of 0.1, 0.3, 0.5, and 0.7%. The survival of *B. polyfermenticus* SCD was the greatest with higher molecular size of methylcellulose, but the viability with 3% sodium alginate/0.7% methylcellulose in the presence of artificial gastric juice was consistently lower than that of 3% sodium alginate/0.5% methylcellulose (Fig. 4). The lower survival rate of 3% sodium alginate/0.7% methylcellulose-encapsulated *B. polyfermenticus* SCD might be due to the increasing concentration of high molecular weight methylcellulose in the alginate matrix. In conclusion, we concluded that the optimal encapsulating material for improving the survival of *B. polyfermenticus* SCD was 3% sodium alginate with 0.5% methylcellulose.

Survival of Encapsulated *B. polyfermenticus* SCD in Artificial Bile Salts

After microorganisms pass through the stomach, they enter the upper intestinal tract where bile salts are secreted into

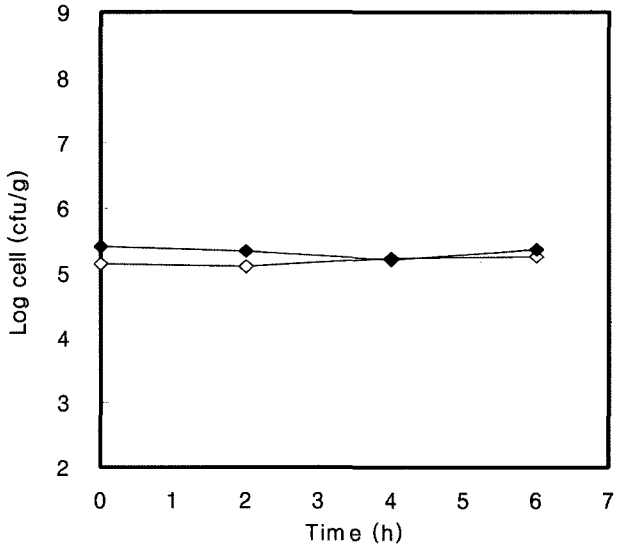

Fig. 4. Survival of encapsulated *B. polyfermenticus* SCD in (◇) non-dried and (◆) freeze-dried 3% sodium alginate with 0.5% methylcellulose capsule after the exposure to artificial bile salt solution for 6 h.

Table 3. Survival of encapsulated *B. polyfermenticus* SCD after more than 6 h of exposure to bile salt solution.

	Bile conc. (%)	Exposure time (h)	Exposure time (h)			
			0	2	4	6
3% Sodium alginate	0.6	Log cfu/g	6.57	6.43	6.39	6.60
		Survival (%)	100	72	66	107
	1.0	Log cfu/g	6.57	6.36	6.33	6.61
		Survival (%)	100	62	58	111
3% Sodium alginate + 0.5% Methylcellulose	0.6	Log cfu/g	6.92	6.93	6.85	7.00
		Survival (%)	100	103	84	120
	1.0	Log cfu/g	6.92	6.96	6.95	7.01
		Survival (%)	100	108	107	122

the gut [5, 12]. Probiotics are reported to tolerate 0.3% oxgall, which is similar to human intestinal conditions [7, 14]. The concentration of bile salts in the human gastrointestinal system is variable, but is usually around 0.6%. To test bile salt tolerance, encapsulated *B. polyfermenticus* SCD was exposed to solutions containing different levels of bile salts (Table 3). Viabilities were recorded at 2-h intervals up to the maximum exposure time of 6 h. The viability of *B. polyfermenticus* SCD encapsulated in sodium alginate decreased with each 2-h interval, although the penetration time was different. However, the viability increased at 6 h. These results show that oxgall as bile salt used nutrient preferably to pass time, except for the initial exposure. Consequently, all 3% sodium alginate, and 3% sodium alginate/0.5% methylcellulose-encapsulated *B. polyfermenticus* SCD exhibited higher growth rates in artificial bile salts than in the control medium after 6 h (Table 3).

Comparison of the Survival of *B. polyfermenticus* SCD in Non-Dried and Freeze-Dried 3% Sodium Alginate/0.5% Methylcellulose Capsules

Scanning electron microscopic observations of freeze-dried 3% sodium alginate/0.5% methylcellulose capsules are shown in Fig. 1: 3% sodium alginate/0.5% methylcellulose-encapsulated *B. polyfermenticus* SCD had a smooth surface, whereas freeze-dried 3% sodium alginate/0.5% methylcellulose-encapsulated *B. polyfermenticus* SCD had a rough surface. The surface morphology of 3% sodium alginate/0.5% methylcellulose capsules was modified with the methylcellulose molecule, even though the overall shape and size were similar.

The survival of *B. polyfermenticus* SCD in the freeze-dried 3% sodium alginate/0.5% methylcellulose capsules in artificial gastric juice (pH 1.5 with pepsin) at 37°C is shown in Table 4. Survival was maintained at 1.2×10^5 cfu/g after treatment for 3 h in artificial gastric juices (60% survival rate). It seemed likely that gastric fluid entered the microparticles through the surface pinholes, resulting in a loss of viability.

The survival of *B. polyfermenticus* SCD in the freeze-dried 3% sodium alginate/0.5% methylcellulose capsules in artificial bile salt solution at 37°C is shown in Table 4. The viability of *B. polyfermenticus* SCD in freeze-dried 3% sodium alginate/0.5% methylcellulose capsules decreased from 2.6×10^5 cfu/ml to 2.3×10^5 cfu/ml after exposure to 0.6% oxgall solution for 6 h (90% survival rate). However, *B. polyfermenticus* SCD in non-dried alginate capsules exhibited a higher survival rate than with freeze-dried 3% sodium alginate/0.5% methylcellulose capsules in artificial gastric juice and bile salt solutions. These results indicate that freeze-drying does not improve the survival of encapsulated *B. polyfermenticus* SCD.

Table 4. Comparison of survival of encapsulated *B. polyfermenticus* SCD in non-dried and freeze-dried 3% sodium alginate with 0.5% methylcellulose capsule after the exposure to artificial gastric juice for 3 h.

Exposure time (min)	Non-dried capsule		Freeze-dried capsule	
	Log cfu/g	Survival (%)	Log cfu/g	Survival (%)
0	5.14	100	5.31	100
60	5.11	93	5.16	70
120	5.06	83	5.05	55
180	5.07	84	5.09	60

Acknowledgment

This work was funded by a research grant received from the Biogreen 21 program in Korea. Also, this work was supported by KRF 2004-F00019.

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