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Improvement of the Stability of *Lactobacillus casei* YIT 9018 by Microencapsulation Using Alginate and Chitosan

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Abstract *Lactobacillus casei* YIT 9018 was microencapsulated within alginate or alginate/chitosan double membrane using an air atomizer. Microbiological analysis revealed that the viability of encapsulated *L. casei* in gastric juice, hydrogen peroxide, and pepsin was 2–3 log cycle higher than that of the nonencapsulated cell. However, the encapsulated cells did not show a significant increase in survival when subjected to *in vitro* high acid and 0.6% bile salt condition. Alginate-encapsulated, alginate/chitosan-encapsulated, and nonencapsulated cells were stored at different temperatures and storage stability was determined. Nonencapsulated and encapsulated cells showed similar stability at 4°C. However, at 22°C, the alginate/chitosan-encapsulated cell was the most stable.

Key words: Microencapsulation, *Lactobacillus casei*, alginate, chitosan, storage stability

Fermented foods have had a long reputation for being beneficial to human health. Ilya Metchnikoff was an early advocate of this idea, based on his theories on disharmonies in nature. The antimicrobial activity of lactic acid bacteria in acidic foods such as yogurt and *kimchi* would inhibit pathogenic and spoilage bacteria, thereby improving the hygienic quality [1, 11]. Lactic acid bacteria also provide flavor and odor in dairy fermented foods [11, 13]. Bacteria used as probiotic adjuncts are commonly delivered in the food system and, therefore, begin their journey to the lower intestinal tract via the mouth [3]. However, a major barrier to the survival of ingested microorganisms is the low pH of the stomach [15]. In addition, the usual starter organisms in yogurt are not bile-tolerant and do not colonize intestines. Hence, there is a need for lactic acid bacteria to be resistant to the stressful conditions of the stomach and upper intestine which contains bile [3].

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Encapsulation may enhance the survival of lactic acid bacteria under such harsh conditions. Several researches have reported a method for the preparation of stable microencapsulated lactic acid bacteria by using sodium alginate [6, 14, 18, 21]. Alginates are salts of alginic acid, which is a linear copolymer composed of 1,4-linked-D-mannuronic acid and L-guluronic acid residues, which are isolated from brown seeds [16]. The simple, mild aqueous-based gel formation of sodium alginate is completed in the presence of divalent cations such as Ca^{2+}. However, Ca-alginate capsules are chemically unstable on contact with various cation-chelating agents such as phosphate, citrate, and lactate which can cause capsule disruption or dissolution [21]. Since alginate is polyanionic, polycationic polymer coatings of polylysine, polyvinylamine, chitosan, etc. have been employed to increase the stability of alginate capsules or to minimize the loss of encapsulated material [4].

Chitosan, a hydrophilic polyelectrolyte prepared by N-acetylation of chitin [16], is a natural polysaccharide, nontoxic, and bioabsorbable [4]. Alginate and chitosan, bearing negatively or positively charged groups, can interact and form three-dimensional networks with molecules of opposite charges [16]. Alginate/chitosan system has been applied for encapsulation of various molecules such as drugs [4, 12, 17] and proteins [5].

Microencapsulation via crosslinking with polymers has been commonly used as a method for viable cells. However, a problem for microencapsulation of viable cells is recognized due to the use of toxic solvents and pH extremes [10]. Recently, an emulsification/internal gelation technique has been reported by Ribeiro *et al.* [17]. This technique did not include any harsh conditions, but resulting microsphere diameters ranged from 120 to about 1,600 μm. An air-atomization technique has been described as a solvent-free technique [9], and this technique was also found to be nontoxic which allowed manufacturing of small-sized and evenly distributed microcapsules.

In this study, the alginate/chitosan system and an air-atomizer were applied to encapsulation of a microorganism, *L. casei* YIT 9108. The viability in harsh conditions and storage stability of microencapsulated *L. casei* were investigated.

MATERIALS AND METHODS

Materials

Wall materials used were sodium alginate (Yakuri Pure Chemical Co., Osaka, Japan) and chitosan (Kittolife, Seoul, Korea). Dihydrate calcium chloride ($CaCl_2 \cdot 2H_2O$) and sodium citrate were purchased from Duksan Chemical (Yongin, Korea). Xanthan gum and hydrogen peroxide were supplied by Sigma Chemical Co. (U.S.A.). Oxbile and pepsin were obtained from Acumedia (U.S.A.) and Junsei (Tokyo, Japan), respectively.

Microorganism and Culture Condition

Freeze-dried *Lactobacillus casei* YIT 9108 was donated by Korea Yakult R&D Center (Yongin, Korea). A loopful of culture was rehydrated in 10% skim milk. The culture was activated by inoculating in lactobacilli MRS broth and incubating for 12–24 h at 37°C.

Preparation of Microcapsules

L. casei YIT 9018 was obtained from culture grown in MRS broth containing 0.001 M $CaCl_2 \cdot 2H_2O$ at 37°C for 20 h. The cells were harvested from the fermented broth by centrifugation at 3,000 ×g for 20 min, and were resuspended in 1% peptone water. This cell suspension was mixed with preservatives (mixture of 3% ascorbic acid, 2% sodium glutamate, 5% lactose, and 0.2% Tween 20) and 4% sodium alginate. Sodium alginate solution containing cells was infused into an air-atomizing device (Saehan Scientific Co., Korea) and sprayed into 1.5% of the calcium chloride solution. The divalent calcium ions crosslinked the droplets of sodium alginate to form temporary microcapsules (alginate-microcapsule). The resulting alginate-microcapsule was obtained by centrifugation at 1,300 ×g for 15 min, and washed with distilled water and buffer solution. Chitosan was dissolved in 0.3 M acetic acid solution, and 0.1, 0.5, and 1.0% chitosan solutions were then prepared and the pH level was adjusted to 5.0. The alginate microcapsule was hardened in the chitosan solution to form permanent microcapsules (alginate/chitosan-microcapsules). After hardening, the resultant alginate/chitosan microcapsule was collected by centrifugation at 1,300 ×g for 15 min and washed with distilled water (Fig. 1). For the storage stability test, encapsulated cells were freeze-dried at 10°C for 20 h using a freeze dryer (model FD-558; Heto, Denmark).

Solubilization of Microcapsules

Encapsulated *L. casei* YIT 9018 was released from the microcapsule to determine its survival rate. Ten ml of encapsulated cells were added to 200 ml of 2% sodium citrate (pH 6.0) or phosphate buffer solutions (concentrations 0.001–0.1 M, pH 4.8–8.5) followed by stirring for 30–60 min. Subsequent serial dilutions were enumerated on MRS agar by the pour-plate method. All plates were incubated at 37°C for 48 h. Survival rate was calculated as follows:

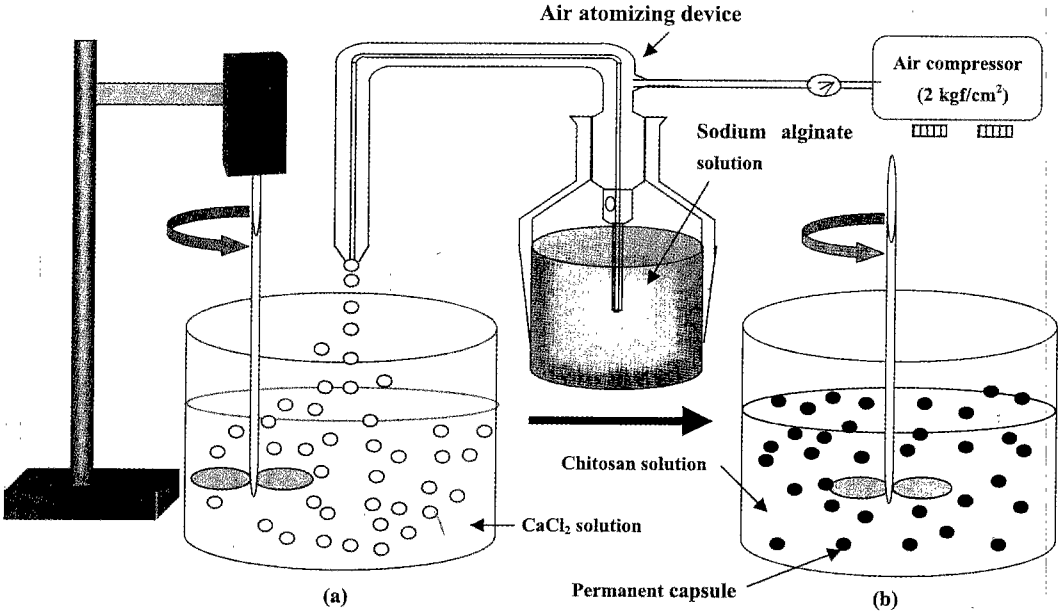

Fig. 1. Schematic diagram of the preparation of alginate/chitosan microcapsules.
(a) Spray drying of sodium alginate solution into calcium chloride solution to form temporary gel microcapsules (alginate microcapsule); (b) formed permanent capsules (alginate/chitosan microcapsule) in chitosan solution.

Survival rate = (B/A)×100

A: colony count of lactobacilli before encapsulation
B: colony count of lactobacilli after encapsulation

Morphology Observation of Microcapsules

The image analyzer (model Diaphot 300; Nikon, Japan) was used to investigate the cell distribution in alginate microcapsule. Encapsulated cells were placed on a slide glass and examined with a 20× and 60× objective lens. Microstructural properties of freeze-dried encapsulated cells were investigated by scanning electron microscopy (model JSM-5410L; Jeol Co., Japan). Encapsulated cells were frozen at −70°C and freeze-dried at 10°C for 20 h using a freeze dryer. Specimens were loaded onto a specimen stub with two-sided adhesive tape. Specimens were coated with pt-pb in an ion sputter (model ε-1030; Hitachi, Japan). Examinations were made at 1,300×, 1,500×, and 3,000× magnifications.

Assay for Tolerance

To determine the tolerance of nonencapsulated and encapsulated cells, various modified MRS agar plates were prepared. For acid tolerance, modified MRS agar was adjusted to four pH levels, 1.0, 1.5, 2.0, and 3.5, with 1.0 N HCl. For the bile tolerance, modified MRS agar was supplemented with 0.6% and 0.8% oxbile, respectively. In the pepsin tolerance test, 0.1 g of pepsin was dissolved in 10 ml of citric acid, then added in the MRS agar, and the pH was adjusted to 2.2 with 1 N NaOH. For the hydrogen peroxide tolerance test, modified MRS agar plates containing 10,000, 30,000, 50,000 ppm hydrogen peroxide, respectively, were prepared. Nonencapsulated and encapsulated cells were inoculated into each modified MRS agar. All plates were incubated at 37°C. At intervals of 0, 2, 4, and 6 h, changes of viability of nonencapsulated or encapsulated cells were assayed.

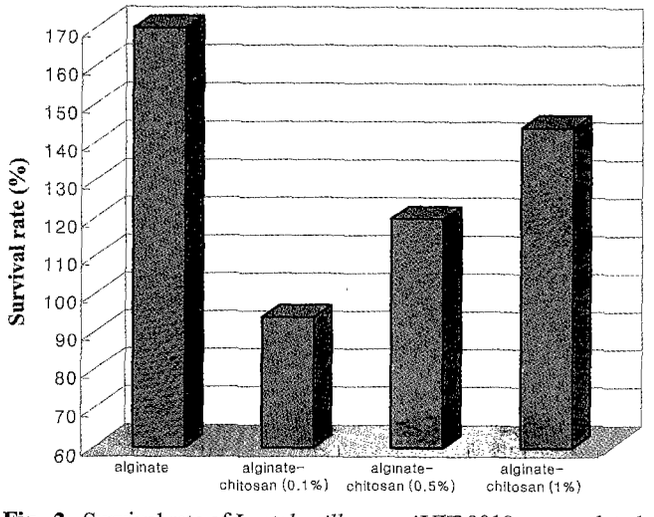

Fig. 2. Survival rate of *Lactobacillus casei* YIT 9018 encapsulated in alginate microcapsule and alginate/chitosan microcapsule.

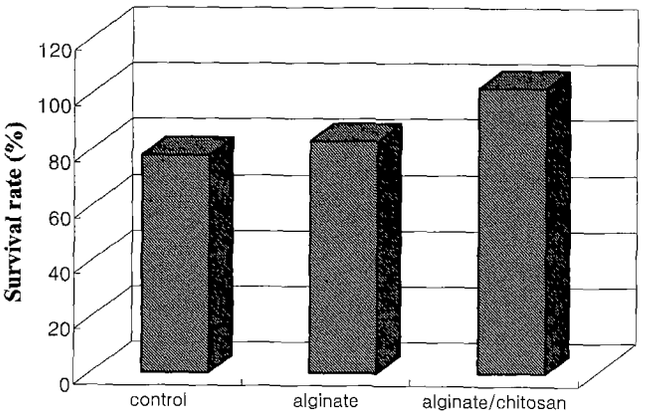

Fig. 3. Survival rate of *Lactobacillus casei* YIT 9018 encapsulated in microcapsules after freeze drying.
Control: nonencapsulated cell; alginate: alginate-encapsulated cell; alg/chitosan: alginate/chitosan-encapsulated cell.

Storage Stability

Before freezing, each of the nonencapsulated and encapsulated cells were mixed with 10% glycerol or 10% milk fat as a cryoprotectant, and then freeze-dried for 20 h. Freeze-dried nonencapsulated cell and encapsulated cell were then

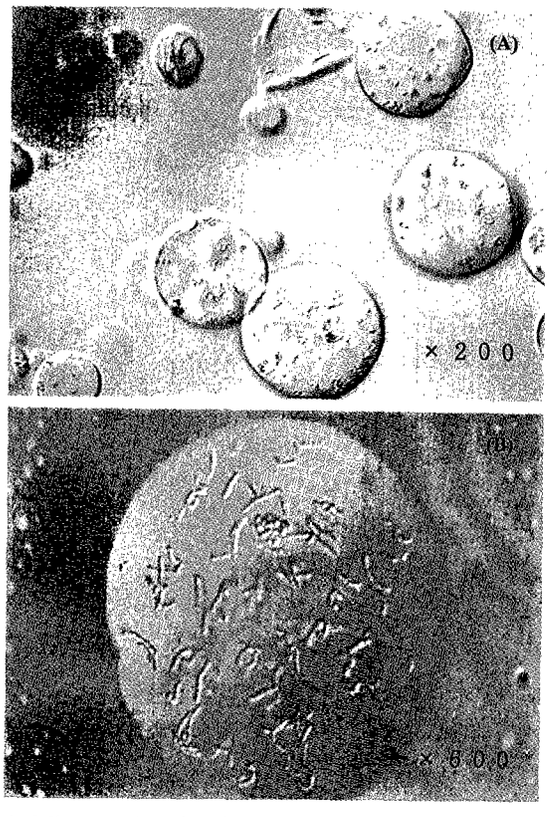

Fig. 4. Micrographs of alginate microcapsules observed through an image analyzer.
(A) Lower magnification image at 1,300×, (B) higher magnification image at 1,500×.

stored at refrigeration temperature (4°C) and room temperature (22°C). Survival of nonencapsulated cell or microencapsulated cell was monitored during 28 days at intervals of 7 days.

Survival rate=(B/A)×100

A: colony count of lactobacilli before freeze drying
B: colony count of lactobacilli after freeze drying

RESULTS AND DISCUSSION

Survival During Encapsulation

Survival rates of alginate-encapsulated cell and alginate/chitosan-encapsulated cell were evaluated (Fig. 2). The colony count was greatly increased after encapsulation. It may be due to the preservatives of which ascorbic acid and lactose can be used as nutrients for the growth of cells. The survival rate of microorganisms greatly increased at higher concentrations of the chitosan solution. The survival rate of alginate-encapsulated cell was increased more then the alginate/chitosan-encapsulated cell. The lower survival rate of alginate/chitosan-encapsulated cell might have been due to the reagent used to dissolve chitosan and the damage of the cells during the breakdown of microcapsule. Because the alginate/chitosan capsule was not readily dissolved in sodium citrate solution, vigorous stirring was required. However, after freeze drying, the number of alginate-encapsulated cell was significantly decreased (Fig. 3), while alginate/chitosan-encapsulated cell showed a higher survival rate, indicating that the double membrane effectively protected the cells from cold damage during freezing and freeze drying.

Microcapsule Morphology

The alginate microcapsules showed a spherical shape and ranged from 30 to 80 μm in size under an image analyzer (Fig. 4a). Larger-sized microcapsules contained more cells. A higher magnification micrograph showed a spatial distribution of cells within the alginatemicrocapsules (Fig. 4b).

Scanning electron photomicrographs of alginate microcapsules and alginate/chitosan microcapsules after freeze drying are shown in Figs. 5 and 6. Dried alginate microcapsules are about 20–50 μm in size, roughly spherical in shape, with a wrinkled surface (Fig. 5A). Kwok *et al.* [9] suggested that this is probably due to the loss of water content during the freeze-drying process.

The surface of alginate/chitosan microcapsules was morphologically modified with the addition of chitosan,

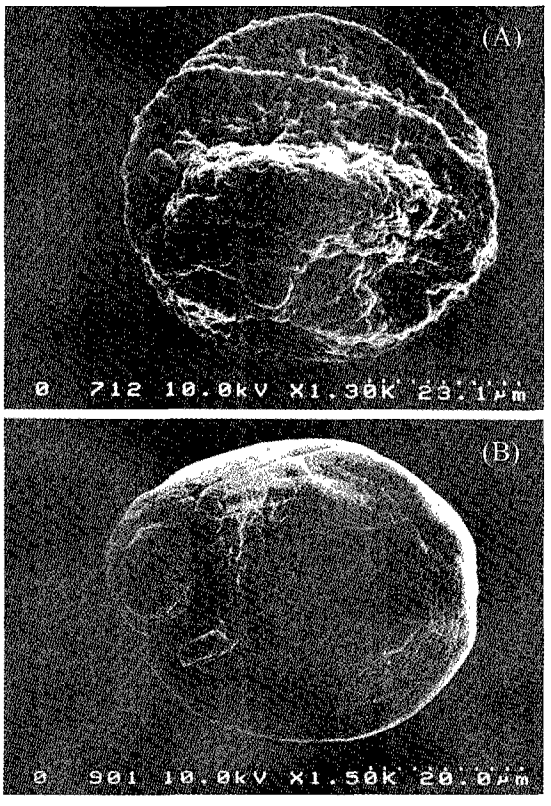

Fig. 5. Scanning electron micrographs of microcapsules after freeze drying.
(A) Alginate microcapsule at 3,000×, (B) alginate/chitosan microcapsule at 3,000× magnification.

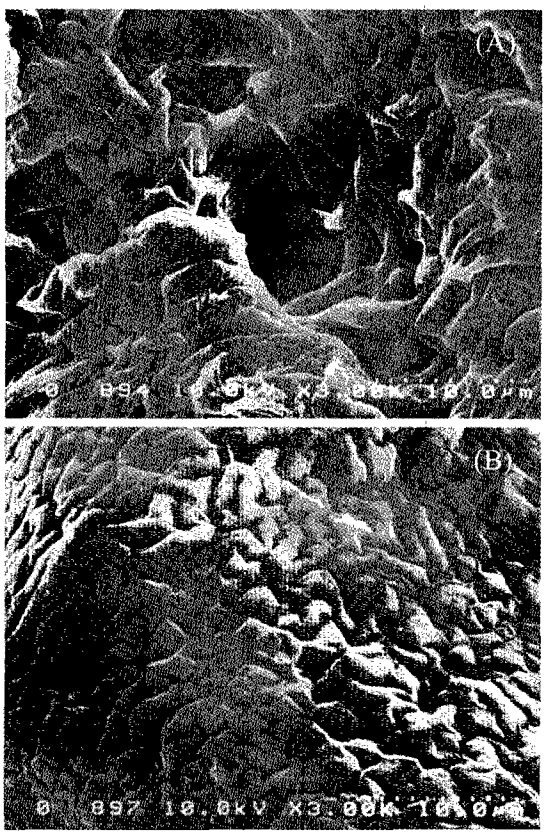

Fig. 6. Scanning electron micrographs of the surface of microcapsules.
(A) Alginate microcapsule, (B) alginate/chitosan microcapsule.

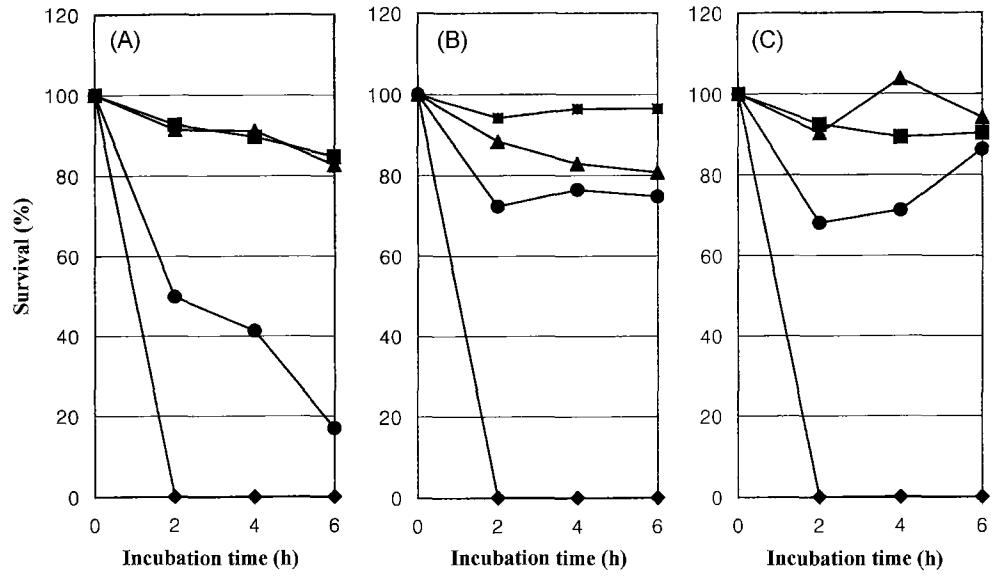

Fig. 7. Acid tolerance of *Lactobacillus casei* YIT 9018 at pH 1.0 (◆), pH 1.5 (●), pH 2.0 (▲), pH 3.0 (■).
(A) Nonencapsulated cell, (B) alginate-encapsulated cell, (C) alginate/chitosan-encapsulated cell.

even though the overall shape and size were not changed (Fig. 5B). The surface of the alginate/chitosan microcapsule seemed much smoother than that of the alginate microcapsule. The results are in agreement with that of Hari *et al.* [4]. They indicated that the presence of chitosan modified the surface as well as the internal structure of the alginate microcapsule.

As seen with higher magnification (Fig. 6), the surface of the alginatemicrocapsule appeared to have micropores that were not observed on alginate/chitosan microcapsules.

Stability of Encapsulated *L. casei* YIT 9018

Following ingestion, the microorganisms must survive a transit through a gastric environment and reach the colon in quantities large enough to facilitate colonization [15]. Cellular stress begins in the stomach, which has pH as low as 1.5. To determine the effect of the acidic pH of the stomach on the survival of *L. casei* YIT 9018, an *in vitro* system with a decreasing pH was utilized (Fig. 7). When both nonencapsulated cells and encapsulated cells were exposed

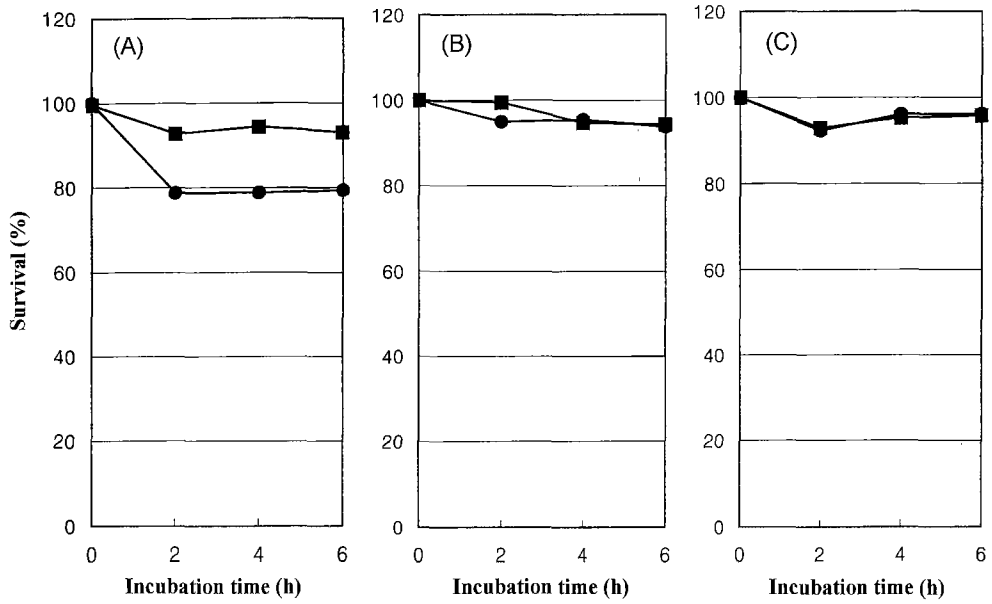

Fig. 8. Bile tolerance of *Lactobacillus casei* YIT 9018 at 0.6% (■), 0.8% (●) oxbile.
(A) Nonencapsulated cell, (B) alginate-encapsulated cell, (C) alginate/chitosan-encapsulated cell.

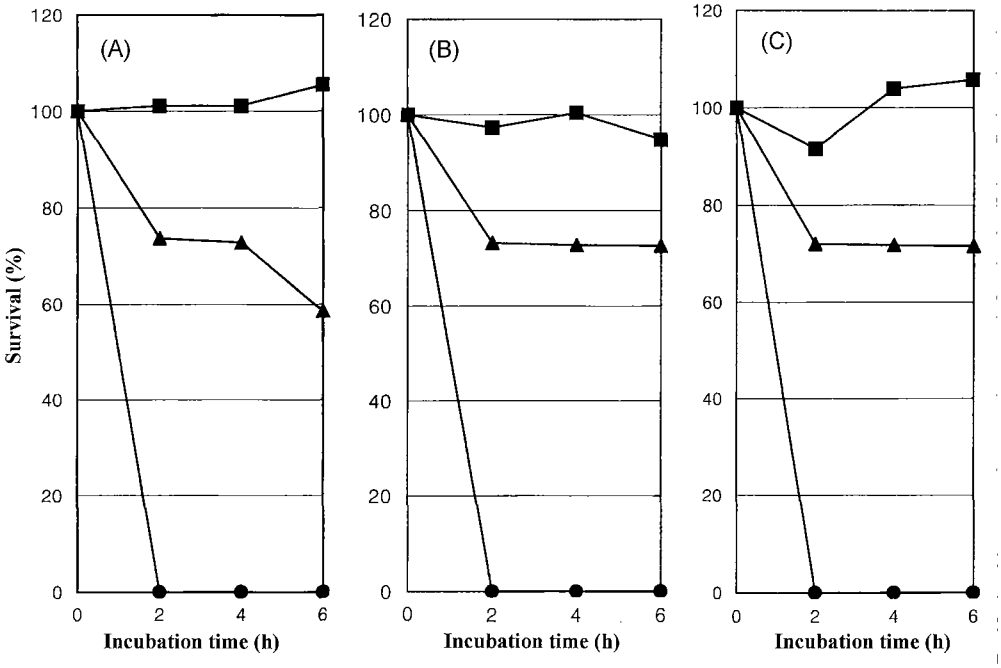

Fig. 9. Tolerance of *Lactobacillus casei* YIT 9018 at 1% (■), 3% (▲), 5% (●) hydrogen peroxide.
(A) Nonencapsulated cell, (B) alginate-encapsulated cell, (C) alginate/chitosan-encapsulated cell.

to pH 1.0, none of the microorganisms survived within 2 h, indicating that there was no protecting effect of microcapsule at pH 1.0. It seemed likely that the HCl solution used to lower the pH had entered into the microcapsule through the surface pinholes [15]. However, the survival of encapsulated cells was greatly increased above pH 1.5. At pH 1.5, 70–80% reduction in the nonencapsulated cell was observed, while encapsulated cells showed 20–30% reduction. However, *L. casei* YIT 9018 itself was shown to have acid tolerance [19], which could explain why there were no significant differences between nonencapsulated cells and encapsulated cells at pHs 2 and 3. These results are in good agreement with the study by Sultana *et al.* [20]. They also indicated that encapsulation of bacteria did not effectively protect from strongly acidic conditions.

After the microorganisms pass through the stomach, they enter the upper intestinal tract where bile is secreted into the stomach [3]. The concentration of bile in the human gastrointestinal system is variable, but usually around 0.6%. Sensitivity of nonencapsulated cells and encapsulated cells to bile was examined on MRS agar containing increasing concentration of bile (Fig. 8). Both alginate-encapsulated cells and alginate/chitosan-encapsulated cells were relatively resistant to bile, observed by maintenance of the population level through the incubation period. Nonencapsulated cells were more sensitive to bile, and significant reduction was observed at 0.8% oxbile concentration. Klaenhammer and Kleeman [8] also described that as the bile concentration increased from 0.6 to 1.0%, a significant reduction in colony-forming ability was observed.

Anaerobes that lack a respiratory system cannot use oxygen as a terminal electron acceptor, and are unable to detoxify some of the products of oxygen metabolism. They are known to be damaged or killed by oxygen when oxygen is reduced to several toxic products, such as hydrogen peroxide (H_2O_2), superoxide (O_2^-), and hydroxyl radical (OH^-). Aerobes have enzymes that decompose these products,

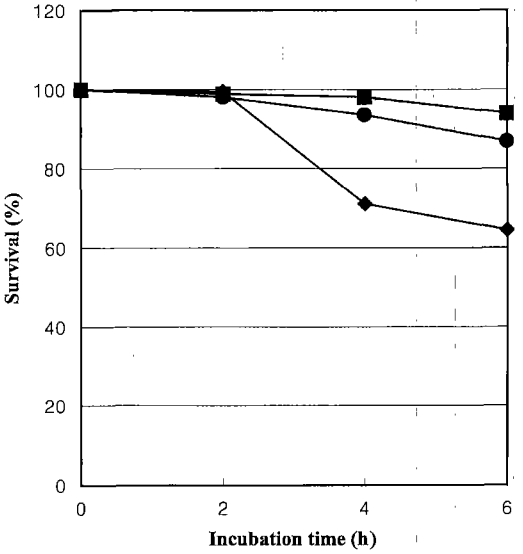

Fig. 10. Tolerance of *Lactobacillus casei* YIT 9018 at 1% pepsin (pH 2.2).
(A) Nonencapsulated cell, (B) alginate-encapsulated cell, (C) alginate/chitosan-encapsulated cell.

Table 1. Viability during storage of nonencapsulated cells and microenapsulated cells.

Days	Viability (CFU/ml)					
	4°C			22°C		
	Nonencapsulated cell	Alginate-encapsulated cell	Alginate/chitosan encapsulated cell	Nonencapsulated cell	Alginate-encapsulated cell	Alginate/chitosan encapsulated cell
0	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}	1.9×10^{10}
7	3.0×10^{10}	1.2×10^{10}	1.8×10^{10}	9.1×10^{8}	1.0×10^{10}	1.2×10^{10}
14	5.6×10^{10}	1.6×10^{10}	1.7×10^{10}	7.7×10^{7}	5.5×10^{9}	7.8×10^{9}
21	1.4×10^{10}	1.4×10^{10}	1.5×10^{10}	6.3×10^{6}	3.2×10^{8}	7.1×10^{9}

whereas anaerobes seem to lack all or some of these enzymes [2]. In this study, the sensitivity of *L. casei* YIT 9018 to hydrogen peroxide was investigated to monitor the effects of encapsulation on survival of cells in aerobic condition. At 1% concentration of hydrogen peroxide, both nonencapsulated and encapsulated cells were stable, while none of the cells survived at 5% concentration (Fig. 9). Therefore, microencapsulation did not give any protection at 1% and 5% hydrogen peroxide concentrations. At 3% concentration, viable counts of the nonencapsulated cell decreased by 10% after 4 h.

Pepsin is an enzyme present in gastric juice and hydrolyzes proteins. Lactic acid bacteria should be resistant to such enzymes to colonize in the lower intestine. Pepsin tolerance of encapsulated cells was maintained through the incubation time. However, the viability of nonencapsulated cells was significantly decreased by 30% after 2 h (Fig. 10).

Storage Stability

The stability of nonencapsulated cells and encapsulated cells during storage at two different temperatures, 4°C and 22°C, is shown in Table 1. The viable counts of nonencapsulated cells and encapsulated cells remained stable through the storage periods at 4°C. At 22°C, the viability of nonencapsulated cells decreased to 2 log-cycle after 7 days and continued to decline until the end of storage as 4 log-cycle. On the other hand, the viable counts in encapsulated cells was maintained upto 7 days of storage. However, the viability of alginate-encapsulated cells and alginate/chitosan-encapsulated cells decreased slightly after 14 days of storage, and reached to 3.2×10^{8} cfu/g and 7.1×10^{9} cfu/g, respectively. The higher survival rate of the encapsulated cells resulted from the protection by the alginate/chitosan-encapsulation. These results are in agreement with the studies by Kim *et al.* [7] who used CMC and sodium alginate as coating materials.

With the above results, it could be concluded that the protective effects of alginate-encapsulation and alginate/chitosan-encapsulation under harsh conditions were similar, but the alginate/chitosan-encapsulated cells showed a higher survival rate after freeze drying, as well as storage stability.

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