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Optimal Thermotolerance of *Bifidobacterium bifidum* in Gellan–Alginate Microparticles

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ABSTRACT: The purpose of this research was to encapsulate *Bifidobacterium bifidum* using gellan, sodium alginate and prebiotics as coating materials, and to maximize the thermotolerance of the probiotics with an optimal combination of the coating materials. The optimal ratio of the coating materials for the microparticles under heat treatments (75°C, 1 min) was obtained by using the response surface method and the sequential quadratic programming technique. Optimization results indicated that 2% sodium alginate mixed with 1% gellan gum as coating materials would produce the highest thermotolerance in terms of *B. bifidum* count. The verification experiment yielded a result close to the predicted values, with no significant difference ($P > 0.05$). The results of heat treatments also demonstrated that the addition of gellan gum in the walls of probiotic microcapsules provided improved protection for *B. bifidum*. These probiotic counts remained at 10^5–10^6 CFU/g for the microcapsules stored for 2 months, then treated in heat and in simulated gastric fluid.

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KEYWORDS: gellan; alginate; microencapsulation; *B. bifidum*; sequential quadratic programming (SQP); response surface methodology (RSM)

Introduction

Good probiotic viability and activity are considered essential for probiotics with maximum functionality (Champagne and Gardner, 2005; Mattila-Sandholm et al., 2002). A suggested minimum level for probiotics in food products is 10^7 CFU/mL (Kurman and Rasic 1991; Robinson 1987) at the time of consumption to benefit the consumer. A major challenge associated with the application of probiotic cultures in functional foods is the retention of viability during processing. Moreover, since viable and biologically active micro-organisms are usually required at the target site in the host, it is essential that probiotics withstand the host's natural barriers such as gastric transit.

Many previous studies have shown low viability of bifidobacteria during processing (Saarela et al., 2005), storage and intestinal transit (Sanders and Huis in't Veld, 1999), due to heat treatment for dehydration probiotics/products, acidity (Klaver et al., 1993; Martin and Chou, 1992; Samona and Robinson, 1994), the presence of hydrogen peroxide (Lankaputhra and Shah, 1996) and the oxygen content (Dave and Shah, 1997). A number of approaches for improving viability of probiotics, including selection of thermal tolerance/acid-resistant strains, control of over-acidification of products, and the addition of cysteine or an oxygen scavenger such as ascorbic acid (Dave and Shah, 1997), have been proposed in various products, but only to a limited extent (Adhikari et al., 2000; Dave and Shah, 1998; Krasaekoopt et al., 2003).

Encapsulation has been investigated for providing protection of micro-organisms in both food products and the intestinal tract (Champagne et al., 1992; Lacroix et al., 1990; Prevost and Divies, 1988). The selection of different types of coating materials usually depends on the functional properties of the probiotics and coating process used (Hegenbart, 1993). For food applications, probiotic encapsulation in food-grade porous matrices has been most widely used (Champagne et al., 1994). Calcium alginate and gellan gum are favored above all other supporting materials for encapsulating probiotics due to its simplicity, non-toxicity, biocompatibility, and low cost (Krasaekoopt et al., 2003; Ogawa et al., 2002; Sheu and

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Marshall, 1993). Solubilization of alginate and gellan gels by sequestering calcium ions and releasing entrapped cells within the human intestines is another advantage. Furthermore, both ionically linked gels are thermostable over the range of 0–100°C. Alginate is a linear heteropolysaccharide of D-mannuronic and L-guluronic acids extracted from various species of algae. Chen et al. (2006) studied prebiotics incorporated with alginate as coating materials for probiotic microencapsulation and demonstrated that the addition of fructooligosaccharides (FOS), isomaltooligosaccharides (IMO) and peptides in the walls of probiotic microcapsules provided improved protection for the active organisms. Gellan gum, an anionic polysaccharide derived from *Pseudomonas elodea*, is widely used in the food and biotechnological industries because it forms a transparent and heat- and acid-resistant gel (Omoto et al., 1999). Sun and Griffiths (2000) encapsulated *Bifidobacterium* spp. with gellan–xanthan gum as the coating material and reported that gellan–xanthan beads were highly acid-stable.

Although various effects of encapsulation on the survival of bacteria under gastrointestinal conditions have been reported and most studies have proven the advantages of encapsulating probiotics over free cells under in vitro gastric conditions, effects of encapsulation on the thermotolerance of bifidobacteria were few. Since alginate and gellan gum are acid-resistant and heat-stable, combination of both gels as coating materials for probiotic capsules may extend the usage of these microorganisms under food processing or as a new functional additive applying in hot beverages such as hot tea and coffee. Thus, the purpose of this research was to develop new *B. bifidum* microparticles using gellan, alginate and prebiotics as the coating materials with the help of a modern optimization technique, and to study the effects of heat treatment and simulated gastric conditions on the survivability and stability of this microencapsulated *B. bifidum*.

Materials and Methods

Experimental Design

The whole concept of this study included (1) experimental design using Box Behnkin Design (BBD), (2) microencapsulation of probiotics according to experimental design, (3) building response surface models and formulation of optimization model, (4) optimization using sequential quadratic program, (5) verification experiments, and (6) storage test.

Experimental Design using BBD

Experimental design preceded commencement of the trials. The Box Behnkin Design (BBD; Box and Behnkin, 1960) is a three-level design based on construction of a balanced incomplete block design. It was assumed that the viability of encapsulated *B. bifidum* is affected by the type and concentration of the coating materials, in this case sodium alginate, gellan, peptide, and FOS (four independent variables). A four-variable BBD with six replicates at the center point was selected to build the response surface models (Table I).

Microencapsulation of B. bifidum According to Experimental Design

Culture conditions. A pure lyophilized culture of *Bifidobacterium bifidum* (CCRC 11844) was purchased from the Culture Collection and Research Center (Hsinchu, Taiwan, ROC). Lithium propionate deMan, Rogosa and Sharp agar (LP-MRS) were used as the media for *Bifidobacterium* spp (Lapierre et al., 1992).

B. bifidum was transferred twice in MRS broth containing 0.05% L-cysteine hydrochloride (Sigma, St. Louis, MO) in an anaerobic incubator and maintained at 40°C. Cultures were harvested after 24 h by centrifugation (3,000*g*, 10 min at 4°C), washed and re-suspended twice in saline solution. The final bacterial counts were adjusted to 10^9 cells/mL.

Probiotic microencapsulation. Probiotic microcapsules were prepared according to the Box Behnkin design shown in Table I (30 combinations of coating materials) by mixing 1% (v/v) of *B. bifidum* with sodium alginate (1–3%, Sigma), gellan gum (1–2%, Sigma) and the previously autoclaved (121°C, 15 min) FOS (0–3%; Cheng-Fung Co., Taipei, Taiwan) and peptides (0–1%; pancreatic digested casein, Cheng-Fung Co.). The extrusion technique of microencapsulation was used (Krasaekoopt et al., 2004). After washing, 1% (v/v) of culture concentrate was mixed with 50 mL of coating material solution, sterilized at 121°C for 15 min. The cell suspension was injected through a 0.11 needle into sterile 0.1 M $CaCl_2$. The beads approximately 0.5 mm in diameter were allowed to stand for 1 h for solidification, and then rinsed with, and subsequently kept in, sterile 0.1% peptone solution at 4°C. Survival of the microencapsulated cells before and after heat treatment (defined as responses) was determined. The two responses were defined as viability of *B. bifidum* before and after heat treatment (75°C for 1 min).

Modeling and Optimization of Coating Materials in Probiotic Microcapsules

To carry out the response surface modeling, regression was performed on the experimental results to construct mathematical models (Table I). The models were then formulated as an objective function in an optimization problem which was subsequently solved by using a sequential quadratic programming (SQP) approach to derive the optimal formulation for the *B. bifidum* microcapsules. Both the response surface modeling and SQP were

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Table I. Factors and responses of the experiment.

Run	Gellan gum (%)	Sodium alginate (%)	Peptides (%)	FOS (%)	Encapsulated B[a] log CFU/g	Heated B[a] log CFU/g
1	1.00	0.50	0.50	1.50	6.56	5.25
2	0.75	1.25	1.00	3.00	6.99	6.46
3	0.75	1.25	0.00	3.00	6.32	6.11
4	0.75	1.25	0.50	1.50	7.18	6.49
5	0.75	1.25	0.00	0.00	5.65	4.13
6	0.50	2.00	0.50	1.50	6.90	6.25
7	0.75	1.25	0.50	1.50	7.03	6.51
8	0.50	0.50	0.50	1.50	7.03	6.44
9	0.75	1.25	1.00	0.00	6.82	6.33
10	1.00	2.00	0.50	1.50	7.00	6.70
11	0.50	1.25	0.50	3.00	7.39	6.71
12	0.50	1.25	0.50	0.00	7.32	6.70
13	1.00	1.25	0.50	3.00	7.16	6.82
14	0.75	0.50	1.00	1.50	7.20	6.33
15	1.00	1.25	0.50	0.00	6.84	6.47
16	0.75	2.00	1.00	1.50	7.57	7.03
17	0.75	2.00	0.00	1.50	7.13	6.66
18	0.75	1.25	0.50	1.50	7.49	6.88
19	0.75	1.25	0.50	1.50	7.31	6.86
20	0.75	0.50	0.00	1.50	6.23	5.91
21	0.50	1.25	1.00	1.50	7.79	6.68
22	1.00	1.25	0.00	1.50	6.38	5.58
23	0.75	1.25	0.50	1.50	7.42	6.67
24	1.00	1.25	1.00	1.50	7.76	6.67
25	0.75	1.25	0.50	1.50	7.35	6.61
26	0.50	1.25	0.00	1.50	6.76	5.92
27	0.75	0.50	0.50	3.00	7.16	6.02
28	0.75	2.00	0.50	0.00	7.53	6.74
29	0.75	2.00	0.50	3.00	7.80	6.61
30	0.75	0.50	0.50	0.00	6.76	6.13

[a]B, *B. bifidum*.

employed in a similar way to the work by Chen et al. (2005, 2006).

Model Verification

After optimal formulation was found by the SQP, experiments based on the formulation were performed and repeated three times. The results were then analyzed using ANOVA from the SAS software package (SAS Institute, Inc., Cary, NC, 1990), with Duncan's multiple range test for significance to detect differences between predicted values and observed values.

Storage Test

In order to understand the survival of microencapsulated *B. bifidum* in heat treatment, simulated gastric fluid test (SGFT) and bile-salt conditions after storage, two kinds of microcapsules (one with the optimal formulation and the other with 3% alginate as the coating materials) were immersed in aseptic water and stored at 4°C for 2 months. The survival of the encapsulated *B. bifidum* in heat treatment, simulated gastric fluid test (SGFT) and bile salt conditions were determined.

Analysis Methods

Survival of Encapsulated B. bifidum in Heat Treatment, SGFT and Bile-Salt Conditions

Survival of encapsulated *B. bifidum* in heat treatment was determined by heating 1 g of the microencapsulated bacteria at 75°C in water bath for 1 min, and then the cell count was measured. Resistance to simulated gastric fluid was determined by adding 1 g of the heated microcapsules into flasks containing 10 mL of the simulated gastric juice, which consisted of 0.3% pepsin (Sigma) and 0.5% sodium chloride (Nakalai, Kyoto, Japan) adjusted to pH 2.0 with 1 N HCl. Resistance to bile salts was determined by adding microcapsules to the bile-salt solution, which consisted of 2% ox gall powder (Sigma). Both resistance treatments took place in agitated flasks (100 rpm) at 25°C for 1 h, and after each treatment the *B. bifidum* viability count was analyzed separately.

Determination of the B. bifidum Viability

To determine the *B. bifidum* viability count, the entrapped *B. bifidum* were released from the microcapsules according

to the method of Sheu and Marshall (1993). One gram of the microcapsules was re-suspended in 9 mL of phosphate buffer (0.1 M, pH 7.0) followed by homogenization in a stomacher (Seward Stomacher 400C, Brinkmann, Westbury, NY) for 15 min. The suitability of the media was tested by plating decimal dilutions of the probiotic cultures. Thus, a 1-g sample was decimally diluted into sterile peptone water (0.1%), and then 0.1-mL aliquot dilutions were plated onto the different media, in triplicate. Plates of LP-MRS agar (GasPak System; Oxoid Unipath Ltd, Basingstoke, Hampshire, England) were incubated anaerobically (72 h at 37°C) before enumeration of the bifidobacteria. The population, in colony-forming units (CFU), and the characteristics of the colonies were recorded for each medium.

Scanning Electron Microscopy

The microstructures of the microcapsules were observed by scanning electron microscope (SEM) according to the method of Lin et al. (1999). Samples were fixed in 30 g/L glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) at 25°C for 4 h. Then, the samples were washed in three changes of buffer and post-fixed with 10 g/L osmium tetroxide in the same buffer at 25°C for 1 h. After washing in distilled water, the samples were dehydrated in an ethanol series: 15, 30, 50, and 70% for 10 min each; 85 and 95% for 15 min each; and, 100% for 1 h. The resulting specimens were critical-point dried (CO_2 Critical Point Dryer Samdri-PVT-3B; Tousimis, Rockville, MD). Eventually, the samples were fixed in stubs on a double-faced metallic tape and covered with a fine layer of gold (Ion Coater JJFC1100E; JEOL Ltd, Tokyo, Japan) while applying a current of 40 mA, and observed using an SEM (JSM-6300, JEOL Ltd).

Results and Discussion

Response Surface Modeling

The results presented in Table I for the viability of microencapsulated probiotics before and after heat treatment were obtained immediately after extrusion. Response surface methodology (RSM) was used in the present work to develop a prediction model for establishing the optimal concentrations of four coating materials for the probiotic microcapsules. The responses, as linear, quadratic and cubic functions of the variables, were tested for adequacy and fitness using analysis of variance (ANOVA). Model analysis (Table II) and the Lack-of-Fit Test were used for selection of adequacy models, as outlined by Lee et al. (2000), Weng et al. (2001), and Chen et al. (2005). Table II compares the validities of the linear, quadratic and cubic models for the four responses according to their *F*-values. A model with *P*-values ($P > F$) below 0.05 was regarded as significant. The highest-order significant polynomial was selected. The Lack-of-Fit Test was used to compare the residual and pure errors at replicated design points. The response predictor was discarded where lack of fit was significant, as indicated by a low probability value ($P > F$). The model with no significant lack of fit was selected. Using ANOVA (Table II), it was demonstrated that one quadratic survival models for *B. bifidum* before heat treatment and one cubic model for the survival of *B. bifidum* after heat treatment appeared to be the most accurate with no significant lack of fit. The two models are given as follows:

Table II. (a) Model analysis (b) Lack of fit tests (c) *R*-square analysis of the viability of microencapsulated *B. bifidum* before and after heat treatment.

	Before		After	
Source	Sum of squares	$P > F$	Sum of squares	$P > F$
(a) Model analysis[a]				
Mean	1,495.64		1,211.67	
Linear	3.90	<0.0001**	4.05	0.0023**
Quadratic	1.14	0.0232*	2.78	0.0385*
Cubic	0.34	0.2250	1.19	0.0059**
Residual	0.10		0.057	
Total	1,502.65		1,221.35	

	Before		After	
Source	Sum of squares	$P > F$	Sum of squares	$P > F$
(b) Lack of fit tests[b]				
Linear	1.55	0.0568	4.03	0.0004**
Quadratic	0.42	0.1309	1.25	0.0008**
Cubic	0.075	0.1496	0.055	0.0094**
Pure error	0.030		2.561×10^{-3}	

	Before		After	
Source	*R*-square	Press	*R*-square	Press
(c) *R*-square analysis[c]				
Linear	0.7113	2.68	0.5012	7.09
Quadratic	0.9187	3.06	0.8450	9.92
Cubic	0.9809	54.33	0.9929	39.61

[b]*Significant at 5% level.
[c]**Significant at 1% level.
[a]Model analysis—select the highest order polynomial where the additional terms are significant.
[b]Lack of fit tests—want the selected model to have insignificant lack-of-fit.
[c]*R*-square analysis—focus on the model minimizing the "Press."

$$f_1 = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j \qquad (1)$$

$$f_2 = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i^2 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ij} X_i X_j + \sum_{i=1}^{n} \beta_{iii} X_i^3 + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{iij} X_i^2 X_j + \sum_{i=1}^{n-1} \sum_{j=i+1}^{n} \beta_{ijj} X_i X_j^2 + \sum_{i=1}^{n-2} \sum_{j=i+1}^{n-1} \sum_{k=j+1}^{n} \beta_{ijk} X_i X_j X_k \qquad (2)$$

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where n is the number of independent variables (i.e., $n = 4$); f_1 and f_2 are the survival of *B. bifidum* before and after heat treatment, respectively; βs are regression coefficients; and Xs are the uncoded independent variables. The regression coefficients for the statistically significant models are presented in Table III. The three-level BBD design is incapable of forming the pure cubic terms, that is, $\beta_{iii} X_i^3$ in Eq. (2), and the coefficients presented in Table III confirms this fact. The two responses are then combined into one composite function (CF) whose maximum can then be sought by optimization techniques.

$$\mathrm{CF} = \frac{f_1 + f_2}{2} \tag{3}$$

Optimizing Combinations of Coating Materials

Since the composite function (Eq. (3)) is a summation of one quadratic function and one cubic analog, it is very likely that multiple local maxima exist. Therefore, a global optimization program consisting of a multi-start SQP was coded to search for the global optimum. The program generates a series of uniformly distributed random points for initial search, and then SQP is applied to find the optimum based on each initial point. If the probability exceeds a preset value (99.99% in this study), the global optimum is considered found. Otherwise, the next random initial point is generated and the SQP re-executed. After 12 sets of randomly generated initial points leading to optimal CF values (local optima) ranging from 7.21 to 7.38, the global optimal CF was found to be 7.38 (99.99% certainty) (Fig. 1). The global optimal CF values corresponded to: 7.42 log CFU for survival of *B. bifidum* before heat treatment and 7.35 log CFU for survival of *B. bifidum* after heat treatment. The highest optimal CF value (7.38) was attained for 9 of 12 sets, with the optimal points, $X_1 = 1.00$ (gellan%), $X_2 = 2.00$ (alginate%), $X_3 = 0.86$ (peptides%) and $X_4 = 0.20$ (FOS%).

To further depict the global optimization results, 3-D response surface plots were generated by fixing two of the four variables. Figure 2a shows three local maxima, including the global analog, in a CF response function produced for the values $X_3 = 0.86$ (peptide%) and $X_4 = 0.20$ (FOS%), while varying X_1 (gellan) and X_2 (alginate) within

Table III. Validation of the optimal composition model recommended by SQP for the *B. bifidum* microcapsules before and after heat treatment.

	B. bifidum (log CFU/g)	
Heat treatment	Pred.[a]	Exp.[b]
Before	7.42	7.49
After	7.35	7.08

[a]Pred., predicted value.
[b]Exp., experimental value.

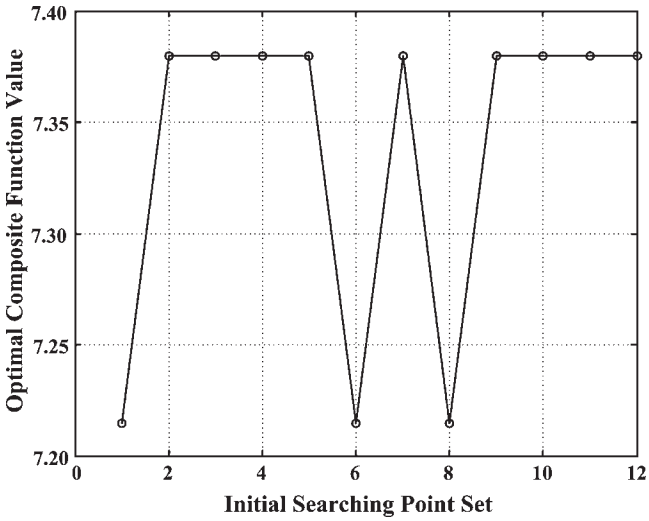

Figure 1. Using SQP to search optimal formulation for microcapsules.

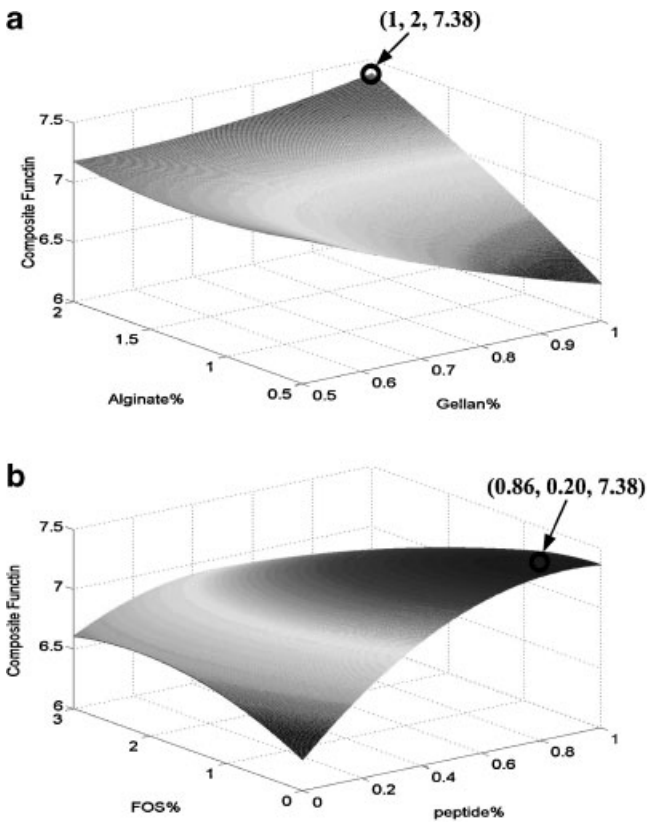

Figure 2. Response surface plots of survivability of *B. bifidum* in microcapsules after heat treatment showing (**a**) the effects of sodium alginate and gellan gum under the conditions of constant levels of 0.86% peptides and 0.20% FOS; and (**b**) the effects of peptides and FOS under the conditions of constant levels of 2% sodium alginate and 1% gellan gum.

their boundaries. The optimization results clearly show that determination of the global optima depends on the initial search points for the response surface models. The optimal combination of coating materials for probiotic microcapsules was 1% gellan gum blended with 2% alginate, 0.86% peptides, and 0.2% FOS.

The reported concentrations of alginate used for gel formation vary from 1.5 to 2.5% with 0.05–1.5 M $CaCl_2$ (Krasaekoopt et al., 2003). Several studies (Chen et al., 2006; Sun and Griffiths, 2000) also reported that the beads made of 0.5% gellan gum or sodium alginate alone were soft and their shapes were irregular. However, our preliminary tests indicated that combination of alginate, gellan and prebiotics as coating materials could improve the shape and strength of probiotic microcapsules when the concentrations of both gels were as low as 0.5%. Therefore, in this study, the concentrations of gellan and sodium alginate were tested within the range of 0.5–1.0% and 0.5–2.0%, respectively.

Our previous study (Chen et al., 2005) incorporated sodium alginate, peptide and FOS as coating materials for probiotic microencapsulation and found that a relatively low level of sodium alginate (1%) with prebiotics could provide a good protection under gastric condition. Conversely, in this research the viability of the encapsulated bacteria under heat treatment increased with increasing gel concentration (Fig. 2a). The optimum value found and subsequently used for preparation of optimum microcapsules was 1% gellan and 2% sodium alginate which are much higher than our previous results. This finding suggested that higher level of gel concentration could provide a shield for encapsulated probiotics under heat treatment.

In addition, the viability of the encapsulated bacteria under heat treatment increased mostly with increasing peptides concentration (Fig. 2b). A number of earlier studies have investigated the effects of peptides upon human gut bacteria (Lourens-Hattingh and Viljoen, 2001, Chen et al., 2003, 2004, 2005). Nitrogen sources, in the form of various peptides and amino acids, probably act by improving the viability of the bifidobacteria present in the gut (Lourens-Hattingh and Viljoen, 2001). The present study confirmed the prebiotic effects of peptides.

Experimental Verification

The optimal production conditions for wall composition were derived from SQP and verified by independent additional experiments. The optimal combination of coating materials for the probiotic microcapsules were 1% gellan gum blended with 2% alginate, 0.86% peptides, and 0.2% FOS. Table III shows that the experimental values were very close ($P > 0.05$) to the predicted values for survival of *B. bifidum* before and after heat treatment with no significant differences.

Survival of Microencapsulated *B. bifidum* in Heat Treatment, SGFT and Bile-Salt Conditions after Storage

Survival of Encapsulated B. bifidum in Heat Treatment after Storage

The effects of microencapsulation on viability of *B. bifidum* in heat treatment after storage are shown in Figure 3. The optimized *B. bifidum* microcapsules produced the highest viable cell counts (Fig. 3a) and survival rates (Fig. 3b) under heat treatment after storage among all samples. *B. bifidum* counts (Fig. 3a) for the optimized microcapsules remained at 10^6 CFU/g after 8-week storage, relative to only 10^3 CFU/g for free cell and no viable count for 3% alginate counterparts. The survival rate results (Fig. 3b) also showed that *B. bifidum* in the optimized microcapsules maintained over 90% survival rates after 8-week storage. Since gellan gum is heat-resistant in the presence of divalent cations (Omoto et al., 1999), addition of gellan gum in microcapsules could increase the gel strength and provide a good protection for probiotics under heat treatment. Although alginate gel is also heat stable, the heat treatment could change the gel structure resulting to a poor gel strength. Thus, the microcapsule matrix prepared by alginate gel alone could only give minimum protection on the encapsulated probiotics in heat treatment.

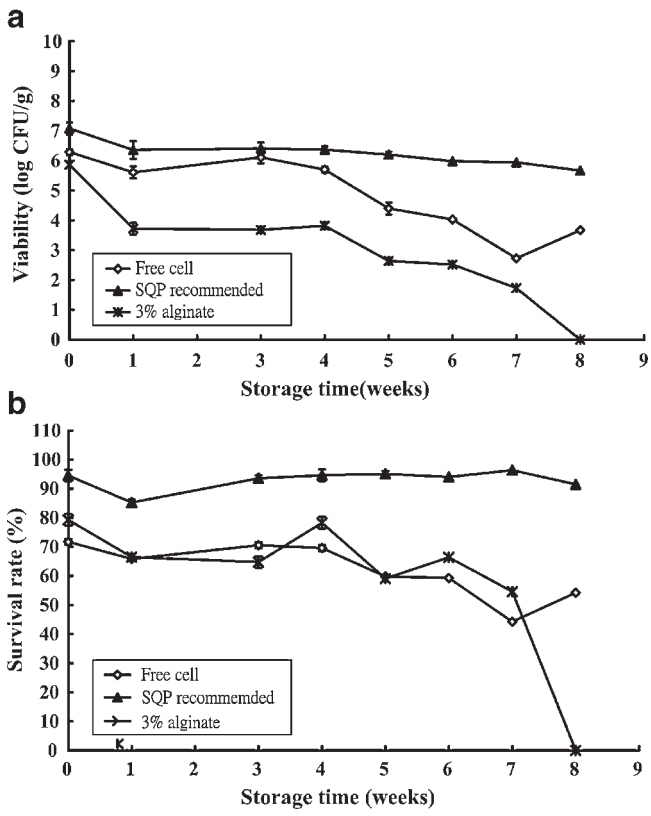

Figure 3. Survival of encapsulated *B. bifidum* in distilled water after 8-week storage and heat treatment. (**a**) Viability and (**b**) survival rate.

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Survival of Encapsulated B. bifidum in SGFT and Bile-Salt Conditions after Storage and Heat Treatment

Tables IV and V show the effects of coating materials on viability of *B. bifidum* under simulated gastric acid fluid and bile salt conditions after storage and heat treatment. The optimized microcapsules produced the highest viable cell counts for *B. bifidum* under the SGFT after storage and heat treatment among all samples (Table IV). *B. bifidum* counts for the optimized microcapsules remained at 10^2–10^3 CFU/g after 8-week storage, compared with no survival for both free cell and 3% alginate microcapsules. However, the bacterial counts and survival rates of *B. bifidum* were decreased with increasing storage time during 8-week storage. The survival of microorganisms is affected by low pH of the environment. Hook and Zottola (1988) reported that most of strains of bifidobacteria were sensitive to pH values below 4.6. Lee and Heo (2000) found that the death rate of the probiotics in the capsules decreased proportionally with an increase in the alginate concentration (1–3%) and initial cell numbers, when the encapsulated *B. longum* was exposed to simulated gastric and intestinal juices. Chen et al. (2005) stated that probiotic counts for the alginate microcapsules with prebiotics remained at 10^5–10^6 CFU/g in SFGT after 8 weeks of storage. Although many studies have reported the alginate beads could protect probiotics under simulated gastric and intestinal juices, heat treatment, as mentioned before, could change the structure of alginate and decrease the strength of alginate gel, causing a poor protection under SFGT for probiotics. Our results demonstrated that microencapsulation with gellan and prebiotics could provide a better protection for probiotics under gastric acid fluid test after heat treatment.

Certain studies (Chou and Weimer, 1999; Vinderola and Reinheimer 2003) showed that probiotics had higher tolerance to acid than to bile salts. In this sense, it is generally considered necessary to evaluate the ability of microencapsulated probiotic bacteria to resist the effect of bile salts. *B. bifidum* counts for the optimized microcapsules remained at 10^5–10^6 CFU/g after 8 weeks of storage, compared to no survival for both free cell and 3% alginate counterparts. This result is similar to that of the SGFT. The survival rates of *B. bifidum* in optimized microcapsules maintained 80–90% after heat treatment and then in the bile salt conditions (Table V).

Table IV. Survival of microencapsulated *B. bifidum* after heat treatment and simulated gastric fluid test.

	Viability* (log CFU/g)		
Sample	0 days	30 days	60 days
Free cell	5.56 ± 0.03^a	0.00 ± 0.00^a	0.00 ± 0.00^a
SQP recommended	6.05 ± 0.01^b	4.43 ± 0.09^c	2.91 ± 0.08^b
3% alginate	5.58 ± 0.03^a	3.45 ± 0.07^b	0.00 ± 0.00^a

*Mean ± SD, $n = 3$.
[a,b,c]Values in the same column with a different letter a,b,c were significantly different ($P < 0.05$).

Table V. Survival of microencapsulated *B. bifidum* after heat treatment and bile salt condition.

	Viability* (log CFU/g)		
Sample	0 days	30 days	60 days
Free cell	6.55 ± 0.02^a	3.58 ± 0.04^a	0.00 ± 0.00^a
SQP recommended	6.69 ± 0.02^b	6.37 ± 0.09^b	5.53 ± 0.04^b
3% alginate	6.51 ± 0.03^a	3.40 ± 0.07^a	0.00 ± 0.00^a

*Mean ± SD, $n = 3$.
[a,b]Values in the same column with different letter a,b were significantly different ($P < 0.05$).

Microstructure of Probiotic Microcapsules

Scanning electron microscopy was used to examine the structure of *B. bifidum* microcapsules. The microcapsules were in spherical shapes (Fig. 4a) with groups of entrapped bacteria evident in the internal voids, and surrounded by the matrix (Fig. 4b). Skjak-Brak et al. (1989) reported that alginate microparticles usually had a core due to the heterogeneous gelation mechanism.

The effects of heat treatment and SGFT on the outer structure of microcapsules observed by SEM are shown in Figure 5. Alginate microparticles (Fig. 5(b1)) were generally smooth with dense surface. Incorporation of 1% gellan gum did not cause any changes in the shape and size but did make the surface of the alginate beads rugged (Fig. 5(a1)). After heat treatment, the structure of alginate microparticles was significantly changed with a rough surface (Fig. 5(b2)). On the other hand, heat treatment smoothed the rugged surfaces of optimized microcapsules (Fig. 5(a2)).

The effects of the SGFT on the outer structure of microcapsules observed by SEM are shown in Figure 5(a3 and b3). Both microparticles retained their shapes with smaller sizes and smoother surfaces after the SGFT. Truelstrup Hansen et al. (2002) also found that the alginate microspheres retained their shapes and had smoother surfaces during exposure to simulated gastric juice at pH 2.0. A possible explanation for this observation is that treating the microparticles with a solution at pH 2 might cause some calcium to be displaced (Fundueanu et al., 1999). After this treatment, surface calcium ions could not contribute anymore to the stability of the beads resulting in smaller beads and smoother surfaces. Instead the microparticles maintained their microscopic structure with mechanical properties similar to those of ionically crosslinked beads.

Conclusion

Optimization results indicated that 2% sodium alginate mixed with 1% gellan gum, 0.86% peptides, and 0.2% FOS as coating materials would produce the highest survival rates in terms of probiotic counts. The verification experiment

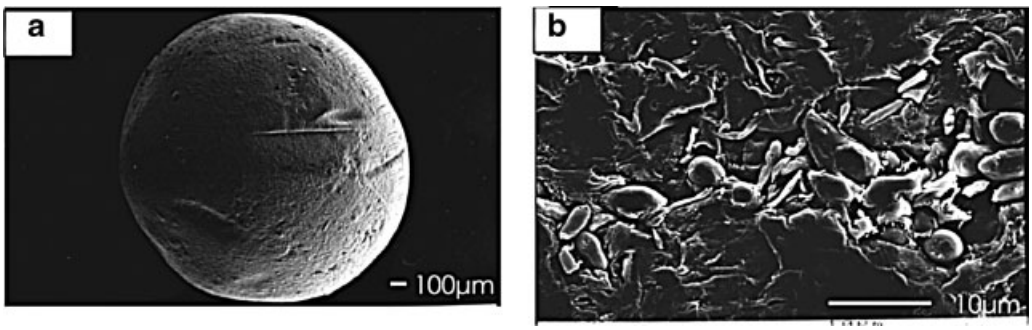

Figure 4. Scanning electron micrograph of optimized *B. bifidum* microcapsules. (**a**) Whole microcapsule, and (**b**) cross section.

yielded a result close to the predicted values, with no significant difference ($P > 0.05$). The results of heat treatments also demonstrated that addition of gellan gum in the walls of probiotic microcapsules provided improved protection for *B. bifidum*. These probiotic counts remained at 10^5–10^6 CFU/g for microcapsules stored for 2-month and then treated in pasteurization, simulated gastric fluid test and bile salt test.

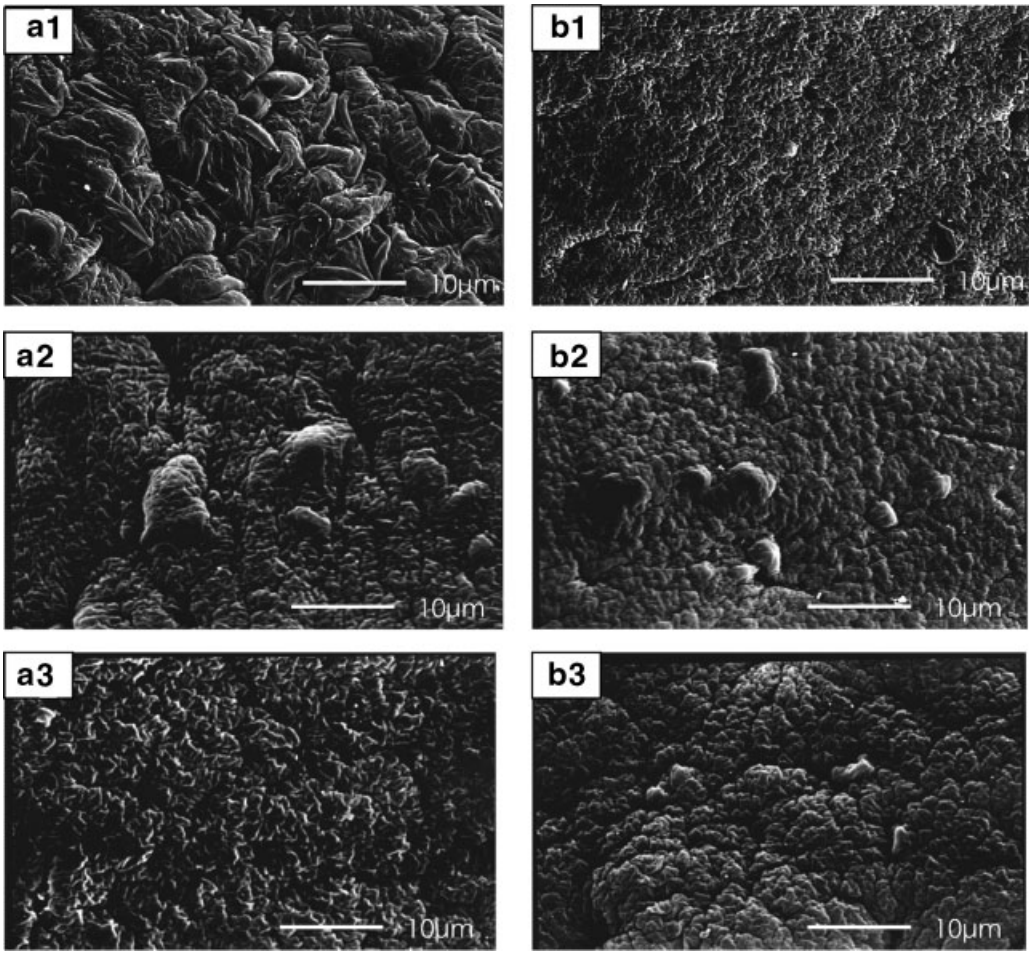

Figure 5. Effects of heat treatment and SGFT on the outer structure of microcapsules observed by SEM. (**a**) Optimal microcapsule, and (**b**) 3% alginate. (1) Control, (2) after heat treatment, and (3) after simulated gastric fluid test.

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