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Development of Probiotic Candies with Optimal Viability by Using Response Surface Methodology and Sequential Quadratic Programming

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ABSTRACT : The objective of this research was to create a new probiotic candy with good flavor and healthy benefits by using the response surface method and a sequential quadratic programming technique. The endpoint was to increase the varieties of dairy products and enhance their market values. In this study, milk was mixed with yogurt cultures (*Lactobacillus bulgaricus, Streptococcus thermophilus*) and probiotics (*L. paracasei, Bifidobacterium longum*) and incubated at 37°C for 20 h. The samples were blended with lyoprotectants (galactose, skim milk powder and sucrose), freeze dried and then mixed with sweeteners (lactose and xylitol) to improve the texture for forming tablets. The processing conditions were optimized in two steps: the first step constructed a surface model using response surface methodology; the second step optimized the model with a sequential quadratic programming procedure. Results indicated that skim milk inoculated with *L. delbrueckii* subsp. *Bulgaricus, S. thermophilus, L. paracasei* subsp. *paracasei* and *B. longum* and blended with 6.9% of galactose, 7.0% of sucrose and 8.0% of skim milk powder would produce a new probiotic candy with the highest viability of probiotics and good flavor. A relatively higher survival of probiotics can be achieved by placing the probiotic candy product in a glass bottle with deoxidant and desiccant at 4°C. These probiotic counts remained at 10^6-10^8 CFU/g after being stored for two months. (**Key Words** : Probiotics, Lyoprotectants, Optimization, Response Surface Methodology, Sequential Quadratic Programming)

INTRODUCTION

In recent years, there has been a worldwide increase in the consumption of fermented milk, especially probiotic products (Carvalho et al., 2004). Probiotics are defined as "the viable microorganisms that exhibit a beneficial effect on the health of the host by improving its intestinal microbial balance" (Mattila-Sandholm et al., 2002). The addition of probiotic bacteria to fermented milk products is made not only as a consequence of certain claimed health-promoting effects of their presence in intestinal tract of consumers, but also due to the expanding variety of products that can be formulated with probiotics bacteria (Gilliland, 1989). It is reasonable to suggest that developing new probiotics-based dairy products may provide added consumer variety, and enhance the robustness of the dairy industry by stimulating demand.

Freeze drying has been a method of choice for the long-term preservation of bioactive materials. This dehydration method causes little shrinkage and results in a completely soluble product that is easily rehydrated. Moreover, lyophilization is frequently used to preserve lactic acid bacterial starter cultures involved in dairy and food fermentations (Lodato et al., 1999). However, not all strains survive the process (Abadias et al., 2001). The major causes of losing cell viability in freeze drying are probably due to ice crystal formation and high osmolarity (Conrad et al., 2000). Microbial cell survival during the freeze drying process is dependent on many factors, including protective additives and conditions during rehydration (Font de Valdez et al., 1983). Many compounds have been tested to improve the survival of lactic acid bacteria during freeze drying, including polysaccharides, disaccharides, amino acids and proteins (Champagne et al., 1991). These compounds were in most cases found to be effective toward protection of different lactic acid bacteria (Leslie et al., 1995; Linders et al., 1997; Carvalho et al., 2002). Zayed and Roos (2004) studied the influence of lyoprotectants on survival of *Lactobacillus salivarius* and found that trehalose and sucrose in addition to skim milk were the most efficient materials. On the other hand, Chen et al. (2006) studied the

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Table 1. Process variables and their levels in the three variables-three levels response surface design

Variables	Symbol	Coded	Nature
Sucrose (%)	X_1	-1	0
		0	3.5
		+1	7
Galatose (%)	X_2	-1	0
		0	3.5
		+1	7
Skim milk (%)	X_3	-1	0
		0	4
		+1	8

effect of freeze drying on the microorganisms in kefir and indicated that addition of 10% galactose or 10% sucrose as lyoprotectants significantly increased the survival rates of both lactic acid bacteria and yeasts ($p<0.05$).

A number of novel fermented dairy products have been developed and marketed under the concept of probiotic products, but few of probiotics were associated with confectionary goods. People loves a sweet treat, but feels quilt when they indulge. Diet and nutrient candy continues to pull their weight in the industry with a 34.3% increase in the 2004. Thus, the objective of this research was to create a new probiotic candy with good flavor and healthy benefits, by using response surface methodology (RSM) and a sequential quadratic programming (SQP) technique. The endpoint was to increase the varieties of probiotic products and enhance their market values.

MATERIALS AND METHODS

Cultivation

Pure lyophilized cultures of *Lactobacillus delbrueckii* subsp. *bulgaricus* (BCRC 10696), *Streptococcus thermophilus* (BCRC 12268), *L. paracasei* subsp. *paracasei* (BCRC 14023) and *Bifidobacterium longum* (BCRC14605) were purchased from the Culture Collection and Research Center, Hsinchu, Taiwan, ROC. Lactobacilli MRS (deMan, Rogosa and Sharp) and lithium propionate MRS agar (LP-MRS) were used as the selective media for *Lactobacillus spp.* and *Bifidobacteria spp.*, respectively (Lapierre et al., 1992).

Preparation of probiotic yogurt candy

Reconstituted skim milk (12% (w/w) skim milk powder, Anchor Foods, New Zealand) in deionized water (pH 6.7, protein 5.9%) was heated at 85°C for 30 min. After cooled to 37°C, the milk was inoculated immediately with *Lactobacillus delbrueckii* subsp. *Bulgaricus*, *Streptococcus thermophilus*, *L. paracasei* subsp. *paracasei* and *Bifidobacterium longum*. When the samples reached to pH values of 4.4-4.5 (fermented approximately 7-8 h), the incubation was halted by cooling down to 4°C and blended with lyoprotectants (galactose, skim milk power and sucrose). Finally, the mixture was dehydrated by freeze drying, blended with sweeteners (44.5% sorbitol and 4.5% xylitol) and compressed in a Manesty F3 tabletting press (Liverpool, UK).

Table 2. Factors and responses of the experiment

Run	Sucrose (%)	Galactose (%)	SMP (%)	Survival L[a] (%)	Survival B[b] (%)
1	3.50	3.50	4.00	76.2	32.7
2	3.50	3.50	4.00	76.3	30.3
3	7.00	0.00	4.00	62.0	41.5
4	7.00	7.00	4.00	75.9	50.7
5	3.50	3.50	4.00	69.9	31.6
6	3.50	7.00	0.00	78.6	46.1
7	0.00	3.50	0.00	60.4	29.5
8	7.00	3.50	8.00	81.2	49.7
9	3.50	7.00	8.00	85.2	53.0
10	0.00	3.50	8.00	73.2	39.6
11	0.00	7.00	4.00	73.7	41.1
12	0.00	3.50	4.00	53.2	26.7
13	3.50	0.00	8.00	73.1	37.1
14	7.00	0.00	0.00	70.3	41.8
15	3.50	0.00	0.00	63.6	30.6
16	3.50	3.50	4.00	75.3	31.8
17	3.50	3.50	4.00	77.6	31.0

[a]L: *Lactobacillus spp.* [b]B: *B. bifidum.*

Optimization of probiotic survival after freeze drying

To optimize probiotic survival after freeze drying, the ratio of lyoprotectants (galactose, skim milk power and sucrose) was determined using the response surface methodology to first construct a response surface model which was subsequently optimized with a sequential quadratic programming approach. Two responses were studied in this research: survival of *Lactobacillus* and survival of *Bifidobacterium*. The experimental design was a Box Behncken Design (BBD; Box and Behncken, 1960) with three factors and three levels. According to our preliminary tests and previous studies (Zayed and Roos 2004; Chen et al., 2006), it was assumed that the viability of probiotics is affected by the type and concentration of the lyoprotectants, in this case galactose, skim milk power and sucrose (three independent variables). A three-variable BBD with six replicates at the center point was selected to build the response surface models. Uncoded and coded levels and experimental design are given in Table 1 and 2. This methodology allows modeling of the results using a second-order equation.

The models were then formulated as an objective function in an optimization problem using a sequential quadratic programming approach to derive the optimal formulation for the probiotic candy. Both response surface modeling and SQP were employed in a similar way to the work by Chen et al. (2004; 2005; 2006).

Table 3. Model analysis, lack of fit tests, R-square of survivability of *Lactobacillus* and *Bifidobacterium* in the probiotic candies after freeze-drying

		Model p[a]>f	Lack of fit p[a]>f	R-square
Lactobacillus [b]	Linear	0.0006*	0.1399	0.7253
	Quadratic	0.0270*	0.5880	0.9477
	Cubic	0.5880	-	0.9661
Bifidobacterium[c]	Linear	0.0010*	0.0959	0.7019
	Quadratic	0.0002*	0.0871	0.9877
	Cubic	0.0871	-	0.9972

* Significant at 5% level.
[a] P: Probability value. [b] Lactobacillus: The survival of Lactobacillus after freeze-drying.
[c] Bifidobacterium: The survival of Bifidobacterium after freeze-drying.

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. 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 effects of packaging on the survival of probiotics after storage, the two different packages (glasses bottle and laminated pouch) were used. The samples were placed in 100 ml glass bottles containing deoxidant (Agelong oxygen absorber, Sand-Tech Enterprise, Taipei, Taiwan) and desiccant (Seca Pax desiccant, Trans Work container). In addition, the products were also placed in a laminated pouch (nylon/aluminum/retort-coated polypropylene, Sun A Enterprise, Taichung, Taiwan) which was vacuum sealed before storage. They were then held at either 25 or 4°C for two months. The viability of test organisms of the samples were measured at predetermined time intervals.

Analysis methods

Determination of probiotic viability : To determine the probiotic viability, the *Lactobacillus spp.* and *Bifidobacterium spp.* populations and growth rates were measured. The suitability of the media was tested by plating decimal dilutions of the probiotic cultures. Thus, a 1-g sample of each pure lyophilized culture 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 MRS agar were incubated aerobically for 72 h at 37°C to inhibit bifidobacteria. Plates of LP-MRS agar (GasPak System; Oxoid Unipath Ltd., Basingstoke, Hampshire, England) were incubated anaerobically (72 h at 37°C) for enumerating the bifidobacteria. The population, in colony-forming units (CFU), and the characteristics of the colonies were recorded for each medium.

The survival of *Lactobacillus* or *Bifidobacterium* was defined in equation (1).

$$\text{Survivability (\%)} = (\text{population (CFU/ml) after freeze drying} / \text{population (CFU/ml) before freeze drying}) \times 100 \quad (1)$$

Sensory evaluation : Trained panelists, composed of 4 adult males and 5 females who were familiar with yogurt, were selected and trained according to the guidelines in ISO 8586 (Sensory analysis-General guidance for the selection, training and monitoring of assessors). Parameters evaluated were appearance/color, body/texture, and aroma/taste on a nine-point hedonic scale (1 = extremely dislike to 9 = extremely like). Samples were presented to the panelists in individual plastic bottles. Each panelist evaluated the sample three times (once per session).

Physical properties : The hardness of the candy was measured on a Pharma Test PTB311 (Hamburg, Germany) hardness tester. The friability of the candy was measured on a Roche Friabilator (Hoffman la Roche, Basel). After weighing, 20 candies were rotated for 20 min and then re-weighed to test for percentage loss of weight due to abrasion and fracture. Dissolution tests were performed according to the basket method described by Rawlins (1977). The rotating speed was 60 rpm and temperature was 37±0.5°C.

RESULTS AND DISCUSSION

Optimization of probiotic survival after freeze drying

Checking of the fitted model : High viable probiotics is an important benefit for this new candy product, but cell survival after the freeze drying process is dependent on many factors and the lyoprotectants ratio may be necessary to maximize the probiotic survival after freeze drying (Font de Valdez et al., 1983). In the process of optimizing the probiotic survival rate in the new candy after freeze drying, a prediction model for the concentrations of three lyoprotectants (galactose, skim milk power and sucrose)

Table 4. Coefficients of survivability of *Lactobacillus* and *Bifidobacterium* in the probiotic candies after freeze-drying

Coefficient\Y	L[a]	B[b]
β0	0.50	0.27
β1	0.53	1.853E-03
β2	0.043	-5.936E-03
β3	2.476E-03	-0.015
β11	-5.201E-03	2.880E-03
β22	-2.051E-03	4.156E-03
β33	1.613E-03	3.269E-03
β12	-1.346E-03	-1.069E-03
β13	-3.469E-04	-3.955E-04

[a] L: *Lactobacillus*.

[b] B: *Bifidobacteri* Table 5 Chemical composition, physical properties and viable counts of the probiotic candy.

was first constructed using RSM in the present work. The results of the viability of probiotics before and after freeze drying were used to build the response surface models according to the BBD presented in Table 2. In addition, the responses, as linear, quadratic and cubic functions of the variables, were tested for adequacy and fitness using model analysis and the Lack-of-Fit test (Table 3), as outlined by Lee et al. (2000), Weng et al. (2001) and Chen et al. (2005). The model analysis compares the validities of the linear, quadratic and cubic models for the two responses according to their f-values and a model with p-values (p>f) below 0.05 is regarded as significant. The highest-order, significant polynomial is selected. The Lack-of-Fit test, measuring the fitness of the model obtained, compares the residual and pure errors at replicated design points. A model with no significant Lack-of-Fit is selected. The analysis results showed that the second-order model was well suited to represent the experimental data with determination coefficients (R^2) between 0.95-0.99. The coefficients of the two responses were showed in Table 4. The two responses (survival of *Lactobacillus* and survival of *Bifidobacterium*) are then combined into one composite function (CF), defined in equation (2), whose maximum can then be sought by optimization techniques.

$$CF = \frac{(f_1 + f_2)}{2} \quad (2)$$

Optimization using SQP : To depict the optimization results (the lyoprotectants ratio for maximum probiotic survival), a 3-D response surface plot was generated by fixing one of the three variables. In our study, survivability of probiotics increased with the increasing three lyoprotectants (Figure 1). Kets et al. (1996) reported that lyoprotectants can be added to maintain the viability of probiotic organisms during freeze drying due to their assistance to the probiotics in the adaptation to the environment. When compatible lyoprotectants accumulate within the cells, the osmotic difference with their external environment is reduced.

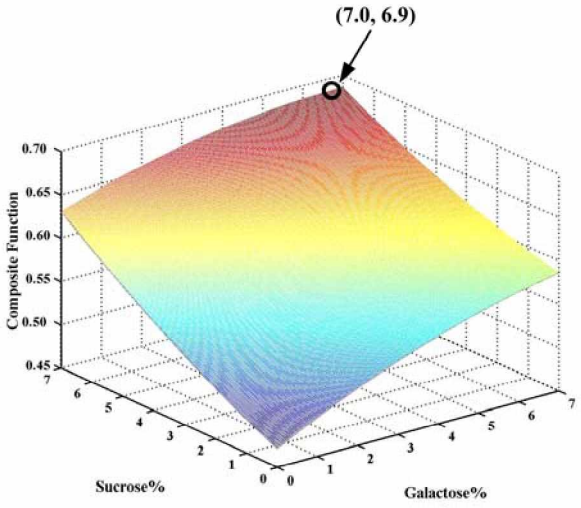

Figure 1. Response surface plot for the composite function of survivability of *Lactobacillus* and *Bifidobacterium* in the probiotic candy after freeze drying, showing the effects of sucrose and galactose under the condition of constant level of 8% skim milk powder.

To search the optimal ratio of lyoprotectants for the probiotic candy from the composite function (equation (1)), an 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, with each point representing an initial design of the ratio of lyoprotectants. Then SQP is applied to find the optimum based on each initial design. If the probability of the optimum being the global one exceeds a preset value (99.99% in this study), the global optimum is considered found. Otherwise, the next random initial design is generated and the SQP re-executed. After 17 sets of randomly generated initial designs leading to 17 sets of optimal CF values (local optima) ranging from 0.631 to 0.685 (Figure 2), the global optimal CF was found to be 0.685 (99.99% certainty). The global optimal CF value corresponded to 0.770 for survival of *Lactobacillus* and 0.601 for survival of *Bifidobacterium*. The highest optimal CF value (0.685) was attained for 10 of 17 sets, with the optimal points, X1 (sucrose) = 7.0% and X2 (galactose) = 6.9% and X3 (SMP) = 8.0% (Figure 1).

Experimental verification : The optimal lyoprotectants ratio for maximum probiotic survival was derived from SQP and verified by independent additional experiments. The optimal combination of lyoprotectants is 7.0% sucrose blended with 6.9% galactose and 8.0% skim milk powder. The experimental values were very close (p>0.05) to the predicted values with no significant differences.

Chemical composition, physical properties, viable

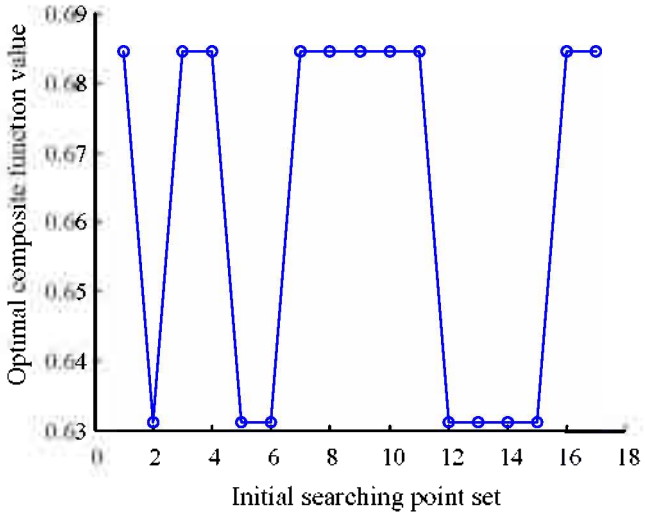

Figure 2. Optimal CF values produced by multi-start SQP for finding the maximum survival rates of probiotics in the probiotic candies.

Table 5. Chemical composition, physical properties and viable counts of the probiotic candy

Chemical composition	
Moisture (%)	1.35
Crude protein (%)	13.81
Ash (%)	2.26
Crude fat (%)	<0.10
Physical properties	
Weight (g)	0.70
Thickness (mm)	5.70
Friability (%)	0.82
Hardness (N)	64.55
Dissolution (min)	15.29
Viable counts	
Lactic acid bacteria (log CFU/ml)	9.04
Bifidobacterium (log CFU/ml)	7.41

counts and sensory evaluation of the optimal yogurt candy

The chemical composition of probiotic yogurt candy is given in Table 5. The ranges of protein levels for the market candy products were from 0% (most of candies including Jelly beans, Taffy etc.) to 5% (milk with choc coated candy). The probiotic yogurt candy contained 13.8% milk protein, which was higher than most of the candies. Adequate numbers of viable cells, namely the "therapeutic minimum," need to be regularly consumed in order to transfer the probiotic effect to consumers. A suggested minimum level for probiotics in yogurt is 10^6 CFU/ml (Robinson, 1987; Kurman and Rasic, 1991). This product had 9.04 log CFU/ml lactic acid bacteria and 7.41 log CFU/ml *Bifidobacterium*. Since this product has high levels of protein and probiotics and contains no fat, it should be beneficial to health.

Physical properties of probiotic yogurt candy are listed

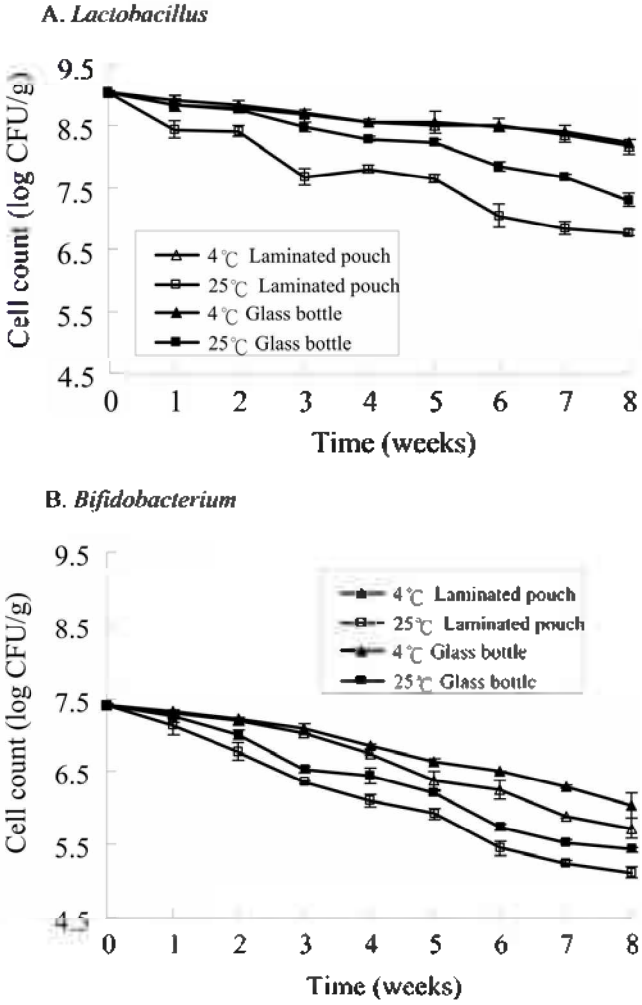

Figure 3. Effects of different packaging materials and storage temperatures on survival of probiotics in the probiotic candy during storage.

in Table 5. The average weight of this product is 0.7 g with 5.7 mm thickness. The hardness and friability of the candy was 64.55 N and 0.82%, respectively. Bertocchi et al. (2005) reported that the compressional force employed in the tableting process greatly influences the hardness, disintegration time and average primary particle size of compressed tablets. The dissolution rate of the candy is mainly related to the surface characteristics of the tablet. In addition, higher friability enhances the rate of dissolution of candy. This candy needed 15.29 min to be totally dissolved in water.

The sensory evaluation results revealed that the probiotic candy in texture and appearance were rated as good to excellent by the judges (Data no shown).

Survival of probiotics during storage

In order to elucidate the effects of the temperature and package on the survival of probiotics during storage, the products were stored in glass bottle and laminated punch for

two months under 4°C or 25°C and the survival of the organisms was measured. Figure 3 shows the survival of probiotics during the storage period. Results of the probiotic counts showed that, as might be expected, viability decreased with increasing storage time for all four treatments. However, a higher viable population of probiotics was noted in the probiotic candy held at 4°C than at 25°C. For example, *Lactobacillus* counts in the probiotic candy sample held in the glass bottle reduced from an initial population of 9.11 to 8.69 log CFU/g with a population reduction of only 0.42 log CFU/g after 2 months of storage at 4°C, compared to a relatively larger population reduction of 1.97 log CFU/g at 25°C. The better survival rate of probiotics in the probiotic candy samples stored at 4°C than 25°C as noted in this study is in agreement with the reports of previous investigators (Champagne et al., 1996).

The results of different packaging indicated that the cell counts for both *Lactobacillus* and *Bifidobacterium* were found to survive better in probiotic candy held in glass bottle with deoxidant and desiccant than in laminated pouch (Figure 3). This might be attributed to the relatively high-oxygen permeability for the laminated pouch. The permeation of oxygen through packaging during storage affected the viability of probiotics. Ishibashi and Shimanura (1993) found that the higher the oxygen permeability of the package, the lower the viability of probiotics is Hsiao et al. (2002) found less survival rate of bifidobacteria held in a PET bottle than in a glass bottle.

CONCLUSIONS

The present study has shown that skim milk was inoculated with *L. delbrueckii* subsp. *bulgaricus*, *S. thermophilus*, *L. paracasei* subsp. *paracasei* and *B. longum* and blended with 6.9% of galactose, 7.0% of sucrose and 8.0% of skim milk powder would produce a new probiotic candy with the highest viability of probiotics and good flavor. A relatively higher survival rate of probiotics can be achieved by placing the probiotic candy product in a glass bottle with deoxidant and desiccant at 4°C. These probiotic counts remained at 10^6-10^8 CFU/g after stored for two months.

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