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ORIGINAL RESEARCH PAPER

APPLICATION OF BOX–BEHNKEN DESIGN FOR OPTIMIZING THE PROCESS OF MICROENCAPSULATION OF *BIFIDOBACTERIUM BIFIDUM* BB28

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The three-level Box-Behnken design, combined with the analysis of variance (ANOVA), was employed in the present study to optimize the process of microencapsulation of *Bifidobacterium bifidum* BB28. The optimization was based on the encapsulation yield. The results showed that the encapsulation yield could be enhanced significantly when the mixture ratio of cell suspension-alginate was 1:11.5, sodium alginate 1.9%, and sodium ascorbate 0.065%. The optimal encapsulation yield of *Bifidobacterium bifidum* BB28 reached 91.52%. The experimental result in terms of encapsulation yield under optimal conditions was very close to the expected value of 91.97%. Therefore, the optimal conditions for encapsulating *Bifidobacterium bifidum* BB28 were accurately predicted through statistical methods.

Keywords: *Bifidobacterium bifidum* BB28, optimization, encapsulation yield, sodium alginate, Box-Behnken design

Introduction

Probiotics are defined as "live microorganisms when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2002). Probiotic cheeses have great potential to be a perfect functional food (Nagpal *et al*., 2012; Sanders *et al*., 2013). *Lactobacillus* and *Bifidobacterium* are the most common probiotics for dairy products (Mohammadi *et al*., 2011). *Lactobacilli* and *Bifidobacteria* species have shown beneficial effects on immunomodulation and on the decrease and prevention of various intestinal diseases (Servin and Coconnier, 2003; Shah, 2007). *Bifidobacteria spp* have been added into some dairy products such as yogurt (Capela et al., 2006; Ramchandran and Shah, 2010), fermented milks (Oliveira *et al*., 2011; Sendra *et al*., 2008). *Bifidobacteria longum* are thought to provide several health related functions including a decrease in severity of the side effects associated with antibiotics, incidence of infection in patients receiving irradiation therapy, in the duration of diarrhea due to various etiologies, improving lactose digestion, reducing

the frequency of allergic reactions, contributing to the normalization of blood lipid composition, and in gut transit time (Chen *et al*., 2010; Dong *et al*., 2010; Zhang *et al*.,2009; Stanton *et al*., 2001; Bermudez-Brito *et al*., 2012). However, for the sake of exerting these beneficial functions, probiotics must be able to resist the acid conditions in the stomach environment and the bile in the small intestine (Doleyres *et al*., 2004; Gardiner *et al*., 2000). In addition, the high concentration of viable cells and the high titratable acidity of the medium make them suitable for incorporation in the composition of probiotic preparations (Teneva *et al*., 2015). The major barriers to the survival of the ingested bacteria are the acidic environment and the secretion of bile salts into the duodenum. The tolerance of *Bifidobacteria lactis* to the pH values of the gastric juice is generally considered low (Matsumoto *et al*., 2004; Takahashi *et al*., 2004; Collado and Sanz, 2006; Charteris *et al*., 1998). Moreover, the survival of probiotics during processing and storage of food is also essential for products (Champagne *et al*., 2005; Mattila-Sandholm *et al*., 2002; Stanton *et al*., 2005).

Microencapsulation is a promising technique. Protection of probiotics by microencapsulation in hydrocolloid capsules prepared either by extrusion, as emulsion, or atomized micro particles, has been investigated (Doleyres and Lacroix, 2005). Microencapsulation by spray drying has been successfully used in the food industry for several decades (Gouin, 2004). Encapsulation of brewing yeast in alginate/chitosan matrix has been applied in beer fermentation (Naydenova *et al*., 2014). In addition, Wall materials which may have an effect on the efficacy of capsules in protecting the encapsulated bacteria, such as gum arabic, alginate, gelatine, malt dextrin, pectin, skim milk, starch, and chitosan, among others, have been used to microencapsulate probiotics (O'riordan *et al*., 2001; Capela *et al*., 2007; Su et al., 2007; Annan et al., 2008; Reddy et al., 2009; Sandoval-Castilla *et al*., 2010; Semyonov *et al*., 2010; Li *et al*., 2011). In general, the viability of the cells in the microcapsules increases with an increase in alginate capsule size and gel concentration (Chandramouli, Kailasapathy, Peiris, & Jones, 2004).

In our previous studies, the significant factors of microencapsulation process of *B. bifidum BB*28 were studied (Chen, et al, 2014a; 2014b). The objective of this study was to optimize the process of microencapsulation of *Bifidobacterium bifidum* BB28 by Box-Behnken design, and then to improve the survival rate.

Materials and methods

Microorganism

The strains of *B. bifidum* BB28, obtained from School of Food and Biological Engineering, Shaanxi University of Science & Technology, were cultured for 24 h in the MRS-broth at 37 °C, the cells were harvested by centrifugation at 4000rpm for 10 min at 4 °C, and then washed twice before being suspended in 5mL of normal saline. The final cell concentration was adjusted to 1.0×10^{11} cfu/mL.

Media

Alginate (Luo Senbo Technology Co., Ltd. Xi'an) was used as carrier agent. MRS-

broth and MRS-agar (Hope Bio-Technogy Co., Ltd.Qingdao) were used to culture and count *B. bifidum* BB28, respectively.

Methods

Microencapsulation

B. bifidum BB28 was encapsulated in sodium alginate matrix. Sodium alginate solutions were prepared, sterilized by autoclaving for 15 min at 120°C and cooled to 38–40°C. Sodium alginate solutions (11mL, 11.5mL, 12mL) and 1mL of cell suspension were transferred into a centrifuge tube and the content was vortexed to homogeneity. Sodium alginate (1.8%, 1.9%, and 2 %), sodium ascorbate (0.06%, 0.065%, and 0.07%), and oil-water ratios 4:1, and Tween 80 0.8% were taken in a beaker (300mL) and the alginate–cell mixture was added dropwise while stirring magnetically. After 15 min, a uniformly turbid emulsion was obtained, into which 2% calcium chloride was quickly added for hardening the microcapsules and breaking the emulsion. The capsules were harvested by centrifuging at 3500rpm for 10 min.

Viable count

After a serial dilution with sterile saline solution (sodium chloride, 0.9% w=v), 0.1 mL diluted bacterial suspension was removed with a syringe and dropped into the anaerobes tubes, shaken for a period of time and the tubes were held at 37°C for 48 h. The viable cells of *B. bifidum* BB28 were determined by pour plating in tubes in triplicate according to Eq. (1)

$$VC=N\times T\times 10 \quad (1)$$

where VC stands for the viable counts of the original suspension on per milliliter (cfu/mL). N is the average colony number in triplicate anaerobes tubes in the same dilution (cfu). T means dilution times.

Encapsulation yield (EY)

Encapsulation yield (EY), which is a combined measurement of the efficiency of entrapment and survival of viable cells during the microencapsulation procedure, is calculated according to Eq. (2)

$$EY= N/N_0\times 100\% \quad (2)$$

Where N is the number of viable entrapped cells released from the microspheres, and N_0 is the number of free cells added to the biopolymer mix during the production of the microspheres.

Box-Behnken design

Based on the determined key factors, three main factors (sodium alginate concentration (X1), cell suspension-alginate ratios (X2), sodium ascorbate (X3)) were chosen and their proper ranges were determined, namely three levels, coded 1, 0 and −1 for high, intermediate and low level, respectively. A Box-Behnken design model was employed. The levels of three variables were given in Table 1. The design matrix of BBD and the results of Y (responses) were listed in Table 2. The design was employed to find the optimal microencapsulation conditions by fitting a polynomial model through response surface methodology (RSM).

Statistical Analysis of the Data

SAS (Version, 9.1.3) software was used for the experiment design and regression analysis of the experimental data to estimate the independent variables. Three-dimensional surface plots and Pareto charts for the effects of the variables on the desirability were also constructed using SAS.

Table 1. The factors levels for the Box-Behnken conditions of microcapsules of *B. bifidum* BB28

Factor level	X1(%) (sodium alginate)	X2 (cell suspension-alginate	X3(%) (sodium ascorbate)
-1	1.8	1:11	0.06
0	1.9	1:11.5	0.065
1	2	1:12	0.07

Results and discussion

The experimental design and results of Box–Behnken

A 15-run Box-Behnken design with three factors and three levels, including three replicates at the Centre point, was used for fitting a second-order response surface. The three centre point is meant to provide a measure of process stability and inherent variability. The design matrix and corresponding results to evaluate the three independent variables including X1(sodium alginate concentration), X2(cell suspension-alginate ratios) and X3(sodium ascorbate) were shown in table 2, and the encapsulation yield of viable cells of *B. bifidum* BB28 was represented by Y (%).

Table 2. The experimental design and results of Box-Behnken design of preparation conditions of monolayer microcapsules of *B. bifidum* BB28

Run	X1	X2	X3	Y (%)
1	-1	-1	0	61.51
2	-1	1	0	69.33
3	1	-1	0	68.53
4	1	1	0	64.21
5	0	-1	-1	71.15
6	0	-1	1	65.24
7	0	1	-1	63.25
8	0	1	1	62.19
9	-1	0	-1	70.85
10	1	0	-1	73.21
11	-1	0	1	68.58
12	1	0	1	76.27
13	0	0	0	92.21
14	0	0	0	92.8
15	0	0	0	90.89

Regression analysis of the data

The BBD data were analyzed by multiple regression analysis using the SAS, and the multivariate quadratic regression model of Eq. (3) was developed for determining the individual effects and mutual interaction effects of candidate variables:

$$Y = 91.967 + 1.494X1 - 0.931X2 - 0.773X3 - 9.651X1^2 - 3.035X1X2 + 1.333X1X3 - 16.421X2^2 + 1.213X2X3 - 10.088X3^2 \quad (3)$$

where Y is the desirability value of the microcapsules of *B. bifidum* BB28, X1, X2 and X3 represent sodium alginate concentration, cell suspension-alginate ratios and sodium ascorbate concentration, respectively.

Table 3. The ANOVA of Box-Behnken Design of monolayer microcapsules of *B. bifidum* BB28

Source	DF	SS	MS	F	Pr > F	sig.
X1	1	17.850	17.850	2.036	0.213	
X2	1	6.938	6.938	0.791	0.414	
X3	1	4.774	4.774	0.544	0.494	
X1*X1	1	343.896	343.896	39.219	0.002	**
X1*X2	1	36.845	36.845	4.202	0.096	
X1*X3	1	7.102	7.102	0.810	0.409	
X2*X2	1	995.608	995.608	113.542	0.000126	***
X2*X3	1	5.881	5.881	0.671	0.450	
X3*X3	1	375.783	375.783	42.855	0.001	**
Model	9	1588.938	176.549	20.134	0.002	**
Linear	3	29.562	9.854	1.124	0.423	
Quadratic	3	1509.548	503.183	57.384	0.00027	***
Cross product	3	49.828	16.609	1.894	0.248	
Error	5	43.843	8.769			
Lack of fit	3	41.930	13.977	14.613	0.065	
True error	2	1.913	0.956			
Total	14	1632.782				

** $p<0.01$, very significant; *$p<0.05$, significant; R^2=97.31%， R^2_{Adj}=92.48%

The Analysis of variance (ANOVA) was used to evaluate the accuracy of the fitted model and to test the significance of the coefficient. The effect of variables was determined by the F-test, and the lower the P value, the more obvious effect on the variables; the R-squared value provided a measure of the variability in the response values that could be explained by the experimental factors and their interactions (Siti Aminah *et al*., 2006). The result of ANOVA was shown in Table 3, the probability value for response Y ($p=0.002<0.05$) demonstrated a high significance for the regression model, and the insignificant probability for the lack of fit ($p=0.065>0.05$) indicated that the regression analysis was effective. This proved that the model equation expressed in Eq. (3) provided a suitable model to describe the response of the value of the encapsulation yield. Furthermore, a value of the coefficient of determination (R^2 =97.31%) calculated and showed that more than 97.31% of

variability in the response could be explained by the second-order polynomial predicted equation already given. Besides, the value of the adjusted determination coefficient (R^2Adj =92.48%) was close to the R^2 value, confirming that the model was highly significant. The P-values of linear coefficients (X1, X2 and X3), interaction term coefficients (X1X2, X1X3 and X2X3) were higher than 0.05, which indicated that they do not have any significant effects on the encapsulation yield of *B. bifidum* BB28, but quadratic term coefficients ($X1^2$, $X2^2$and $X3^2$) less than 0.01 indicated that they have very significant effects on encapsulation yield, which illustrated that both encapsulation yield and variables were not a simple linear function, so the experimental method was reliable.

It is well known that the conventional optimization technique, e.g. one-factor-at-a-time method, is not only tedious and time-consuming, but also misleading in terms of result interpretation, especially for the interactions among different factors, which are unable to be detected. The orthogonal array method, coupled with variance analysis, has proved to be a cost-effective optimization strategy that can be used to assign experimental factors in a series of experimental trials (Wang & Yang *et al*., 2003), but it cannot fit the results into a regression equation to locate the optimum level through the entire space of the tested independent variables. The response surface methodology is an efficient statistical technique for the optimization of multiple variables to predict the best conditions with a minimum number of experiments. In comparison with orthogonal design and variance analysis, Box-Behnken design, one of the designs of the response surface methodology, allows calculations to be made of the response at intermediate levels which were not experimentally studied (Dong *et al*., 2009).

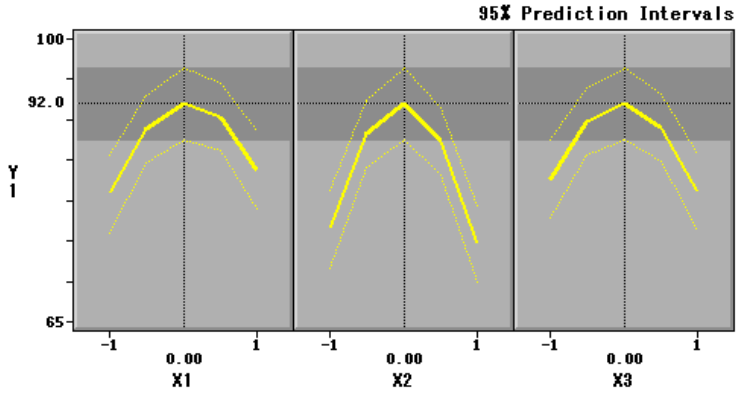

Figure 1. The trends of entrapped yield Y1 with the factors of the concentration of sodium alginate (X1), cell suspension-alginate ratios (X2) and Sodium ascorbate (X3)

The 95% confidence interval of these variables implied a positive effect on the responses, in the range of tested concentration. The sodium alginate (X1) impacted the corresponding variables in the same trend depending on its concentration, so that the response values increased at first and then decreased following the increase of sodium alginate. Cell suspension-alginate ratios represented by X2 impacted the

increased of both Y1, and then decreased the responses value gradually. Sodium ascorbate (X3) showed a positive trend to Y1 based on its concentration, but both responses reduced sharply when the concentration was beyond the scope.

Three-dimensional response surface plots and two-dimensional contour plots were generated to obtain a better understanding of the interactive effects of the independent variables on the corresponding variables (Zhang *et al*., 2015). As is shown in Fig. 2-4, the encapsulation yield of microcapsules *B. bifidum* BB28 was investigated when two varieties kept in experimental range and other variety fixed at zero. The two-dimensional contour plots seemed to be a circle, which indicated that the mutual interaction of terms X1 × X2, and X1 × X3 was not significant for responses (Figure 2 and 3). Moreover, the oval in the contour plots of X2 × X3 implied that the interaction effect on the corresponding variables between X2 and X3 was significant (Figures 4). SAS software was used to analyze the regression equation for Y, by solving the regression equation and analyzing the response surface contour plots, the optimal encapsulation conditions were obtained as follows: X1 (sodium alginate) 1.9%, X2 (cell suspension-alginate ratios) 1:11.5, X3 (sodium ascorbate) 0.065%. the predicted encapsulation yield of monolayer microcapsules of *B. bifidum* BB28 was 91.97% under the optimal conditions.

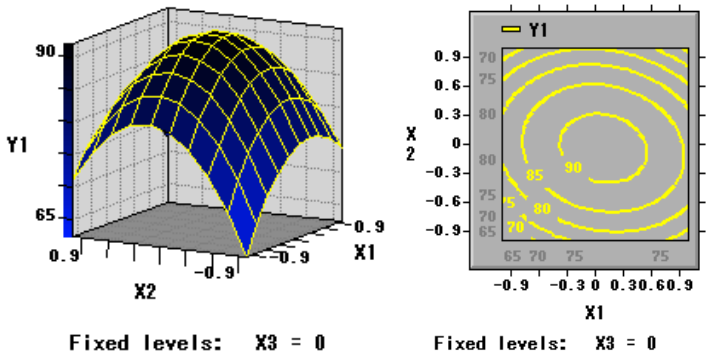

Figure 2. Response surface and contour plots of the concentration of sodium alginate (X1), proportion of *B. bifidum* BB28 and sodium alginate (X2) to encapsulation yield (Y1)

Yang *et al*. (1996) used sodium alginate as wall material to prepare microcapsules whose encapsulation rate was 70%, which increased the stability of *Bifidobacterium* in adverse environments. Shi *et al*. (2005) reported the viable cell count of *BifidobacteriumB1* microcapsules of 10^{11}cfu/g on cell suspension-alginate ratios 1:1. Chen *et al*. (2014) found that the optimal cell suspension-alginate ratios for *Bifidobacterium* BB28 were 1:10, and the entrapped yield of 76% was lower than the present study. The phenomenon may be due to the high proportion of sodium alginate and bacteria suspension, which made the microcapsule membrane thickening and reduced the viable count in microcapsules.

The encapsulation yield of microcapsules of *B. bifidum* BB28 from the models was verified to the react value. At the end of the experiments performed in triplicate under the optimum conditions (sodium algianate1.9 %, cell suspension-alginate ratios 1:1.5, sodium ascorbate 0.065%), the result showed that the encapsulation yield of

B. bifidum BB28 microcapsules were 91.25%, 92.14% and 91.18%, respectively. The average value was 91.52% which was very close to the estimated value of 91.97%. This result suggested that statistical methods were successfully used to determine the optimum lyoprotectant formulations for the encapsulation yield of microcapsules of *B. bifidum* BB28.

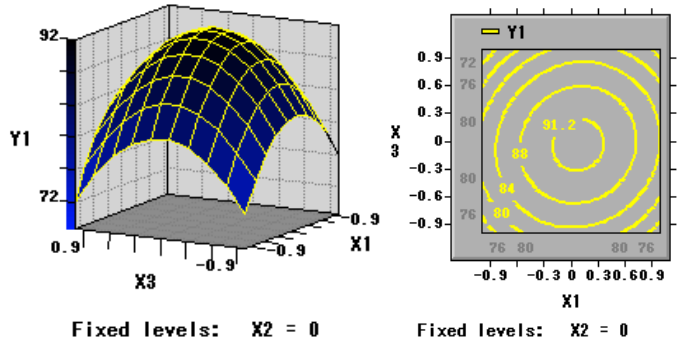

Figure 3. Response surface and contour plots of the concentration of sodium alginate (X1), Sodium ascorbate (X3) to encapsulation yield (Y1)

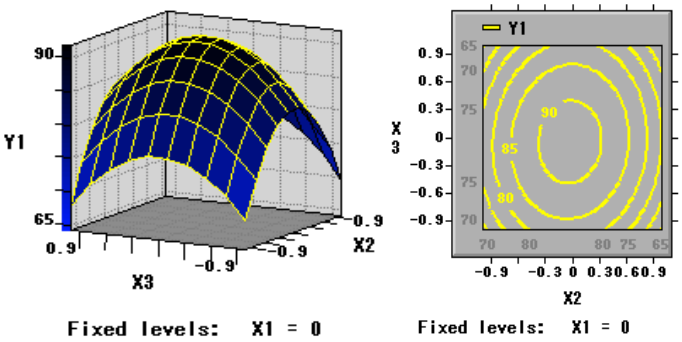

Figure 4. Response surface and contour plots of the concentration of proportion of *B. bifidum* BB28 and sodium alginate (X2), Sodium ascorbate (X3) to encapsulation yield (Y1)

Bifidobacterium microcapsules are now mainly used for dairy products. Homayouni *et al*. (2008) who found probiotics in fermented dry sausages showed that microencapsulated *Bifidobacterium* survival rate was higher than the non-microencapsulated one, but it weakened the *Bifidobacterium* inhibition on *E. coli*. Kailasapathy *et al*. (2006) investigated the behavior of alginate microencapsulated *Bifidobacterium lactis* in ice milk, and reported that the survival of *Bifidobacteria lactis* was improved by 40% in the freezing process. Gomes *et al.* (2011), R. Altamirano-Fortoul *et al*. (2012) used carrageenan immobilized by *Lactobacillus acidophilus* gel beads into the banana puree to improve the fermentation efficiency.

Wall materials are very important for microencapsulation, they should have high solubility, good emulsification, film forming, drying properties, and provide low

viscosity of an emulsion (Gharsallaoui *et al*., 2007). Ismail *et al*. (2013) found that the combination of modified starch/Arabic gum/whey protein concentrate (4/0/1, w/w/w) provided the highest efficiency in flaxseed oil microencapsulation, and the microencapsulation efficiency of the microcapsules was 91%. K. O'Riordan *et al.* (2001) used starch as wall material to prolong viability of *Bifidobacterium* PL1 during storage, because this starch appeared to be an inherent property that free cells were observed to be clumped when spray dried. This paper used sodium alginate as wall material to encapsulate *Bifidobacterium* BB28 whose encapsulation yield was 91.52%.

Conclusions

In this study, the encapsulated conditions of microcapsules of *B. bifidum* BB28 were successfully formulated and optimized by employing Box–Behnken Design. The developed microencapsulation formulation showed that 1.9% sodium alginate, 1:11.5 cell suspension-alginate ratios, 0.065% sodium ascorbate had a significant impact on the encapsulation yield of *B. bifidum* BB28 during microencapsulation, and the encapsulation yield of microcapsules of *B. bifidum* BB28 was 91.52% under the optimal conditions. The actual measured responses of encapsulation yield from the optimal formulation were close to the predicted responses generated by the design, which means that it was possible to determine the optimal microencapsulated concentration to obtain a higher encapsulation yield by the method of experimental factorial design and response surface analysis.

Acknowledgments

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