OPTIMIZATION THE PROCESS OF MICROENCAPSULATION OF *BIFIDOBACTERIUM BIFIDUM* BB01 BY BOX-BEHNKEN DESIGN

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Abstract: The effects of cell suspension-alginate ratios, sodium erythorbate, and inulin on encapsulation yield of microcapsules of *Bifidobacterium bifidum* BB01 were studied by Box-Behnken design. The experimental results indicated that cell suspension-alginate ratios, sodium erythorbate and inulin had a significant impact on encapsulation yield, and the embedding yield could be enhanced significantly in the condition of 1:3 cell suspension-alginate ratios, 0.12% sodium erythorbate, and 6% inulin. The optimal embedding yields of microencapsulation of *B. bifidum* BB01 were observed to be 81.52%, that values were very close to the expected values 81.81%, so the method was effective.

Keywords: *Bifidobacterium bifidum* BB01, optimization, microencapsulation, Box-Behnken design

INTRODUCTION

Probiotics are a group of living microorganisms that are beneficial to human health (FAO/WHO, 2002; Guarner & Schaafsma, 1998). *Lactobacillus* and *Bifidobacterium* are the most common probiotics for dairy products and that have been added into yogurt and fermented milks (Mohammadi et al., 2011; Ramchandran & Shah, 2010; Oliveira et al., 2011; Sendra et al., 2008; Capela

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et al., 2006). *Bifidobacterium* is one of the beneficial bacteria in the human gut, which has antibacterial, anti-aging, strengthening the immune system and many other effects. The numbers of *Bifidobacterium* are a sign of health, so it has been widely used in the food industry. However, for the sake of exerting these beneficial functions, probiotics must be to resist the adverse environment in the stomach and small intestine (Doleyres et al., 2004). The secretion of acid and bile salts into the duodenum is the biggest obstacle to the survival of probiotic bacteria and the resistance of *Bifidobacterium* to gastric juice is weak (Collado & Sanz, 2006; Matsumoto et al., 2004; Takahashi et al., 2004). What's more, in the process of processing and storage, the number of living probiotics are very important for food. (Champagne et al., 2005; Stanton et al., 2005; Mattila-Sandholm et al., 2002).

Microencapsulation can improve the survival of probiotics in adverse environment for functional food (Brinques and Ayub, 2011; Homayouni et al., 2008; Kailasapathy et al., 2006). Shi et al., (2013), Capela et al., (2006), Chen et al., (2006) and Heidebach et al., (2010) demonstrated by microencapsulation to protect probiotics.

Spray drying has been used to produce microencapsulation with probiotics for a long time. Especially, in spray drying process, the water content of microcapsules containing probiotics is an important factor that impacts the viability of probiotics in the process of storage and processing (Meng et al., 2008; Chan et al., 2011).

In general, the survival rate of microorganisms is the highest under the optimum water activity, with the decline in water activity, resulting in reduced survival, similar views were proved by Golowczyc et al. (2010). Nevertheless, excessive drying can also reduce the survival rate of probiotics in spray drying (Li et al., 2011). In addition, the most common wall material used in microencapsulation is calcium alginate. Using different materials to make wall material, the survival rate of probiotics in microcapsules is also different. At present, the most commonly used sodium alginate and chitosan as wall material.

Embedding rate is an index to measure the efficiency of microencapsulation, Chen et al. (2014) found the optimum ratio of cell suspension-alginate for *B. bifidum* BB01 were 1:5, and the entrapped yield were 64% through single factor experiment, its entrapped yield was lower than the present study. It is mainly because of the high ratio of cell suspension-alginate make the microcapsule membrane thickening, and inclusion of microcapsule is few, so the entrapped yield and viable counts contained in microcapsules declined. Zou (2012) used emulsification/ internal gelation to encapsulated microcapsules, and entrapped yield was approximately 43%-50%, which was lower than Krasaekoopt et al., (2003). That is because the embedding method

of emulsification/internal gelation need to add ice acetic acid that have some damage to probiotics.

Furthermore, the survival rate of probiotics in microcapsules is also a very important indicator under adverse circumstances. (Lotfipour et al., 2012) used polysaccharide psyllium as protective agent to encapsulate probiotics of *L. acidophilus DMSZ 20079*, and demonstrated that the survival rate of probiotics increased with the increase of polysaccharide psyllium concentration when exposed to simulated gastric acid. Nag et al., (2011) used sodium caseinate with gellan gum as protective agent to encapsulate *Lactobacillus casei*, and experimental results showed that the number of living bacteria in formed microcapsules by 3.1 log reductions compared to control by 4.6 log reductions.

Several factors may have an effect on the viability of the cells in the process of microencapsulation. In general, with the increase of alginate capsule size and gel concentration, the number of living bacteria will be increased (Chandramouli, Kailasapathy, Peiris, & Jones et al, 2004). In addition, the optimal processing conditions for new dairy products were studied by combining Response Surface Methodology (RSM) with advanced optimization techniques (Chen et al., 2006).

In our previous works, the significant factors about microencapsulation of *B. bifidum BB*01were studied (Chen, et al 2014). The purpose of this paper was to optimize the process of microencapsulation of *Bifidobacterium* BB01 by Box-Behnken design, and to improve the stability of *B. bifidum BB*01 in spray drying.

MATERIALS AND METHODS

Materials: the strain of *B. bifidum* BB01 was obtained from School of Food & Biological Engineering, Shaanxi University of Science & Technology. Sodium alginate (Luo Senbo Technology Co., Ltd. Xi'an) was used as carrier agents. MRS broth (Hope Bio-Technogy Co. Ltd., Qingdao) was used to culture the cells. Centrifuge (LG10-2.4) was used to obtain bacterial suspensions. All the chemical reagents used were of analytical grade.

Microorganism: MRS medium were sterilized for 15 min at 121°C, after cooling to room temperature *B. bifidum* BB01 were inoculated in the activated culture medium for 24 h at 37 °C. The activated bacteria were inoculated into the fermentation medium by 5%, cultured 18h at 37°C, and all cells in the fermentation broth using a centrifuge to collect at 4500r for 10 min at 4 °C. The concentration of the bacterial suspension was adjusted to 1.0×10^{11} cfu/mL by using 0.85-0.9% saline.

Microencapsulation: *B. bifidum* BB01 were 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 (2.7mL, 3.0mL, 3.3mL) and 1mL of cell suspension were transferred into 3 centrifuge tubes, respectively, and the content was vortexed to homogeneity. Sodium erythorbate (0.11%, 0.12%, and 0.13 %), inulin (5.7%, 6.0%, and 6.3%), oil-water ratios 4:1 and sodium alginate 2%, containing Tween 80 0.4% was taken in 3 volume 300mL beakers, respectively. and the alginate–cell mixture was added dropwise to beakers. After 15 min while stirring magnetically, a uniformly and stably turbid emulsion was obtained, and extruding emulsion into 2% calcium chloride solution using sterile pressure nozzles. Finally, all the microcapsules were obtained by centrifuging at 3500r for 10 min.

Viable count: The bacteria suspension was diluted with sterile saline solution, until the concentration of the bacterial suspension is adjusted to 10^{-7} to 10^{-8} cfu/mL, then take 1mL the bacteria suspension inoculation into the agar medium to count. The average value of the count was determined after the bacterium were cultured for 48h at 37°C, and the number of viable bacteria was converted into the unit volume. The number of viable bacteria in bacterial suspension were measured according to Eq. (1)

$$VC = N_0 \times T \times 10 \qquad (1)$$

where VC is the number of viable bacteria per milliliter of bacterial suspension. N_0 the mean values of 3 parallel count experiments in the same dilution. T is times of dilution.

Encapsulation yield (EY): Embedding yield is a parameter to measure the bacteria be trapped in the microcapsules, evaluated according to Eq. (2)

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

where N is the number of viable bacteria released from microcapsules; N_0 is total number of viable bacteria in the polymer matrix before embedding.

Box-Behnken design: In order to study optimal process about microencapsulated *B. bifidum* BB01, a Box-Behnken model was used. Three factors, cell suspension-alginate ratios, sodium erythorbate concentration and inulin concentration, and three levels, coded 1, 0 and−1 for high, intermediate and low level, respectively. The levels of these three variables were given in Table 1. Then, a total 15 runs BBD experiment was used to optimize the optimal encapsulation conditions. Three variables including X1 (cell suspension-alginate ratios), X2(sodium erythorbate concentration) and X3(inulin concentration) and were presented in Table 1. The design matrix of

BBD and results of Y1 (responses) were listed in Table 2. Encapsulation yield of microencapsulation of *B. bifidum* BB01 was represented by Y1 (%).

Table 1. The factors levels for conditions of Box-Behnken of microencapsulation of *Bifidobacterium* BB01

Factor level	X1(cell suspension-alginate ratios)	X2(%) (sodium erythorbate)	X3(%) (inulin)
-1	1:2.7	0.11	5.7
0	1:3.0	0.12	6.0
1	1:3.3	0.13	6.3

Table 2 Box–Behnken design and results of preparation conditions of microencapsulation of *Bifidobacterium* BB01

Formulations	X1	X2	X3	Y (%)
1	-1	-1	0	37.72
2	-1	1	0	44.91
3	1	-1	0	46.32
4	1	1	0	54.56
5	0	-1	-1	41.84
6	0	-1	1	54.04
7	0	1	-1	58.95
8	0	1	1	51.93
9	-1	0	-1	58.42
10	1	0	-1	38.95
11	-1	0	1	53.42
12	1	0	1	55.61
13	0	0	0	85.26
14	0	0	0	80.18
15	0	0	0	80.00

Statistical Analysis of the Data: SAS (Version, 9.1.3) software was applied to the experiment to design and regression analysis, and the influence of each variable could be represented by a three-dimensional surface plots.

RESULTS AND DISCUSSION

To determine the optimal encapsulation conditions of cell suspension-alginate ratios (X1), sodium erythorbate concentration (X2) and inulin concentration (X3), a total 15 runs BBD experiment was applied to evaluate the effects of three variables on the experiment. The experimental design and results are

shown in table 2.

The BBD data were analyzed by applying multivariate quadratic regression model; the predictive values Y is described by equation (3):

$$Y = 81.813 + 0.121X_1 + 3.804X_2 + 2.105X_3 - 18.0130X_1^2 + 0.263X_1X_2 + 5.415X_1X_3 - 17.923X_2^2 - 4.805X_2X_3 - 12.200X_3^2 \qquad (3)$$

where Y is the predictive values of the single-embedded microencapsulation of *B. bifidum* BB01, X_1, X_2 and X_3 represent cell suspension-alginate ratios, the content of sodium erythorbate and inulin, respectively.

The result of ANOVA is demonstrated in Table 3. The predict Y P-values are 0.013 less than 0.05 implied that regression model was significant. Moreover, the P-values of factors X_1, X_2, X_3, X_1 and X_2, X_2 and X_3, and X_1 and X_3 were higher than 0.05, implying not significant effects on encapsulation yield, but quadratic term coefficients (X_1^2, X_2^2 and X_3^2) are lower than 0.05 indicating the not significant effects on Encapsulation yield of these items, which illustrates both encapsulation yield and variables are not a simple linear function.

Table 3. The ANOVA of Box-Behnken design of monolayer microencapsulation of *Bifidobacterium* BB01

Source	DF	SS	MS	F	Pr > F	sig.
X1	1	0.118	0.118	0.957	0.957	
X2	1	115.748	115.748	0.134	0.134	
X3	1	35.448	35.448	0.368	0.368	
X1*X1	1	1198.025	1198.025	0.002	0.002	**
X1*X2	1	0.276	0.276	0.934	0.934	
X1*X3	1	117.289	117.289	0.132	0.132	
X2*X2	1	1186.083	1186.083	0.002	0.002	**
X2*X3	1	92.352	92.352	0.171	0.171	
X3*X3	1	549.601	549.601	0.011	0.011	*
Model	9	2921.523	324.614	0.957	0.013	*
Linear	3	151.314	50.438	0.134	0.347	*
Quadratic	3	2560.292	853.431	0.368	0.002	**
Cross product	3	209.917	69.972		0.242	
Error	5	180.905	36.181			
Lack of fit	3	163.070	54.357	6.095	0.144	
Pure error	2	17.835	8.918			
Total	14	3102.428				

** ($p<0.01$), very significant; *($p<0.05$), significant, $R^2=94.17\%$, $R_{adj}^2=83.67\%$

What's more, the coefficient of determination (R^2) was 94.17%, which meant

94.17% of the variability in the response, is explained by the model. In addition, the value of adjustment coefficient ($Radj^2$) was 83.67%, which was close to the R^2 value also confirmed that the model was significant, so the experimental method was reliable.

The trends of entrapped yield Y1 with the factors of proportion of *Bifidobacterium bifidum* BB01 and sodium alginate(X1), sodium erythorbate content(X2) and inulin content(X3) are presented at Figure 1. The 95% confidence interval indicated that these factors had a positive effect on entrapped yield within a certain range of concentration. The suspension-alginate ratios (X1) influences the entrapped yield in the same trend relying on its ratios, so that the entrapped yield first increases and then decreases along with the increase of ratios of suspension-alginate; sodium erythorbate (X2) and inulin(X3) influenced entrapped yield through adjusting the concentration gradually, but entrapped yield reduced distinctly when the concentration was out of range.

The regression equation is represented by the response surface and contour plots, which shows the relationship between the dependent variable and the independent variables (Zhang, Lu et al., 2015).
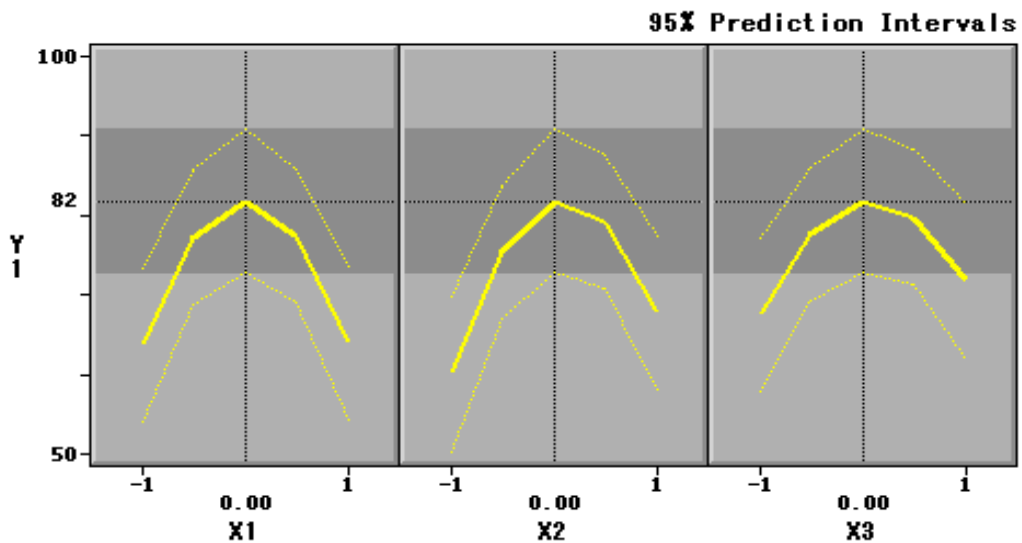

Figure 1. The trends of entrapped yield Y with the factors of proportion of
Bifidobacterium bifidum BB01 and sodium alginate(X1), sodium erythorbate
content(X2) and inulin content(X3)

As shown in Figures 2-4, the encapsulation yield of microencapsulation of *Bifidobacterium* BB01 is evaluated by fixing one variable and changing the other two variables.

For cell suspension-alginate ratios and sodium erythorbate concentration (Figure 2), the contour plot was close to be a circle, which indicates the

mutual influence between cell suspension-alginate ratios and sodium erythorbate concentration is not significant. The same trend (Figure 3) was also observed for cell suspension-alginate ratios and inulin concentration. Close to the circular contour plot displayed in Figure 4 demonstrates that the mutual influence between sodium erythorbate concentration and inulin concentration is not noticeable.
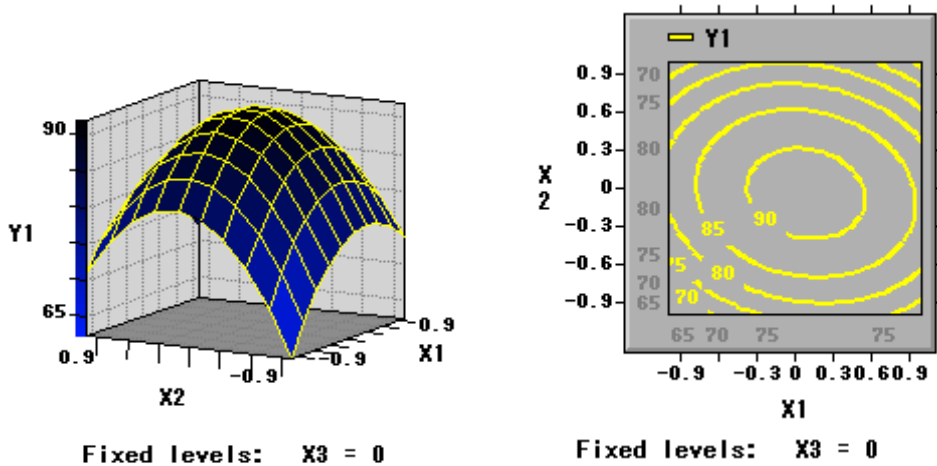

Figure 2. The influence of variables cell suspension-alginate ratios(X1), sodium erythorbate content(X2) to encapsulation yield (Y) were demonstrated by response surface and contour plots
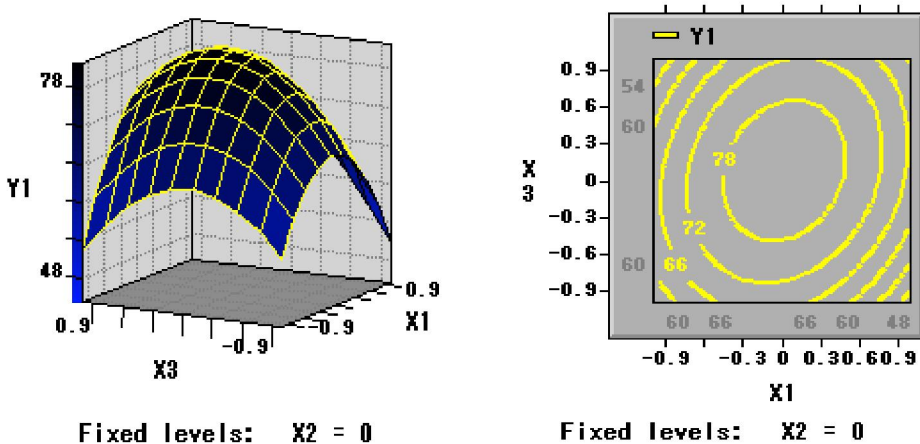

Figure 3. The influence of variables cell suspension-alginate ratios(X1), inulin content(X3) to encapsulation yield (Y) were demonstrated by response surface and contour plots.
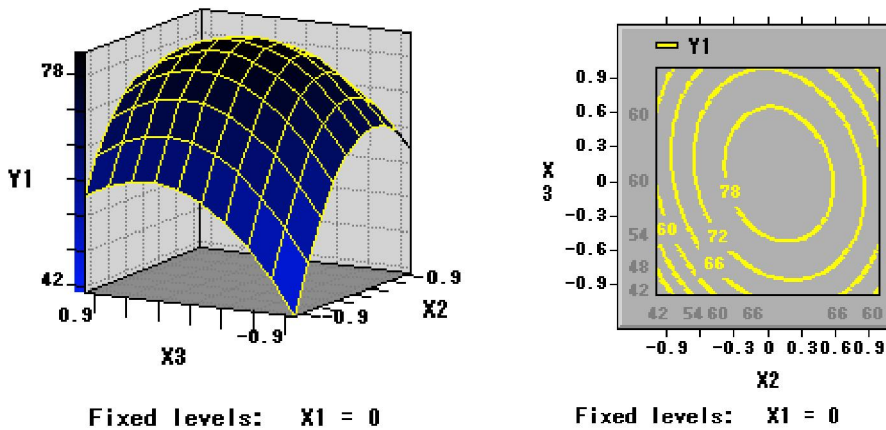
Figure 4. The influence of variables sodium erythorbate content(X2), inulin content(X3) to encapsulation yield (Y) were demonstrated by response surface and contour plots.

Through the analysis of regression and the response surface contour plots, the optimal encapsulation conditions were found as follow: X1 (cell suspension-alginate ratios) 1:3, X2 (sodium erythorbate) 0.12%, X3 (inulin) 6%. Under the optimal conditions, the predicted embedding yield of monolayer microencapsulation of *B. bifidum* BB01 was 81.81%.

The embedding yield of microencapsulation of *B. bifidum* BB01 from the models was verified to be close. Applying to the optimum conditions (cell suspension-alginate ratios1:3, sodium erythorbate 0.12%, inulin 6%) to finish repeated experiments, the result showed the embedding yield of *B. bifidum* BB01microcapsules were 81.25%, 82.14% and 81.18%, respectively, and the average values were 81.52%, which was very close the estimate values 81.81%. These results mean that the optimal encapsulated conditions of microencapsulation of *B. bifidum* BB01 could be determined successfully by statistical methods.

CONCLUSION

In this paper, Box-behnken design was used to optimize the process of microencapsulation of *B. bifidum* BB01, and it showed that 1:3 cellsuspension-alginate ratios, 0.12% sodium erythorbate and 6% inulin had a significant impact on the embedding yield of *B. bifidum* BB01 during spray drying, and the embedding yield of monolayer microencapsulation of *B. bifidum* BB01 was 81.52% under the optimal conditions.

Moreover, it was effective to determine optimal microencapsulated

concentration by the method of factors design and response surface analysis. In the model equation, it also proved the validity of the model by fitting the values of variables.

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