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OPTIMIZATION OF PREBIOTICS AND OXYGEN SCAVENGERS FOR *BIFIDOBACTERIUM BIFIDUM* BB01 MICROCAPSULES BY RESPONSE SURFACE METHODOLOGY

– Research paper –

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ABSTRACT: The effects of different prebiotics and oxygen scavengers on making the process of xanthan gum and chitosan (XC) *Bifidobacterium bifidum* BB01 microcapsules were studied by single factor experiment and Plackett-Burman screening test, ascorbic acid, sodium erythorbate and xylo-oligosaccharide had significant effects. Based on the previous studies, the process XC *B. Bifidum* BB01 microcapsules were further optimized by Box-Behnken model in this study. Response surface analysis showed that the best additive amoumt of ascorbic acid, sodium erythorbate and xylo-oligosaccharide were 3.0%, 2.36% and 4.99%, respectively. The viable counts of *B. Bifidum* BB01 microcapsules reached to 1.52×10^{10}CFU/g from 1.25×10^{10} CFU/g, the encapsulation yield reached to 94.88% from 90% under the optimum conditions. It provided the research foundation for the afterward production and exploration of the process XC *B. Bifidum* BB01 microcapsules.

Keywords: optimization; encapsulation yield; Box-Behnken design; viable counts; oxygen scavengers; prebiotics; *Bifidobacterium bifidum* BB01.

INTRODUCTION

Probiotics have been viewed as food supplements traditionally. However, in recent years, probiotics have been increasingly prescribed as nutraceuticals in light of their therapeutic effects, which range from alleviating symptoms of lactose malabsorption and irritable bowel syndromes to suppressing colon cancer and enhancing resistance to gut infections (Kailasapathy & Chin, 2000; Sanders et al., 2013). Probiotics have been primarily selected from the genera *Lactobacillus* and *Bifidobacterium*, which are part of lactic acid bacteria (LAB) group. Some of the usual *Bifidobacterium* microorganism are *Bifidobacterium bifidum, Bifidobacterium breve, Bifidobacterium infantis, Bifidobacterium longum, Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus plantarum, Lactobacillus rhamnosus and Lactobacillus GG* (Timmerman et al., 2004). *Bifidobacterium* is a very crucial type of intestinal probiotics, which has important physiological and healthy functions on the human body (Wean et al., 2014). It can be employed to inhibit the growth of saprophytic bacteria, lower cholesterol and synthetic vitamins and promote calcium absorption and other functions. Some researchers have suggested that the viable counts of intestinal *B. Bifidum* can reflect a person's health status in a way.

Probiotics provide their health benefits to maintain health by inhibiting harmful microbe growths, that also can promote gut microflora and stimulate the host's immune response (Figueroa-Gonzalez et al., 2011). The probiotics must maintain viable counts to provide these

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benefits, however after the probiotics reach the small intestine, the high concentrations of bile solutions can cause a substantial reduction in the activity of probiotics (Huiyi et al., 2013; Wean et al., 2014), which are difficult to achieve in their healthful effects. Therefore, the increase of viable counts in probiotic products and the improvement of the acid resistance and bile tolerance for the bacterial cells have becoming urgent problems needed to be solved.

One effective method to protect probiotic cells from the stresses encountered during processing and gastrointestinal transit is by microencapsulation of probiotic cells into polysaccharide carrier matrices such as Sodium Alginate, Xanthan Gum, Gelatin, Carrageenan, Chitosan, Starch, Proteins. Microencapsulation has been found to enhance the cell survival rate upon freeze drying (Heidebach et al., 2010), meanwhile protect the cells from the acidic environment of the stomach and facilitating the cell release gradually in the intestinal sections of the gut Later (Cook et al., 2011; Guerin et al., 2003). Microencapsulation is a promising technique. Protection of probiotics by microencapsulation in hydrocolloid capsules prepared has been investigated, which method including by extrusion, emulsion, or atomized micro particles (Doleyres & Lacroix, 2005). Microencapsulation by spray drying has been used prosperously 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).

The encapsulation material was changed based on the study of probiotic *B. Bifidum* BB01 microencapsulation, the probiotics *B. Bifidum* BB01 was embedding by the combination of xanthan gum and chitosan. Xanthan gum and chitosan are natural biological macromolecules with good biocompatibility with non-toxic side effects (Graziela et al., 2011). Chitosan is soluble in acidic aqueous solution and its amino group is positively charged in acidic solution. The anionic polysaccharide xanthan gum is co-gel with chitosan through polyelectrolyte complexing, which can be used as microcapsule wall material.

Oxygen scavenger can eliminate the oxygen for the strain *B. Bifidum* BB01, prebiotics as *B. Bifidum* BB01 growth promoting factor, promote the growth of *B. Bifidum* BB01 to a certain extent (De et al., 2012). The effects of different prebiotics and oxygen scavengers on viable counts and encapsulation yield of making the process of XC *Bifidobacterium bifidum* BB01 microcapsules were performed by single factor experiments and Plackett–Burman designed experiment. ascorbic acid, sodium erythorbate and xylo-oligosaccharide were selected as main factors, which had positive effects on viable counts and encapsulation yield. According to the results of the steepest ascent experiment, 3.0%, 2.4%, 5.0% were set as the centers of Box–Behnken design (data unpublished). The aim of this study is to optimize the levels of the significant factors (ascorbic acid, sodium erythorbate and xylo-oligosaccharide) for the viable counts and encapsulation yield of making the process of XC *Bifidobacterium bifidum* BB01 microcapsules by using Box–Behnken design based on the previous study.

MATERIAL AND METHODS

Strain, media and previous preparation: Starter bacteria of *B. Bifidum* BB01 was obtained from the School of Food and Biological Engineering, Shaanxi University of Science and Technology. They were inoculated in MRS-broth and cultured three generations at 37°C for 24 hours, the cells were collected by centrifugation with 4000 rpm for 10 min at 4°C, and then the cell was washed twice and adjusted viable counts to 10^{11} CFU/ mL by suspending 0.1 ml into 0.9 g/mL sterilized saline at last. MRS-broth and MRS-agar (Hope Bio-Technogy Co., Ltd. Qingdao) were used to culture and count *B. Bifidum* BB01. Xanthan (Zhongxuan biological chemistry Co., Ltd., Shandong, China) was dissolved in deionized water (DIW) to the concentration of 0.68% (w/v), which weight of molecular was 1.02

million, then this solution was sterilized for 10min at 110 ℃. Chitosan (Xingcheng Biological Co., Ltd., Jiangsu, China), which weight of molecular was 0.37 million, was dissolved into stirring HCL (1 mol/L) solution to the 0.76% (w/v) concentration.

Microencapsulation procedure and encapsulation yield: Prebiotics or oxygen scavengers were mixed with the bacterial suspension proportionally that was decentralized in xanthan solution thoroughly. Then the mixture solution was dripped into chitosan solution which placed on the magnetic stirrer. The mixed solution was stirred continuously until the wet capsule was fully formed. The XC (xanthan gum and chitosan) beads loaded with *B. Bifidum* BB01 was obtained thought the wet capsules were filtered and washed 3 times by sterilized saline water (Shoji et al., 2013).

According to the previous experiment, *B. Bifidum* BB01 microcapsules were prepared with chitosan 0.87%, pH 4.24, xanthan gum 0.5%, bacteria suspension and xanthan gum ratio of 1: 3.8 (v/v); the ratio of xanthan gum to chitosan was 1: 7.7 (v/v).

Measurement of viable counts: *Bifidobacteria* were counted using a high-layer agar medium. The test sample was diluted 10 times with sterile saline, 1mL different dilutions of *Bifidobacterium* suspension was injected into the high-level agar medium, incubated at 37℃ for 48-72h, observe the colony growth and counts. Calculate the viable cell number per unit volume of *B. Bifidum* BB01 microcapsules. Viable counts were count according to Eq. (1):

$$Vc = N \times T \quad (1)$$

where V_C represents the viable counts in per milliliter of the original suspension (CFU/mL), N is the average colony number in triplicate anaerobes tubes in the same dilution (CFU). T means dilution times.

Measurement of encapsulation yield: 1g microcapsules were dispersed in 10mL of simulated intestinal juice, after being vibrated with 200 rpm for 35 min at 37 °C. The encapsulation yield would be counted according to Eq. (2):

$$E_Y(\%) = N_1 \times W / (N_0 \times V_0) \times 100 \quad (2)$$

where N_1 (CFU/mL) was viable counts of microcapsules after simulated intestinal juice treatment. N_0 (CFU/mL) was the viable counts in the cell suspension at the outset. W (g) was the weight of the wet microcapsule. V_0 (mL) was the original volume of bacterial suspension used for microcapsule.

Box-Behnken Design (BBD) and statistical analysis: The Box-Behnken design under the response surface methodology (RSM) developed by the Design Expert software (Version 8.0.6) was used to further optimized the process of XC *B. Bifidum* BB01 microcapsules based on the results of single factor and Plackett-Burman experiments. The three significant factors, ascorbic acid (A), sodium erythorbate (B), xylo-oligosaccharide (C) were independent variables, which were studied at three different levels (−1, 0, 1). The actual factors levels were shown in Table 1.

Table 1. The actual factors levels table of BBD of oxygen scavengers and prebiotics

Variable	Factor, %	level		
		-1	0	1
A	ascorbic acid	2.9	3.0	3.1
B	sodium erythorbate	2.3	2.4	2.5
C	xylo-oligosaccharide	4.9	5.0	5.1

RESULTS AND DISCUSSION

Experimental design using BBD of three independent variables including three replicates at the central point is given in Table 2, showing the experimental and predicted response.

Table 2. The experimental design and results of BBD for oxygen scavengers and prebiotics

Run	A	B	C	$Y_1(10^{10}CFU/ml)$	Y_2（%）
1	-1	-1	0	1.03	85
2	-1	1	0	1.27	79
3	1	-1	0	0.89	76
4	1	1	0	1.16	81
5	0	-1	-1	0.84	76
6	0	-1	1	1.12	72
7	0	1	-1	0.93	68
8	0	1	1	0.97	66
9	-1	0	-1	1.27	87
10	1	0	-1	1.18	79
11	-1	0	1	1.35	74
12	1	0	1	1.23	83
13	0	0	0	1.52	95
14	0	0	0	1.49	93
15	0	0	0	1.51	96

Regression analysis of the data

Design-Expert software was applied for regression analysis and model fitting of experimental data based on the experimental results of Table 2. The experiment results analysis was performed by Design-Expert software to fit out equations:

$$Y_1=1.51+0.10A-0.058B+0.0087-0.03C+0.0075-0.03AB-0.06AC-0.0075-0.03BC-0.36A^2-0.063B^2-0.19C^2 \quad (3)$$

$$Y_2=94.67-0.88A-0.75B-2.88C+2.75AB+0.5AC+4.25BC-12.33A^2-2.08B^2-11.83C^2 \quad (4)$$

where Y_1 represents the predicted viable counts of XC *B. Bifidum* BB01 microcapsules (CFU/ml), Y_2 is the encapsulation yield of XC *B. Bifidum* BB01 microcapsules, while A, B and C represents ascorbic acid (w/v), sodium erythorbate (w/v) and xylo-oligosaccharide (w/v), respectively.

Variance analysis

The adequacy of the model was checked using analysis of variance (ANOVA) which was tested using statistical analysis and the results are shown in Table 3. The effects of variables were determined by the F-test, and the more obvious effect on the variables with the lower *p*-value. The R-squared value provided a measure of the variability of the response values that could be explained by the experimental factors and their interactions.

The *p*-value could test the significance of each coefficient and reveal the interaction pattern of independent variables (Mkadmini et al., 2016), if the *p*-value less than 0.05, the corresponding variables would be more significance.

As shown in Table 3, the *p*-value of viable counts (Y_1) demonstrates a high significance for regression equation ($p = 0.0004 < 0.001$), and the *p*-value of lack of fit was 0.0714 ($p > 0.05$), which indicates that the model adequately fitted the experimental data and proves the adequacy of the regression model. What's more, the results showed that for three responses, two independent variables including the ascorbic acid (A) , sodium erythorbate (B) and had significant quadratic effects ($p<0.05$). The order of variables affecting viable counts was as follows: ascorbic acid (A)>sodium erythorbate (B)> xylo-oligosaccharide (C). Simultaneously, the *p*-values of A^2, C^2 indicated the significant effects on viable counts, ($p_{A^2} < 0.0001$, $p_{C^2}=0.0005$) showing that the correlation were not a simple linear relation between response value and variables. The value of determination coefficient R^2,

which was 98.62%, indicated that only 1.38% of the total variations could not be explained by the response model. The value of adjustment coefficient (R_{adj}^2=96.13%) was close to R^2 value showed the significance of the model, which indicated a good relationship between the predictive and the measured value of viable counts. Hence, the model was valid and convenient for predicting viable counts of *B. Bifidum* BB01 microencapsulation under any combination of values of the variables.

Table 3. The ANOVA of viable counts (Y_1) and encapsulation yield (Y_2) as response values

Source	Viable Counts				Encapsulation Yield		
	DF	MS	F	Pr > F	MS	F	Pr > F
Model	9	0.0768	39.6141	0.0004	131.38	34.73	0.0006
A	1	0.0861	44.4261	0.0011	6.13	1.62	0.2592
B	1	0.0265	13.6457	0.0141	4.50	1.19	0.3252
C	1	0.0006	0.3160	0.5983	66.13	17.48	0.0086
AB	1	0.0002	0.1161	0.7472	30.25	8.00	0.0368
AC	1	0.0144	7.4291	0.0415	1.00	0.26	0.6291
BC	1	0.0002	0.1161	0.7472	72.25	19.10	0.0072
A^2	1	0.4675	241.1919	< 0.0001	561.64	148.45	< 0.0001
B^2	1	0.0148	7.6407	0.0396	16.03	4.24	0.0947
C^2	1	0.1275	65.7835	0.0005	517.03	136.66	< 0.0001
Residual	5	0.0019			3.78		
Lack of fit	3	0.0031	13.1786	0.0714	4.75	2.04	0.3462
Pure error	2	0.0002			2.33		
Cor Total	14						

DF: Degree of freedom; MS: mean square; SS: sum of squares.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

The optimized mathematical model about the encapsulation yield of xanthan and chitosan XC *B. Bifidum* BB01 beads showed the effects of ascorbic acid, sodium erythorbate and xylo-oligosaccharides on the response value. It could be used to indicate the interaction between two variable factors as well. According to the results of ANOVA in Table 3, it could be seen that the effect of ascorbic acid (A) on the encapsulation yield of microcapsules was significant. The A^2 and C^2 were very significant, which demonstrated that the simple linear relationship couldn't be used to explain the relevance of each factor. The *p*-value of the model was inferior to 0.001, and the lack of fit *p*-value was superior to 0.05, which showed that the model was available. The regression coefficient R^2 of the obtained equation was 98.43% with a negligible experimental error, showing that the response model could explain that 98.43 % of the variation in the specific activity was explained by the model. The adjusted R^2 (95.59%) also implied statistical significance of the model, which suggested a good relationship between the predictive and the measured value of encapsulation yield with the regression equation.

Two-dimensional contour plot could describe the interactions between independent variables (ascorbic acid, sodium erythorbate, xylo-oligosaccharide) that were significant or not. And the three-dimensional response surface plots were plotted to assess the effect of independent variables on the response value (Zhang et al., 2016). The viable counts of XC *B. Bifidum* BB01 microcapsules was investigated when two varieties kept in experimental range and other variety fixed at zero (Figure 1).

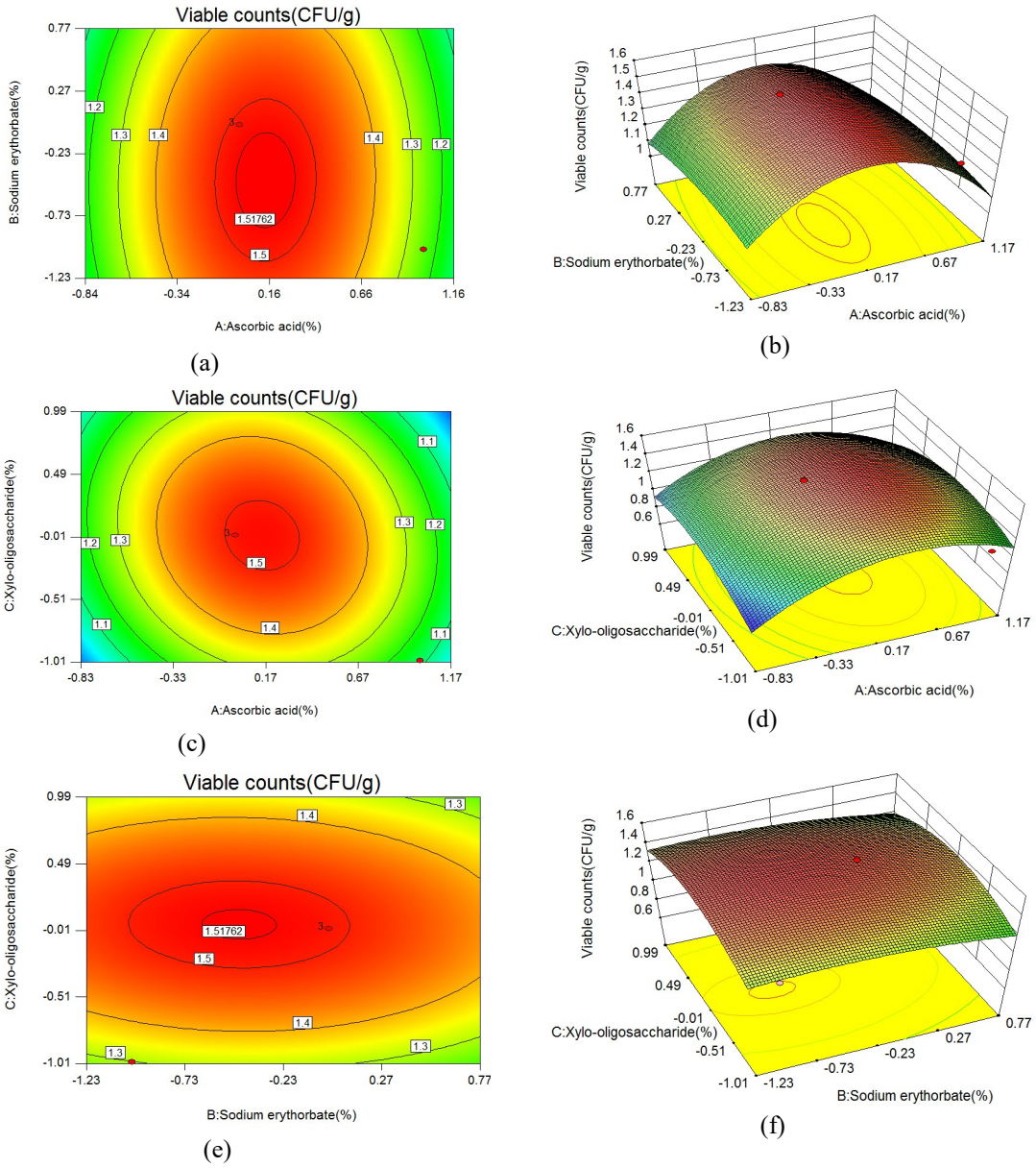

Figure 1. Response surface and contour plots of proportion of ascorbic acid (A), sodium erythorbate (B), xylo-oligosaccharide (C) to viable counts (Y_1)

Elliptical contour plots demonstrated significant interaction between the independent variables (Ghavi, 2015). For ascorbic acid (A) and sodium erythorbate (B) (Figure 1a), the contour plot was oval prominently, which showed strong mutual interaction between parameters A and B, (p_{AB}=0.0368) fit well with the results of interaction terms in variance analysis. The same trend was noticed between parameters B and C (Figure 1e), the mutual interaction between sodium erythorbate (B) and xylo-oligosaccharide (C) was strong, (p_{BC}=0.0072) which indicated the mutual influence between sodium erythorbate and xylo- oligosaccharide was significant.

As shown in Figure 2a that the effect of the addition of ascorbic acid (A) and sodium erythorbate (B) on the encapsulation yield of XC *B. Bifidum* BB01 microcapsules. When the ascorbic acid at a certain level, the encapsulation yield increased with the amount of sodium erythorbate additive increased firstly and then decreased. When the sodium erythorbate at a certain level, the encapsulation yield increased with the amount of ascorbic acid additive increased firstly and then decreased.

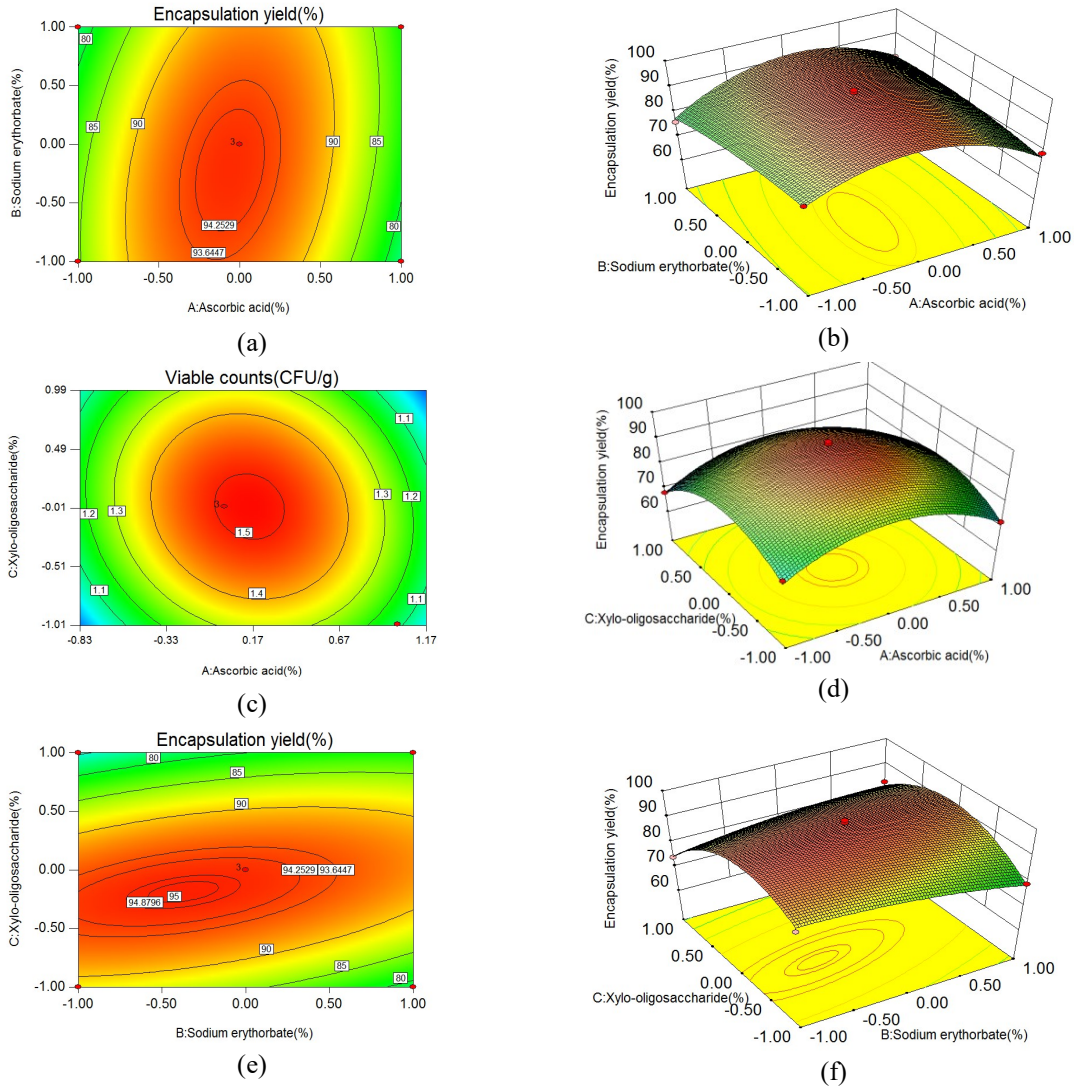

Figure 2. Response surface and contour plots of proportion of ascorbic acid (A), sodium erythorbate (B), xylo-oligosaccharide (C) to encapsulation yield (Y_2)

As the relationship between ascorbic acid (A) and xylo-oligosaccharide (C) (Figure 2c), the contour plot was close to be a circle, showing weak mutual interaction between parameters A and C.

Verification of the model

The obtained ternary quadratic regression equation was analyzed by using the Design-Expert software. The optimal values of independent variables and response variable were as follows: the addition of ascorbic acid (A), sodium erythorbate (B) and xylo-oligosaccharide (C) were 3.0%, 2.36% and 4.99%, respectively. Triplicate confirmatory experiments were carried out under these optimal conditions of addition. The average viable counts and encapsulation yield were $1.50\pm0.12\times10^{10}$ CFU/g and 95±0.8%, respectively, which has been significantly improved compared with un-optimized and provides a reference for the follow-up test. The measured values were in agreement with the predicted ones (viable counts was 1.52×10^{10} CFU/g and encapsulation yield was 94.88%).

The probiotic strains used in foods are usually anaerobic or microaerophilic. Hence, the presence of oxygen in the product may lead to microbial poisoning, death and loss of function (Cruz et al., 2007). Cui added L-Cysteine which usually were used for the culture of *bifidobacteria* as an oxygen scavenger in the process of encapsulation of microcapsules,

increasing the viable counts of microcapsule-embedded greatly (Cui et al., 2000). The oxygen scavenger may promote a more favorable anaerobic environment that has a protective effect on probiotic cells during storage (Shah et al., 2010). The effects of ascorbic acid and sodium erythorbate on viable counts were mainly due to which can eliminate the stress of remaining oxygen in solution. Probiotics can regulate the intestinal microbiota through selectively fermentable prebiotics that play a beneficial role in the growth of bacteria in the colon (Gibson et al., 2004; McCartney & Gibson, 2006; Roberfroid, 2007; Wells et al., 2008). There is a synergistic relationship between probiotics and prebiotics in which the prebiotics as sources of carbon and energy are used by probiotics, promoting the colonization of probiotics in the intestine (Vernazza et al., 2006; Homayouni et al., 2008). Xylo-oligosaccharide is one of the primary prebiotic components that could to provide beneficial health effects to hosts associated with modulation of their microbiota in the market (FAO/AGNS, 2007). The reason is that xylo-oligosaccharide could be the substrates for the metabolism of the probiotic cultures. Therefore, xylo-oligosaccharide could increase the viable counts in processing of XC *B. Bifidum* BB01 microcapsules.

The analysis results of the resistance and stability for probiotics to exposed to simulated gastric fluids (SGF) and solid lipid microparticles (SLM) suggested that the addition of prebiotic components during embedding had increase viable counts compared with free probiotic cells which by the study of Okuro *et al*. (2013). Chen et al. (2017) found good encapsulation systems XC and XCX in yogurt, which protected probiotic *B. Bifidum* BB01 effectively during exposure to adverse environment conditions and the single-layer microcapsules XC showed better release profile in SIF. A study by Chen et al. (2014) showed that sodium erythorbate was used in the production process of XC *B. Bifidum* BB01 microcapsule, the corresponding viable counts and encapsulation yield of microcapsules were 2.9×10^9 CFU/ml, 82%, respectively. The addition of 3.2g/L sodium erythorbate during the fermentation process could effectively reduce the stress caused by oxygen on *B. bifidum*, and the viable counts in the fermentation broth reached 1.06×10^9 CFU/ml, which was demonstrated by Li et al. (2008). The results of all above studies were lower than my research. The reason was the synergistic effect of three factors occurred probably in my study, which result to a better performance.

CONCLUSION

According to the results of BBD, three groups of repeated experiments were carried out on the optimized conditions (3.0% ascorbic acid addition, 2.36% addition of sodium erythorbate and 4.99% xylo-oligosaccharide added). The viable counts of XC *B. Bifidum* BB01 reached to 1.52×10^{10}CFU/g from 1.25×10^{10} CFU/g and the encapsulation yield reached to 94.88% from 90% under the optimum conditions, which were increased 21.6% and 5.4%, respectively. There was no significant difference between the verification value and the predicted value, which proved the effectiveness of the model. There were few researches on adding probiotics and oxygen scavengers during the process of preparing microcapsules and increase the viable counts of microcapsules was a difficult problem in microcapsule processing simultaneously at present. This research provides the basis for future research on XC *B. Bifidum* BB01 microcapsule encapsulation technology.

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