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ORIGINAL RESEARCH PAPER

OPTIMIZATION OF PREBIOTICS AND OXYGEN SCAVENGERS FOR MICROCAPSULES OF *BIFIDOBACTERIUM BIFIDUM BB01* BY TWO-LEVEL FRACTIONAL FACTORIAL DESIGN

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Abstract: In this paper, protection effects of four oxygen scavengers including sodium erythorbate, ascorbic acid, sodium ascorbate, cysteine hydrochloride and five prebiotics including stachyose, Fructo-oligosaccharide (FOS), Isomalto-oligosaccharide (IOS), Xylo-oligosaccharide (XOS), Galacto-oligosaccharides (GOS) on the microencapsulation of xanthan gum / chitosan *Bifidobacterium bifidum BB01* were investigated by single factor experiment and two-level fractional factorial design. The single factor experiment showed that ascorbic acid, sodium erythorbat in oxygen scavengers and XOS, GOS in prebiotics could get higher viable counts and encapsulation yield for preparation of *Bifidobacterium bifidum BB01* microcapsules. It also indicated that the variable factors such as sodium erythorbate, XOS and ascorbic acid were significant both for viable counts and encapsulation yield by two-level fractional factorial design. Therefore, these three factors can be further optimized to improve the protection of probiotics by microcapsule technology. So that more probiotics reach the intestines and have a beneficial effect on the human body.

Keywords: *Bifidobacterium bifidum, encapsulation yield, microencapsulation, single factor experiment, two-level fractional factorial design, viable counts*

INTRODUCTION

Probiotics are generally divided into two broad categories, one of which refers to living probiotic products and the other to dead probiotic products including bacterial components and their metabolites [1]. For example, adding probiotics to goat milk powder can be used to obtain probiotic goat milk tablets [2], also Kefir was obtained by probiotic fermentation [3]. All of these probiotic products help the body's flora to maintain balance, which is beneficial to human health [4].

A large number of publications reported that the probiotics are widely distributed and have many kinds, which can be divided into the following three categories in general [5 – 8]. (1): probiotic bacteria *Lactobacillus*, including *L. acidophilus*, *L. plantarum, L. casei* etc.; (2): probiotics of *bifidobacteria*, mainly including *B. bifidum, B. adolescentis, Bifidobacterium infanfis* etc.; (3): part of Gram-positive cocci probiotics, including *S. thermophilus, E. faecalis* and so on. There are two kinds of probiotics *Lactobacillus* and *Bifidobacterium* commonly used by people at present [9]. *Lactobacillus* and *Bifidobacterium* have shown beneficial effects on immunomodulation and the decrease and prevention of various intestinal diseases [10, 11]. *Bifidobacteria* [12] belong to the genus *Bifidobacterium* of the actinomycetes family, anaerobic, Gram-positive. *B. Bifidum* is a very important type of intestinal probiotics, which has important physiological and healthy functions on the human body [13]. It can synthesize a variety of digestive enzymes and vitamins. And it is one of the important signs of human health.

Probiotics must be kept in sufficient quantity in the human body to perform their health functions [14]. However, most probiotics strains are easily affected by the surrounding environment because of their own characteristics. Therefore, it is difficult to maintain the quantity and activity of probiotics. The activity and quantity of probiotics are greatly reduced after most living probiotics pass through the stomach and small intestines. So improving the activity and quantity of probiotics has become the problem which we need to solve. Technology of embedding probiotics with microcapsule has become an effective method to solve the above problems. Microcapsule technology can effectively protect probiotics from adverse environmental effects. Thereby, a large number of active probiotic bacteria enter the intestinal tract to exert a healthy effect [15, 16].

Extrusion method was first proposed by Schultz in 1956 [17]. In the study of probiotic microencapsulation, the basic operation of extrusion method was to mix the probiotic with hydrophilic colloid [18]. Then, the mixed suspension of the bacteria was put into the fixed solution as a liquid drop by the injection needle. The microcapsule wall material prepared by the extrusion method is generally a water-soluble or fat-soluble polymer. Wall materials may affect the efficacy of capsules in protecting the encapsulated bacteria. Some materials such as arabic gum, alginate, gelatin, malt dextrin, pectin, skim milk, starch and chitosan had been used to microencapsulate probiotics [19 – 25]. The extrusion method has the advantages of simple process, easy operation and scalability.

This topic changed the wall material based on the previous research and used the compounding of xanthan gum and chitosan as the wall material to embedded the *Bifidobacteria* and *L. acidophilus*. Chitosan and xanthan gum [26] are natural biological macromolecules with good biocompatibility and non-toxic side effects. Chitosan is soluble in acidic aqueous solution; which amino group is positively charged in acidic

solution. It can be used to form a co-gel with the negatively charged anionic polysaccharide xanthan gum through polyelectrolyte. This co-gel can be used as a microcapsule wall material.
This subject used a single factor experiment to study the effect of four oxygen scavengers (sodium erythorbate, ascorbic acid, sodium ascorbate, cysteine hydrochloride) and five prebiotics (stachyose, FOS, IOS, XOS, GOS) on the microencapsulation of xanthan gum / chitosan *Bifidobacterium bifidum BB01*. Two-level fractional factorial design test was used to screen out the main factors influencing the xanthan gum / chitosan preparation of *Bifidobacterium bifidum BB01* microcapsules.

MATERIALS AND METHODS

Materials

Bifidobacterium bifidum BB01 was supplied by Shaanxi University of Science and Technology. (Xi'an, China). MRS-broth and MRS-agar (Hope Bio-Technogy Co., Ltd. Qingdao, China). Xanthan (Zhongxuan biological chemistry Co., Ltd., Shandong, China). Chitosan (Xingcheng Biological Co., Ltd., Jiangsu, China). Four oxygen scavengers including sodium erythorbate, ascorbic acid, sodium ascorbate and cysteine hydrochloride (Sigma Co., Ltd. USA). Five prebiotics including stachyose, FOS, IOS, XOS, GOS (Robertson Technology Co., Ltd. Xi'an, China).

Preparation of xanthan gum/chitosan *Bifidobacterium bifidum BB01* microcapsules

The *Bifidobacterium bifidum BB01* activated to the third generation were collected by centrifugation (10000 rpm, 12 min). Then the bacterial sludge of *Lactobacillus acidophilus* and *Bifidobacterium bifidum BB01* was prepared into a bacterial suspension by using 1 mL of 0.9 % sterile saline. Prebiotics or oxygen scavengers were mixed in proportion with the bacterial suspension, which was dispersed in xanthan solution thoroughly. The mixture was dripped into chitosan solution placed on magnetic stirrer through a manually operated syringe with 0.7 mm cannula. The chitosan solution was stirred constantly to make capsules cross bonding. The mixed solution was stirred continuously for 47 min until the wet capsule is fully formed. Then wet capsules were filtered and washed by sterilized saline water for 3 times. The XC (xanthan and chitosan) beads loaded with *Bifidobacterium bifidum BB01* was obtained [27]. The initial conditions for the preparation of *Bifidobacterium bifidum BB01* microcapsules were chitosan 0.87 %, *p*H 4.24, xanthan gum 0.5 %, the ratio of bacterial suspension to xanthan gum was 1:3.8 (v / v) and xanthan gum to chitosan was 1:7.7 (v / v).

Determination of viable counts and encapsulation yield

Bifidobacterium bifidum BB01 were counted by using a high-layer agar medium. The test sample was diluted 10 times with sterile saline. Then 1 mL different dilutions of *Bifidobacterium bifidum BB01* suspension was injected into the high-level agar medium, incubated at 37 °C for 48-72 h, observed the colony growth and counts. The viable counts were calculated according to (Equation 1):

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

where: VC represents the viable counts per milliliter of the original suspension (CFU·mL^{-1}), N is the average colony number in triplicate anaerobes tubes in the same dilution (CFU), T means dilution times.

Encapsulation yield: One gram of fresh microcapsules was dispersed in 10 mL of simulated intestinal juice, after being shaked at 37 °C for 40 min under 210 rpm. The encapsulation yield was calculated according to (Equation 2):

$$EY(\%) = \frac{N1\times M}{N0\times V0} \times 100\% \quad (2)$$

where: N1 (CFU·mL^{-1}) was viable counts of microcapsules after subjected to simulated intestinal juice, M (g) was the weight of the wet microcapsule, N0 (CFU·mL^{-1}) was the initial viable counts in the cell suspension, V0 (mL) was the volume of original bacteria liquid using for microcapsule.

Data statistical analysis

The statistical analysis was performed by Origin (Version 9, Origin Lab Inc., Alexandria, VA, USA) and Design-Expert (DOE Version 8.0.6, Stat-Ease. Inc, Minneapolis, MN, USA) to identify the significant factors and determine the best factors species finally.

RESULTS AND DISCUSSION

Effect of different deoxidants and prebiotics on viable counts and encapsulation yield of *Bifidobacterium bifidum BB01* of microcapsules

Four oxygen scavengers (ascorbic acid, sodium ascorbate, cysteine hydrochloride, sodium erythorbate) are respectively mixed with the bacteria glue added in the preparation process of xanthan gum/polysaccharide chitosan BB01 microcapsules. The amount of oxygen scavenger which is added is 1 % of the total volume of the gelatin. Then measured the viable counts and encapsulation yield of *Bifidobacterium bifidum BB01*. As exhibited in Figure 1, ascorbic acid and sodium erythorbate have a good effect on xanthan/chitosan *Bifidobacterium bifidum BB01* microcapsules under the same concentration conditions.

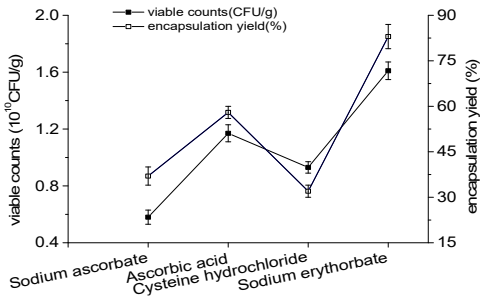

Figure 1. *Effect of oxygen scavengers on viable counts and encapsulation yield of Bifidobacterium bifidum BB01 of microcapsules*

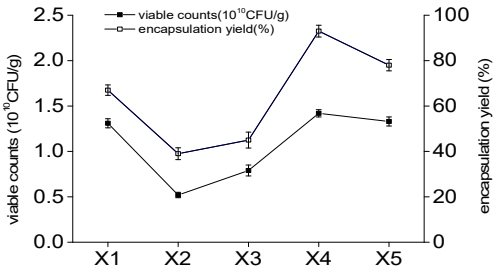

Figure 2. *Effect of prebiotics on viable counts and encapsulation yield of Bifidobacterium bifidum BB01 of microcapsules*

And the following five kinds of prebiotics (stachyose, FOS, IOS, XOS, GOS) were added during the preparation of xanthan gum/chitosan *Bifidobacterium bifidum BB01* microcapsules respectively, each prebiotic were mixed with the bacterial suspension of *Bifidobacterium bifidum BB01* suspension in an amount of 5 %. Then measured the viable counts and encapsulation yield of *Bifidobacterium bifidum BB01*. As exhibited in Figure 2 (X1 was stachyose, X2 was FOS, X3 was IOS, X4 was XOS, X5 was GOS), XOS and GOS have a great effect on the xanthan/chitosan *Bifidobacterium bifidum BB01* microcapsules under the same concentration.

Effect of ascorbic acid and sodium erythorbate on xanthan gum/chitosan *Bifidobacterium bifidum BB01* microcapsules

In the light of the optimized encapsulation yield conditions of xanthan gum/chitosan *Bifidobacterium bifidum BB01* microcapsules, the ascorbic acid and sodium erythorbate were mixed with the bacterial glue according to the addition amount of 1, 2, 3 and 4 % respectively. The method of microcapsule preparation followed by mentioned previous. The results were shown in Figures 3 and 4.

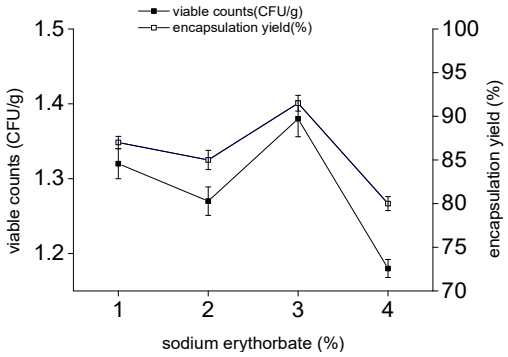

Figure 3. *Effect of sodium erythorbate on viable counts and encapsulation yield of Bifidobacterium bifidum BB01 of microcapsules*

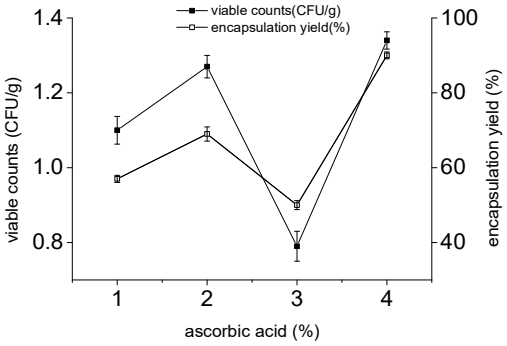

Figure 4. *Effect of ascorbic acid on viable counts and encapsulation yield of Bifidobacterium bifidum BB01 of microcapsules*

The viable counts and encapsulation yield of the xanthan/chitosan *Bifidobacterium bifidum BB01* microcapsules were correlated with the amount of sodium erythorbate negatively on the beginning. But the range of variation of this trend was not large and then followed a positive trend. The encapsulation yield and viable counts of *Bifidobacterium bifidum BB01* microcapsules approached the maximum when the 3 % sodium erythorbate was added，which can be seen from Figure 3.

The results showed that when the amount of ascorbic acid added was less than 3 %, the oxygen in the bacterial gum mixture was not removed due to the amount of ascorbic acid added was relatively small. However, increasing the amount of sodium erythorbate would change the concentration structure of xanthan gum, which leads to reductions of viable counts and encapsulation yield of *Bifidobacterium bifidum BB01* microcapsules.

As shown in Figure 4, the viable counts and encapsulation yield of the *Bifidobacterium bifidum BB01* microcapsules increased slightly and then decreased with the amount of ascorbic acid added increases, which was different from sodium erythorbate. The viable counts and encapsulation yield showed a greater blessing with the further increase of ascorbic acid. The reason was the increasing amount of ascorbic acid would remove oxygen in the bacteria gum, which made *Bifidobacterium bifidum BB01* far from away oxygen poisoning.

The *Bifidobacterium bifidum BB01* microcapsules with oxygen scavenger had higher viable counts and encapsulation yield compared with the control. Therefore, in the procedure of the microencapsulated *Bifidobacterium bifidum BB01*, the optimal addition of sodium erythorbate and ascorbic acid were initially determined to be 3 % and 4 % respectively, which corresponding viable counts and encapsulation yield were 1.38 × 10^{10} CFU·g^{-1}, 91.5 % and 1.34 × 10^{10} CFU·g^{-1}, 90 % respectively.

Effect of GOS and XOS on xanthan/chitosan *Bifidobacterium bifidum BB01* microcapsules

Under the optimum encapsulation conditions of xanthan/chitosan *Bifidobacterium bifidum BB01* microcapsules, the GOS and XOS were respectively mixed with the *B. bifidum* according to the addition amount of 1, 2, 3 and 4 %. The result was shown in Figures 5 and 6.

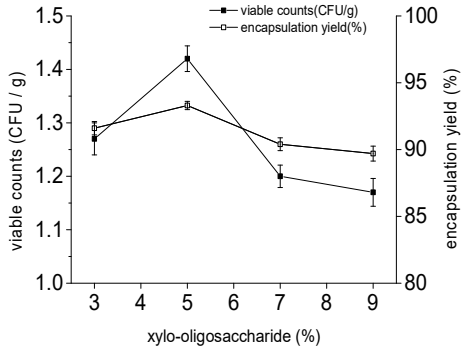

Figure 5. *Effect of xylo-oligosaccharide on viable counts and encapsulation yield of Bifidobacterium bifidum BB01 of microcapsules*

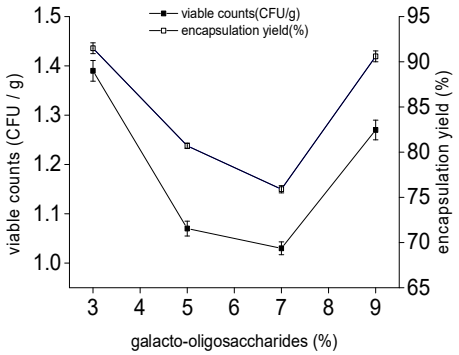

Figure 6. *Effect of galactooligosaccharide on viable counts and encapsulation yield of Bifidobacterium bifidum BB01 of microcapsules*

The viable counts and encapsulation yield of xanthan/chitosan *Bifidobacterium bifidum BB01* microcapsules varied with the addition of the XOS, which could be seen from Figure 5. The viable counts and encapsulation yield of the microcapsules were positively correlated the addition of XOS from 3 % to 5 %. The viable counts and encapsulation yield of *Bifidobacterium bifidum BB01* microcapsules reached the maximum when the amount of XOS addition was 5 %. The small addition relatively of XOS made the viable counts and encapsulation yield of microcapsules small. Probably because XOS not played a good role in the proliferation and promotion of probiotics when the addition of XOS was less than 5 %. However, increasing the amount of XOS may be decreasing the viable counts of *Bifidobacterium bifidum BB01* because of the large amount of prebiotics changing the concentration structure of xanthan gum.
The viable counts and encapsulation yield of *Bifidobacterium bifidum BB01* microcapsules were negatively correlated with the addition of GOS, which would be shown from Figure 6. The reason was that with the increases of the GOS, the concentration structure of xanthan gum was changed, which further affected the encapsulation yield and viable counts of microcapsules.
B. bifidum microcapsules with prebiotics had higher viable counts and encapsulation yield compared with the control. Therefore, in the procedure of the microencapsulated *Bifidobacterium bifidum BB01*, the optimal addition of XOS and GOS were initially determined to be 5 and 3 % respectively, which corresponding viable counts and encapsulation yield were 1.42×10^{10} CFU·g^{-1}, 93.3 % and 1.39×10^{10} CFU·g^{-1}, 91.5 % respectively.

Screening of the main factors of oxygen scavenger and prebiotics for preparation xanthan / chitosan *Bifidobacterium bifidum BB01* microcapsules

In order to screen out the factors that have a significant impact on the test results, the two-level fractional factorial design was be conducted on the basis of the oxygen scavenger and prebiotic single-factor test. The level coding of each factor was shown in Table 1.

Table 1. *The factors levels for oxygen scavengers and prebiotics of two-level fractional factorial design of Xanthan gum/chitosan Bifidobacterium bifidum BB01 microcapsules*

Symbols	Factors	Level	
		-1	+1
A	Sodium erythorbate [%]	2.4	3
C	GOS [%]	2.4	3
E	XOS [%]	4	5
G	Ascorbic acid [%]	3	4

The two-level fractional factorial design test design and results were shown in Table 2.

Table 2. *The experimental design and results for oxygen scavengers and prebiotics of two-level fractional factorial design of Xanthan gum/chitosan Bifidobacterium bifidum BB01 microcapsules*

RUN	A	B	C	D	E	F	G	Y1 [10^{10} CFU·g^{-1}]	Y2 [%]
1	-1	-1	-1	1	1	1	-1	1.48	94.1
2	1	-1	-1	-1	-1	1	1	1.32	92.2
3	-1	1	-1	-1	1	-1	1	1.41	93.7
4	1	1	-1	1	-1	-1	-1	1.37	92.7
5	-1	-1	1	1	-1	-1	1	1.36	93.2
6	1	-1	1	-1	1	-1	-1	1.45	93.9
7	-1	1	1	-1	-1	1	-1	1.47	93.7
8	1	1	1	1	1	1	1	1.39	92.8

The response values Y1 and Y2 represent the viable counts and encapsulation yield of *Bifidobacterium bifidum BB01* microcapsules respectively. The software was used to analyze the above test results. And choosing the factors with higher significance to performed the variance analysis. Tables 3 and 4 showed the analysis of variance.

Table 3. *Variance Analysis of Selected Factors for Y1*

Source	SS	DF	MS	F Value	p-value (Prob > F)	
Model	0.022	4	0.005	15.6	0.0239	*
A-A	0.005	1	0.005	13.0	0.0364	*
C-C	0.001	1	0.001	2.9	0.1856	
E-E	0.006	1	0.006	15.9	0.0281	*
G-G	0.011	1	0.011	30.4	0.0117	*
Residual	0.001	3	0.000			
Cor Total	0.023	7				

SS: sum of squares; DF: Degree of freedom; MS: mean square; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

It was demonstrated from Table 3, the p value of the experimental model is 0.0239, indicating that the model has a significant effect on the Y1. Analysis of various factors found that sodium erythorbate (A) ($p = 0.0364$), XOS (E) ($p = 0.0281$) and ascorbic acid (G) ($p = 0.0117$) were significant. The significance factors were: G> E>A.

Table 4. *Variance Analysis of Selected Factors for Y2*

Source	SS	DF	MS	F Value	p-value (Prob > F)	
Model	2.995	4	0.749	14.6	0.0262	*
A-A	1.201	1	1.201	23.4	0.0168	*
C-C	0.101	1	0.101	2.0	0.2545	
E-E	0.911	1	0.911	17.8	0.0244	*
G-G	0.781	1	0.781	15.2	0.0298	*
Residual	0.154	3	0.051			
Cor Total	3.149	7				

SS: sum of squares; DF: Degree of freedom; MS: mean square; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

It was illustrated from Table 3, the p-value of the experimental model is 0.0262, indicating that the model was significant. Analysis of various factors found that sodium

erythorbate (A) (p = 0.0168), XOS (E) (p = 0.0244) and ascorbic acid (G) (p = 0.0298) were significant. Sort the significance of factors: A>E>G.

The purpose of this experiment was to choose the factors that affect the viable counts and the encapsulation yield simultaneously. Therefore, based on the influence of the viable counts and the encapsulation yield, the factors A, E and G which have a higher influence on Y1 and Y2 were chosen. The effects of these three factors on Y1 and Y2 were shown in Figures 7 and 8 respectively. The positive or negative of the slope of the trend line indicates the positive or negative effect of this factor on the response value. Therefore, C and E had a positive effect on Y1 and Y2 and A and G had a negative effect on Y1 and Y2 within the selected concentration range. Hence, A and G should be reduced in subsequent experiments, C and E should be increased in subsequent trials.

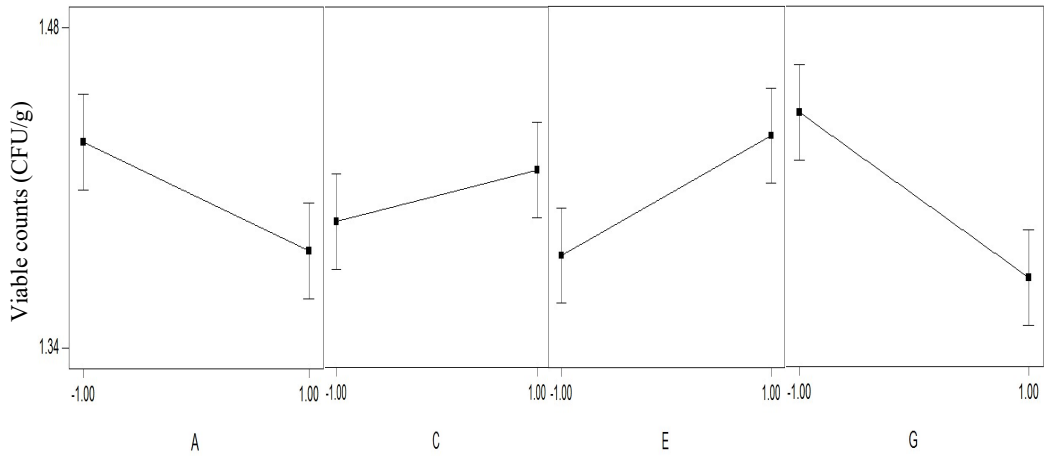

Figure 7. *The 95 % confidence interval of the variable factor to viable counts Y1*

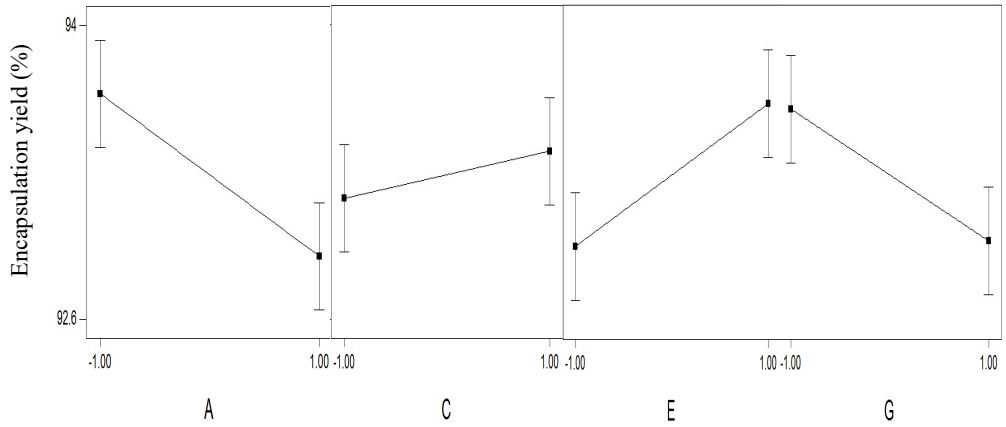

Figure 8. *The 95 % confidence interval of the variable factor to encapsulation yield Y2*

From Figures 7 and 8, it can be seen that the variable factors Ascorbic acid (G), Sodium erythorbate (A) and XOS (E) were significant both for viable counts and for encapsulation yield. As determined by Figures 7 and 8, the variable factor XOS (E) has a positive effect on response (Y1 and Y2), indicated the response values increases with increasing E. All remaining variables the factor was a negative effect, imply the response values Y1 and Y2 decrease as their concentration increases. For the remaining factors B, D and F which could be ignored were dummy entries. Therefore, to screen

out the more significant factors were A (Sodium erythorbate), E (XOS), G (Ascorbic acid) through the two-level fractional factorial design screening test, which can be considered important factors for further optimization test.

DISCUSSION

Probiotics are endowed with the ability to modulate the intestinal microbiota. And the presences of prebiotics, ingredients that are selectively fermentable, exert a beneficial effect on the growth and activity of bacteria in the colon [28 – 31]. There is a synergistic relationship between probiotics and prebiotics. The prebiotics are consumed by probiotics as sources of carbon and energy. So probiotics are easier to colonize in the intestine than pathogenic microorganisms [32, 33]. XOS is one of the main prebiotic components available in the market, being capable to provide beneficial health effects to hosts associated with modulation of their microbiota [34]. The reason for this trend is that they are substrates available for the metabolism of the probiotic. Therefore, XOS could increase the viable counts in processing of microencapsulation.
According to the results of single factor test and two-level fractional factorial design, main factors affecting the oxygen scavengers and prebiotics of the preparation of the microencapsulated *Bifidobacterium bifidum BB01* were screened. The result showed A (Sodium erythorbate), E (XOS), G (Ascorbic acid) had a significant effect on the viable counts and encapsulation yield of xanthan/chitosan *Bifidobacterium bifidum BB01* microcapsules, and the *p*-value of the above-mentioned factors were all less than 0.05. In statistics, the factor whose confidence level is greater than 95 % ($0.01< p <0.05$) was defined as a significant factor. Thus, A (Sodium erythorbate), E (XOS), G (Ascorbic acid) were important factors. A study by Chen *et al* [35] showed that sodium erythorbate was used in the production process of microencapsulated *Bifidobacterium bifidum BB01*, the corresponding viable counts and encapsulation yield of microcapsules were 2.9×10^9 CFU·mL^{-1}, 82 % respectively. 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 probiotics compared with free probiotic cells which by the study of Okuro *et al* [36].

CONCLUSION

Based on the result of single factor experiment, the effects of four kinds of oxygen scavengers and five kinds of prebiotics on viable counts and encapsulation yield of *Bifidobacterium bifidum BB01* microcapsules were studied by using two factorial design. The results showed that A (Sodium erythorbate), E (XOS), G (Ascorbic acid) had a significant effect on viable counts of BB01 microcapsules. Among the above three factors, the influence of these three factors on the viable counts of BB01 microcapsules: G (Ascorbic acid)> E (XOS)> A (Sodium erythorbate). At the same time, A, E and G had a positive effect on the encapsulation yield of *Bifidobacterium bifidum BB01* microcapsules. In addition, the influence of these three factors on viable counts of *Bifidobacterium bifidum BB01* microcapsules: A (Sodium erythorbate)> E (XOS)> G (Ascorbic acid). Therefore, these three factors can be further optimized to improve the

protection of probiotics by microcapsule technology. So that more probiotics reach the intestines and have a beneficial effect on the human body.

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