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OPTIMIZATION OF NITROGEN SOURCE FOR *BIFIDOBACTERIUM BIFIDUM* USING RESPONSE SURFACE METHODOLOGY

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Abstract: In order to improve the viable counts of *Bifidobacterium bifidum* BB01 in the liquid medium, the Central Composite Design (CCD) was used to optimize the nitrogen source in the medium of *B. bifidum* BB01. The results showed that the nitrogen source composition of *B. bifidum* BB01 was: peptone 0.9%, yeast extracts 0.3%, beef paste 0.7%. Under the optimal conditions, the viable counts of *B. bifidum* BB01 reached (2.49±0.06)×109CFU/mL after cultured at 18h, which was 42.97% higher than MRS (lactose), and 12.85% higher than the optimized MRS medium (carbon source and prebiotics were optimized). Therefore, the CCD used in this study is workable for promoting the growth of *B. bifidum BB01*.

Keywords: *Bifidobacterium bifidum*, response surface methodology, optimization, nitrogen source

INTRODUCTION

Bifidobacterium bifidum was originally isolated from the feces of healthy infants fed by the French Pasteur Research Institute of the French Tissier in 1899 (Annan et al., 2007). *B. bifidum* was important constituents of the intestinal macrobiotic for humans (Rada et al., 2006; Homayouni et al., 2008). It has been

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reported that the physiological functions of *B. bifidum* could be got as follows: maintain the intestine bacterial flora balance and prevent constipation; reduce blood cholesterol levels and prevent hypertension; improve lactose tolerance of dairy products; enhance the tolerance of host to radiation (Mokarram et al., 2009; Guerin et al., 2003; Weinbreck et al., 2010), it also can enhance the activity of the immune system, as well as decreased on the level of serum cholesterol (Altieri et al., 2008). At present, the production of *B. bifidum* mainly concentrated upon drugs, health products and foods. However, the development of these products were main lie in the viable counts of the bacteria, thus, the optimization of the culture medium was the key technology to improve quality of product.

Response surface methodology (RSM) is a method of optimizing the experimental conditions developed in the back lobe in twentieth Century (Almeida et al., 2008). It can evaluate interaction relationship between the factors by constructing and exploring multivariate quadratic function relationship for response variables and design variables, and obtain the optimal process parameters through the analysis of the regression equation. The single factor and orthogonal experiment may not find the optimal combination of the factors and the optimal response value in the whole region (Ye et al., 2011; Sun et al., 2010). The RSM has been recently used on model and optimization of biological process, such as optimization of medium composition (Manikandan et al., 2009; Gao et al., 2005), and cultivation process for biomass production (Nikerel et al., 2006; Zhang et al., 2015).

B. bifidum has strict nutritional requirement, the culture medium not only needs amino acids, vitamins, sugar and buffer salts, but also the carbon source and nitrogen source. Janer et al (Janer et al., 2004) found that 2% whey protein and 2% casein added into milk can increase viable counts of *B. bifidum* both, Espinosa-Martos et al (Espinosa et al., 2009) found that utilization efficiency of nitrogen source (bean cake powder) for *B. bifidum* was much higher than the *Lactobacillus acidophilus*. Zeng et al (Zeng et al., 2006) optimized the production of new type ketal compounds and allenic ethers from a mangrove fungus, using response surface methodology to optimized of nitrogen source composition of medium (glucose, peptone and yeast extract).To promote cell growth, shorten the growth cycle and improve economic benefits, The purpose of this study is to optimize the medium nitrogen source using central composite design based on the previous work. And provide theoretical basis for the industrialized production of *B. bifidum* freeze-dried powder.

MATERIALS AND METHODS

Microorganism and medium: *Bifidobacterium bifidum* BB01 obtained from School of Food and Biological Engineering, Shaanxi University of Science & Technology. MRS (Lactose) medium was used for strain activation and fermentation; MRS agar medium was used for the determination of the viable counts.

Strain activation: The *B. bifidum* freeze dried powder was added in MRS (lactose) broth and fully mixing, and cultured at 37℃ for 24h. The microscopic examination was conducted to make sure there was no bacteria in culture medium, and then 5% samples was added in the liquid medium to activate three times, eventually cultured at 37℃ for 18h. The samples were storage in the refrigerator when OD value met certain requirements.

The determination of OD value, pH and viable counts: The absorbance determination of OD value: using spectrophotometer to measure the OD value of the medium under the condition at 600nm. The medium was not inoculated bacteria or not add the test factors which used as a blank sample. The pHs-3c acidimeter was used for the determination of pH. The viable counts were determined by the spread plate technique using MRS agar medium.

Experimental design and date analysis: The parameters of peptone, yeast extract and beef paste which based on previous test results were the independent variable, and the viable counts of *B. bifidum* BB01 was used as the response value in the medium. A three-factor, five-level CCD (N=23) was conducted to determined the optimal medium nitrogen compound composition for *B. bifidum* BB01. All the data analysis and model constructed were using software SAS 9.1.

RESULTS AND DISCUSSIONS

Optimization of the screened variables: The factors level coding values of CCD were presented in Table 1.

Table 1. The factors level coding table of CCD

factors level	X1 (%)	X2 (%)	X3 (%)
-1.682	0.7318	0.1318	0.5318
-1	0.8	0.2	0.6
0	0.9	0.3	0.7
1	1	0.4	0.8
1.682	1.0682	0.4682	0.8682

The experimental design and the result of response value Y1 (the viable counts of *B. bifidum* BB01 in the medium) were presented in Table 2.

Table 2 The experimental design and results of CCD

RUN	X1	X2	X3	Y1 ($\times 10^9$CFU/mL)
1	-1	-1	-1	2.16
2	-1	-1	1	2.28
3	-1	1	-1	2.34
4	-1	1	1	1.86
5	1	-1	-1	1.92
6	1	-1	1	1.85
7	1	1	-1	2.24
8	1	1	1	1.92
9	-1.682	0	0	2.27
10	1.682	0	0	2.15
11	0	-1.682	0	1.93
12	0	1.682	0	1.90
13	0	0	-1.682	2.12
14	0	0	1.682	2.13
15	0	0	0	2.55
16	0	0	0	2.51
17	0	0	0	2.44
18	0	0	0	2.44
19	0	0	0	2.41
20	0	0	0	2.45
21	0	0	0	2.58
22	0	0	0	2.44
23	0	0	0	2.62

The CCD data were analyzed by multiple regression analysis, and then got a polynomial equation as follows:

$$Y1=2.491033-0.066403\times X1+0.008021\times X2-0.0053316\times X3-0.097107\times X1\times X1+0.07675\times X1\times X2-0.0045\times X1\times X3-0.2026434\times X2\times X2-0.1055\times X2\times X3-0.12609\times X3\times X3$$

where Y1(10^9CFU/mL) represents the predicted value of viable counts of *B. bifidum* in the culture medium; X1, X2 and X3 represent peptone, yeast extract and beef paste, respectively.

Analysis of variance (ANOVA) is used to analyze the significance of the

multiple groups. The *p*-value represents the influence of the variables, and the R-squared value indicates the fitting degree of polynomial equation. (Siti Aminah et al., 2006). The analysis of variance results of the regression equations were shown in Table 3.

Table 3 The ANOVA of Central Composite Design

SOURCE	DF	SS	MS	F-value	*P*-value	Sig.
X1	1	0.0602	0.0602	7.5140	0.0168	*
X2	1	0.0009	0.0009	0.1097	0.7458	
X3	1	0.0388	0.0388	4.8441	0.0464	*
X1*X1	1	0.1498	0.1498	18.6962	0.0008	**
X1*X2	1	0.0471	0.0471	5.8802	0.0306	*
X1*X3	1	0.0002	0.0002	0.0202	0.8891	
X2*X2	1	0.6525	0.6525	81.4168	0.0001	***
X2*X3	1	0.0890	0.0890	11.1106	0.0054	*
X3*X3	1	0.2527	0.2527	31.5262	0.0001	***
Model	9	1.2788	0.1421	17.7295	0.0001	***
Linear	3	0.0999	0.0333	4.1560	0.0286	*
Quadratic	3	1.0425	0.3475	43.3623	0.0001	***
Cross product	3	0.1363	0.0454	5.6703	0.0104	*
Error	3	0.1042	0.0080			
Lack of fit	5	0.0589	0.0118	2.0791	0.1707	
Pure error	8	0.0453	0.0057			
Total	2	1.3830				

Note. ***p<0.0001, extremely significant; **p<0.001, very significant; *p<0.05, significant; DF refers to degrees of freedom, SS refers to sum of squares, MS refers to mean square.

The variance analysis shown that the regression model's F-value was 17.7295, it was extremely significant, indicating that the test method was reliable; the correlation coefficient R^2 was 92.47%, which indicated the regression equation was fitted well. So it can be used to predict the response value. Besides, the linear effect of factors X1 and X3 on the response value of *B. bifidum* was significant, and squared terms were significant, which indicated that the response value and factors were not a simple linear relationship.

The higher F-value of interactive items X1*X2 and X2*X3, the more significant interaction between X1 and X2, X2 and X3, while low F-value of interactive item X1*X3, which indicated that the interaction between X1*X3 was not significant. Lack of fit for F-value (P=0.17>0.05) indicating that the equation fitted well with the experiments.

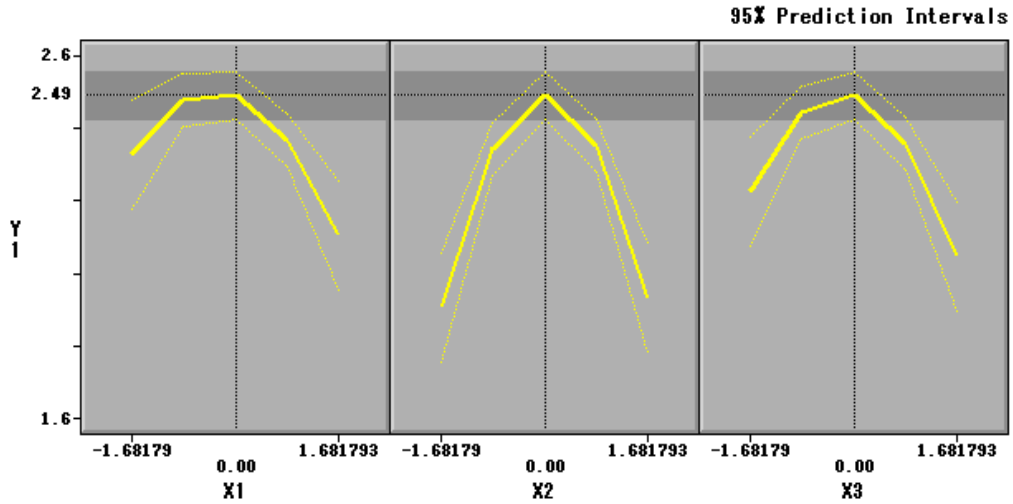

Figure 1. The trends of Y1 with factors.

X1*X1 was very significant (p=0.0008<0.001), both X2*X2 and X3*X3 were extremely significant (p=0.0001). The effects of the three factors response value of Y1 were very significant, respectively, which were shown in Figure 1, and the extreme value appeared in the inflection point. The response Y1 increased slowly with the increase of parameters of X1; but when it reached the biggest value at the central point, the response Y1decreased with the increase of parameters of X1. The effects of X3 on the response Y1 were the same with parameter of X1. While the effects of X2 on the response value of Y1 were stable, and the increase and decline of Y1 were similar, reached the biggest value pre and post.

According to the equation, the 3D response surface plots and 2D contour plots were obtained by using the software SAS 9.1 (Figures 2-4).

Figure 2 and 4 shown that the 2D contour plots seemed to be an ellipse, this indicates that the interaction of terms X1*X2, and X2*X3 were significant; Furthermore, the 2D contour plot seemed to be a circle, this implied that the mutual interaction between X1 and X3 was not significant (Figure 3) (Patil et al., 2013).

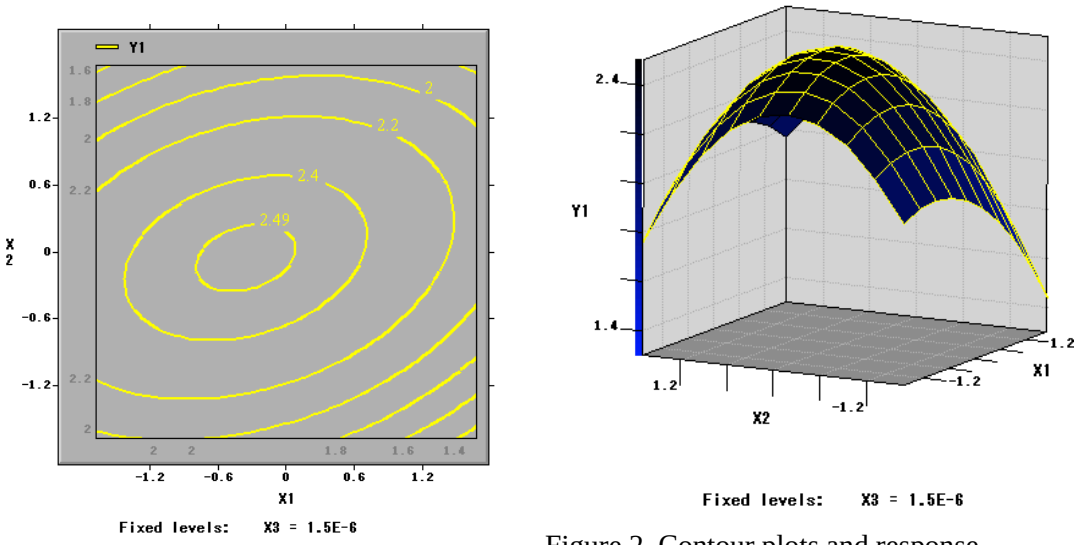

Figure 2. Contour plots and response surface of peptone and yeast extract on the viable counts of BB01

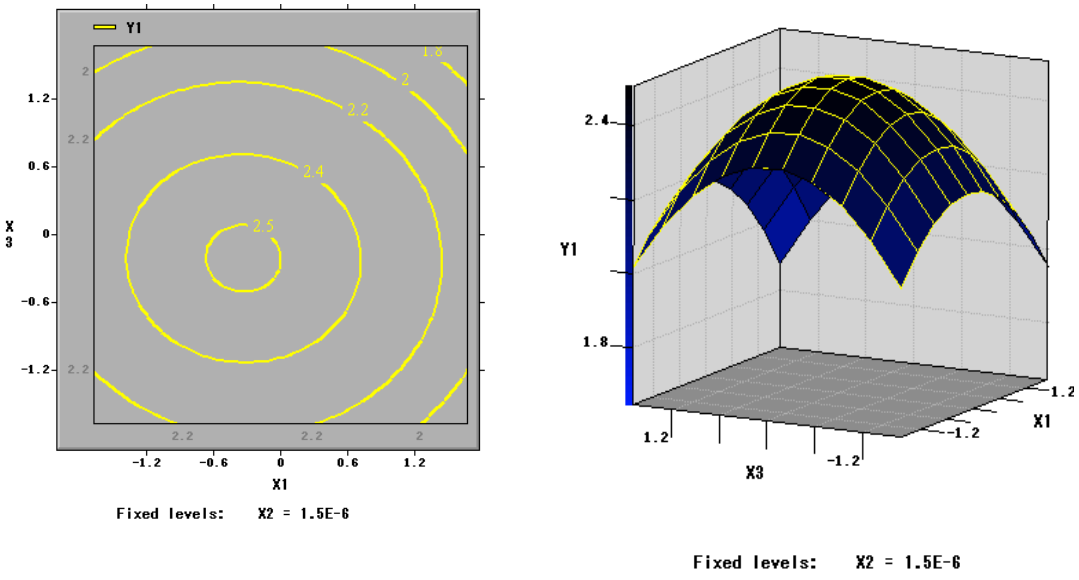

Figure 3. Contour plots and response surface of peptone and beef paste on the viable counts of BB01

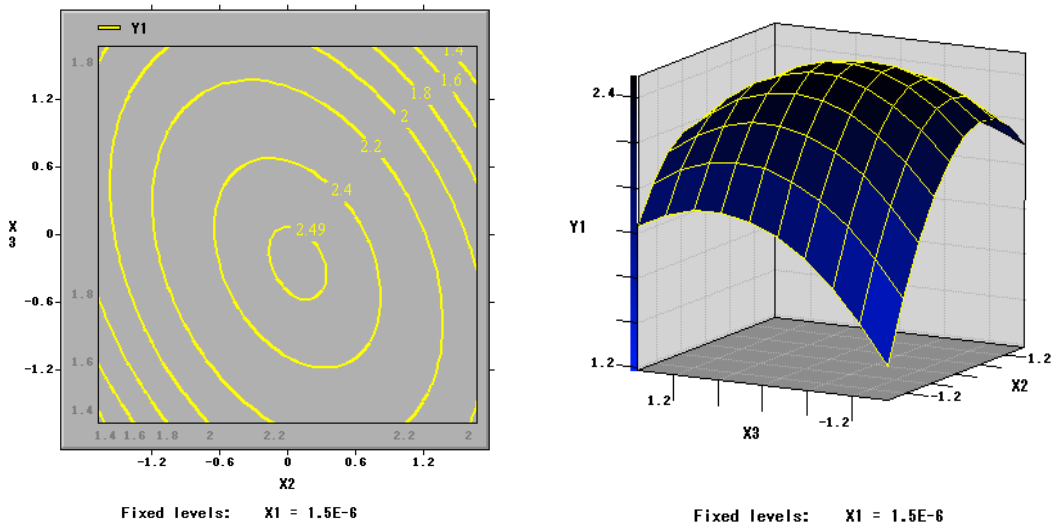

Figure 4. Contour plots and response surface of yeast extract and beef paste on the viable counts of BB01

Based on the SAS software to analyze the regression equation, and the partial derivatives of X1, X2 and X3 were obtained, respectively, and then get maximum points. The code value was converted into the actual value of the concentration: peptone 0.9%, yeast extract 0.3%, beef paste 0.7%. Under the conditions, the maximum response value can reach high at 2.51×10^{9}CFU/mL by the established model.

Validation of the model

Added *B. bifidum* of 5% inoculation in MRS (lactose) medium, then mixing them fully and evenly, cultivate it at 37℃ for 24h, then determined the sample's OD (at 600nm), the pH of culture medium value and the number of viable counts every 3h. using MRS (lactose) medium (M0) and the optimized MRS medium (M1)(carbon source and prebiotics were optimized) as two control groups, three parallel experiments were processed in these two control groups and experimental group (M2), respectively, then regard the average value as the experimental value and repeat the confirmatory experiment to the optimize results.

The determination of OD value, pH and the viable counts of the medium at 12h, 15h, 18h, 21h, respectively，which data were shown in Figure 5.

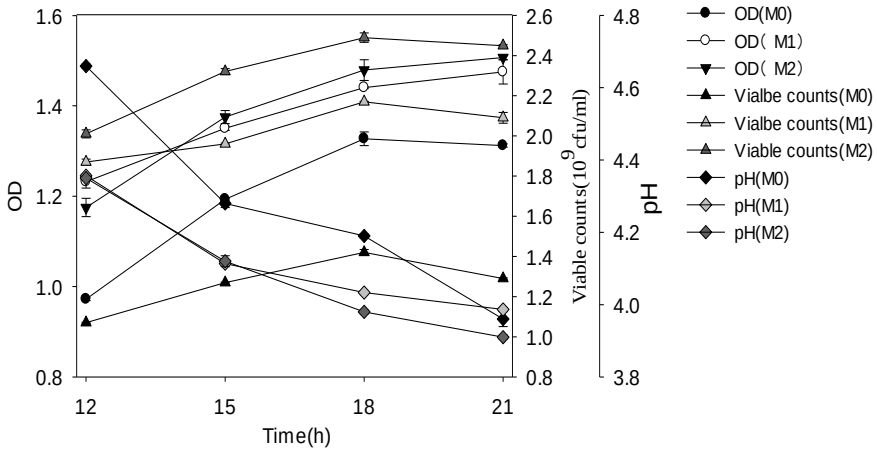

Figure 5. The verification test on Central Composite Design of composite nitrogen.

Figure 5 shows that cultivation of compound nitrogen source after optimization, the growth of *B. bifidum* BB01 has improved significantly, the logarithmic phase was ahead and prolonged in the whole culture period. After optimization of the compound nitrogen source of the medium, the OD value and the viable counts of the bacteria reached 1.479, (2.49 ± 0.06)×10^9CFU/mL, respectively. While control groups (M1, M2) were 1.341, 1.441 for OD value and (1.74 ± 0.06)×10^9CFU/mL, (2.2 ± 0.06)×10^9CFU/mL for viable counts, respectively. The pH decreased slightly, but compared to the carbon source and prebiotics optimized medium little change, may be because add various of nitrogen sources with a number of small peptide and free amino acids into culture medium that can neutralize acid due to excessive carbon source added.

CONCLUSIONS

The CCD was used to determine the interactions between three different factors (peptone, yeast extracts and beef paste) in this study. It was recognized that these three nitrogen sources had a significant effect on the viable counts of *B. bifidum* BB01. The optimized nitrogen sources were: peptone 0.9%, yeast extracts 0.3%, beef paste 0.7%. Based on the optimized medium, the OD value of the bacteria reached 1.479, which was 10.28% higher than MRS (lactose), and 2.64% higher than the optimized MRS medium (carbon source and prebiotics were optimized). While the viable counts reached (2.49±0.06)×10^9CFU/mL, which was 42.97% higher than

MRS (lactose), and 12.85% higher than the optimized MRS medium (carbon source and prebiotics were optimized).The predicted values and actual values of regression equation were not significantly different. Thus, response surface methodology was feasible and effective to optimize nitrogen source in the medium of *B. bifidum* BB01.

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