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10.1515/aucft-2017-0009

# RESPONSE SURFACE OPTIMIZATION OF LYOPROTECTANT FROM AMINO ACIDS AND SALTS FOR BIFIDOBACTERIUM BIFIDUM DURING VACUUM FREEZE-DRYING

– Research paper –

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**Abstract**: High quality probiotic powder can lay the foundation for the commercial production of functional dairy products. The freeze-drying method was used for the preservation of microorganisms, having a deleterious effect on the microorganisms viability. In order to reduce the damage to probiotics and to improve the survival rate of probiotics during freeze-drying, the Response Surface Methodology (RSM) was adopted in this research to optimize lyoprotectant composed of amino acids (glycine, arginine) and salts ($NaHCO_3$ and ascorbic acid). Probiotic used was *Bifidobacterium bifidum* BB01. The regression model ($p<0.05$) was obtained by Box–Behnken experiment design, indicating this model can evaluate the freeze-drying survival rate of *B. bifidum* BB01 under different lyoprotectants. The results indicated these concentrations as optimal (in W/V): glycine 4.5%, arginine 5.5%, $NaHCO_3$ 0.8% and ascorbic acid 2.3%, respectively. Under these optimal conditions, the survival rate of lyophilized powder of *B. bifidum* BB01 was significantly increased by 80.9% compared to the control group (6.9±0.62%), the results were agreement with the model prediction value (88.7%).

**Keywords**: *Bifidobacterium bifidum*, amino acids and salts, freeze-drying, lyoprotectants, optimization

## INTRODUCTION

Probiotics are living microorganisms, which can confer a health benefit to the host (WHO, 2001). Therefore, probiotics are more and more widely added to food for human beings health. Meanwhile, the research and application of probiotics have gained much attention, such as the United States, Germany, Japan, Russia, etc.

Most commonly, probiotics are mainly added to dairy products, such as fermented milk, yoghurt, cheese ice cream and fruit juice (Fritzenfreire et al., 2010; Ramchandran & Shah., 2010; Ranadheera et al., 2010). Fruit juices have the better advantage that suitable for people with lactose intolerance. Freeze-drying method has been widely used in microbiology for many decades to stabilize and store cultures (Santivarangkna et al., 2007). However, the numbers of viable *bifidobacterium bifidum* reduce rapidly owing to the bactericidal effects of the low pH, temperature (Sun & Griffiths., 2000), oxygen and limiting nutrient conditions during freeze-drying process and storage (Ji & Guo., 2008). Microorganisms vary greatly in their tolerance to freeze-drying and the addition of lyoprotectant can reduce cell death. It is neccessary to optimize lyoprotectant formula for obtaining the maximum viability and stability of strains (Morgan & Vesey., 2009). More and more lyoprotactants of bifidbacteria have been reported. Research found that carbohydrates have a protective effect on probiotics (Ljm et al., 1997). Carbohydrates as a protective agent increases the stability of cellular protein by forming hydrogen bonds, thus reducing the risk of exposure to stressful conditions (Champagne et al., 1991). Some studies also indicated that some salt buffer solutions such as sodium chloride or potassium chloride, sodium citrate, phosphate(Kurtmann et al., 2009; Ohtake et al., 2004), protect cells from injury during the freeze drying process.

The mechanism of cell damage in the freezing process has been studied for decades, currently, considered to be mainly by solute effects and mechanical effects (Crowe et al., 2002). The loss of bacteria cell viability is

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mainly due to hostile environmental conditions such as ice crystallization and high osmotic pressure (Yang et al., 2012). Low temperature and water evaporation will cause a lot of damages to the bacteria during freeze drying process, the number of living bacteria will be greatly decreased when the direct freezing of the liquid. Removal of water during freeze-drying may cause structural integrity of these cell components to be unstable, resulting in loss or impairment of function (Leslie et al., 1995). Besids, sample surface is easy to dehydrate faster which causes sample local dry, thus leads to biological macromolecules material protection layer destruction (Iaconelli et al., 2015).

Significant improvement survival of probiotics during freeze-drying has been found in the research of whey protein (Weinbreck et al., 2009). High survival of probiotic bacteria in skim milk as protectants have been demonstrated (Thomas et al., 2009). Besides, proteins, sugars and their interaction had significant effects on acid tolerance, bile tolerance and maintenance of β-aminoglutaric acid, lactic dehydrogenase (LDH) and adenosine triphosphatase (ATP) activities (Dianawati et al., 2013). Poly vinyl pyrrolidone (PVP) and bovine serum albumin (BSA) can also protect the bacteria from the freezing and drying process (Liu & Meng., 2006). Mechanisms of protective agent are not similar, some protective agents as fillers or as an anti-freeze, antioxidant, pH regulator, buffer etc. Generally, interaction of various agents showed better effects during freeze-drying. Therefore, several protectants are mixed together according to a certain formula for a better performance (Wang, 2000).

Amino acid is one of the most common protein lyoprotectants during vacuum freeze drying, in the freezing process, the low concentration of glycine can inhibit the protein denaturation by inhibiting the change of pH value (Mattern et al., 1999). Ascorbic acid as a reducing agent sometimes to the freezing and drying processes of the protein also played a protective role because of anaerobic characteristic of *B. Bifidum* BB01, which could lower the oxidation-reduction potential and consume the oxygen in experiment (Carpenter et al., 1997). In some freeze dried products, adding salt and amine can obtain the stable effect of freeze drying, $NaHCO_3$ as a buffering agent can effectively reduce the damage caused by the dehydration of the cells. Besides, glycine, arginine, $NaHCO_3$ and ascorbic acid can cooperate to protect the cell during vacuum freeze drying.

The development of probiotic lyoprotectants become the focus in relevant reasearch (Saadatzadeh et al. 2013; Tripathi & Giri. 2014). Generally, each probiotic have its own lyoprotectants, the Response surface analysis is also an optimization method, which is the use of graphics technology to show a function of relations, so that we rely on intuitive observation to select the optimal formula of lyoprotectants for probiotic in the design. In our previous work, glycine, arginine, $NaHCO_3$ and ascorbic acid were screened from amino acids and salts as lyoprotectants on *B. bifidum* BB01 during freeze-drying by single factor experiment and Plackett-Burman experiment (Chen et al., 2014; Chen et al., 2013). Based on the results above, RSM is adopted in this research to optimize lyoprotectants formula of *B. bifidum* BB01 during freeze-drying.

## MATERIALS AND METHODS

**Microrganism culture and preparation of lyoprotectants:** *Bifidobacterium bifidum* BB01 was preserved by school of Food and Biological Engineering, Shaanxi University of Science & Technology. *B. bifidum* BB01 was grown in MRS medium with 5% (v:v) inoculum at 37 °C for 16-18 h. Bifidobacteria activates three generations and centrifugates at 8000 rpm for 20 min. After wet cell mixs with different lyoprotectants solution at a ratio of 1:1(W/V), pre-frozen at -20 °C for 12 h and freeze-drying (-51 °C) for 24 h.

**Treatment of lyoprotectants:** All the lyoprotectants used in the experiment were dissolved in distilled water and formulated into various concentrations.

**Vacuum freeze-drying:** The cells were prefrozen at -20 °C for 12 h after protective agents were added and then frozen at -51°C, 6.93Pa for 24 h by using a vacuum freeze dryer.

**Calculation of survival rate:**

Survival rate (%) $=N/N_0 \times 100\%$ (1)

where N and $N_0$ represent the viable counts (cfu/g ) after freeze-drying and the initial count (cfu/g ) before freeze-drying. The viable count method is as follows, the frozen

concentrate is diluted 10 times in the PBS buffer. The total number of viable bacteria is calculated by the method of plate counting, 48 h anaerobic incubation.

**Statistical analysis of the data:** SAS software was used for experiment design and regression analysis. Three-dimensional surface plots and Pareto charts were constructed by using SAS.

**Box–Behnken experiment design:** The significant factors (arginine, glycine, $NaHCO_3$, ascorbic acid) were obtained from PB design. The single factor experiment results showed that the concentration of single protective agent of *B. bifidum* BB01 during freeze-drying was with 4-6% (w/v) arginine, 3-5% (w/v) glycine, 0.6-1% (w/v) $NaHCO_3$, 2-2.6% (w/v) ascorbic acid, and the maximum survival rate was 44.23, 44.02%, 47.92%, 36.38%, respectively. And then according to the results of ascent experiment to determine the range of factors for Box–Behnken design (BBD). The lyoprotectant composition was further optimized using a four-factor, three-level (BBD) and three levels of N=27 test with the Y (freeze-dried survival rate of *B. bifidum* BB01) as response value. Factors encoding table are shown in Table 1.

Table 1. Experimental factor levels of Box-Behnken design

| factors/ level | X1 arginine (%) | X2 glycine (%) | X3 $NaHCO_3$ (%) | X4 ascorbic acid (%) |
|---|---|---|---|---|
| -1 | 5 | 4 | 0.75 | 2.4 |
| 0 | 5.5 | 4.5 | 0.8 | 2.3 |
| 1 | 6 | 5 | 0.85 | 2.2 |

Note: % indicates the mass concentration of each substance, g/100ml.

A polynomial equation was used as follows:

$$Y=a_0+a_1X1+a_2X2+a_3X3+a4X4+a_{12}X1X2+a_{13}X1X3+a_{14}X1X4+a_{23}X2X3+a_{24}X2X4+ a_{34}X3X4+a_{11}X1X1+a_{22}X2X2+a_{33}X3X3+ a_{44}X4X4 \quad (2)$$

In the equation (2), Y is the predicted responses of the dependent variable, X1, X2 X3 and X4 are the values of significant variables, $a_0$ is the second-order reaction constant, $a_1$, $a_2$ $a_3$ and $a_4$ are the linear coefficients, $a_{12}$, $a_{13}$, $a_{14}$ $a_{23}$ $a_{24}$ and $a_{34}$ are the interaction coefficients and $a_{11}$, $a_{22}$, $a_{33}$ and $a_{44}$ are the quadratic coefficients.

## RESULTS AND DISCUSSIONS

### RSM optimization of formulation of lyoprotectant

RSM was used to optimize the formula of lyoprotectants. The experiment design and results are shown in Table 2, response value Y1 with a decimal representation, which indicates the survival rate of freeze-drying powder. According to the test results of Table 2, the regression model (equation) obtained by using SAS software is Eq.3. In the equation (3), Y1 is the predicted value of survival rate vacuum freeze-dring *B. bifidum* BB01 cells. X1, X2, X3 and X4 were arginine, glycine, $NaHCO_3$ and ascorbic acid encoding value.

$$Y1=0.8880-0.0641\times X1+0.0225\times X2+0.0159\times X3-0.0455\times X4-0.0990\times X1\times X1-0.0592\times X1\times X2-0.0020\times X1\times X3+0.0797\times X1\times X4-0.0767\times X2\times X2-0.0934\times X2\times X3+0.0761\times X2\times\times X4-0.1644\times X3\times X3+0.1012\times X3\times X4-0.1678\times X4\times X4 \quad (3)$$

Variance analysis and coefficient significance test of the regression equations were obtained by SAS software. The results are shown in Table 3. Analysis of variance (ANOVA) is used to evaluate the adequacy of the fitted model and test the significance of the coefficient. From Table 3, variance analysis showed that the regression equation mode is significant ($P<0.05$). The regression coefficient $R^2$ is 93.79%, which shows that the fitting degree of the equation is better. The F value of the equation is 8.3941 and Prob>F (0.01), which shows that the linear relationship between the dependent variable and the independent variable is significant, that is, the experimental method is reliable.

Table 2. Design and results of Box-Behnken design (N=27)

| RUN | X1 | X2 | X3 | X4 | Y1 |
|---|---|---|---|---|---|
| 1 | -1 | -1 | 0 | 0 | 0.69 |
| 2 | -1 | 1 | 0 | 0 | 0.72 |
| 3 | 1 | -1 | 0 | 0 | 0.71 |
| 4 | 1 | 1 | 0 | 0 | 0.51 |
| 5 | 0 | 0 | -1 | -1 | 0.61 |
| 6 | 0 | 0 | -1 | 1 | 0.28 |
| 7 | 0 | 0 | 1 | -1 | 0.52 |
| 8 | 0 | 0 | 1 | 1 | 0.59 |
| 9 | -1 | 0 | 0 | -1 | 0.83 |
| 10 | -1 | 0 | 0 | 1 | 0.59 |
| 11 | 1 | 0 | 0 | -1 | 0.48 |
| 12 | 1 | 0 | 0 | 1 | 0.56 |
| 13 | 0 | -1 | -1 | 0 | 0.46 |
| 14 | 0 | -1 | 1 | 0 | 0.64 |
| 15 | 0 | 1 | -1 | 0 | 0.82 |
| 16 | 0 | 1 | 1 | 0 | 0.64 |
| 17 | -1 | 0 | -1 | 0 | 0.74 |
| 18 | -1 | 0 | 1 | 0 | 0.73 |
| 19 | 1 | 0 | -1 | 0 | 0.65 |
| 20 | 1 | 0 | 1 | 0 | 0.63 |
| 21 | 0 | -1 | 0 | -1 | 0.79 |
| 22 | 0 | -1 | 0 | 1 | 0.58 |
| 23 | 0 | 1 | 0 | -1 | 0.68 |
| 24 | 0 | 1 | 0 | 1 | 0.78 |
| 25 | 0 | 0 | 0 | 0 | 0.89 |
| 26 | 0 | 0 | 0 | 0 | 0.89 |
| 27 | 0 | 0 | 0 | 0 | 0.89 |

Table 3. The ANOVA of Box-Behnken design

| SOURCE | DF | SS | MS | F | P | Sig. |
|---|---|---|---|---|---|---|
| X1 | 1 | 0.0494 | 0.0494 | 5.2860 | 0.0403 | * |
| X2 | 1 | 0.0061 | 0.0061 | 0.6512 | 0.4354 | |
| X3 | 1 | 0.0030 | 0.0030 | 0.3259 | 0.5786 | |
| X4 | 1 | 0.0248 | 0.0248 | 2.6544 | 0.1292 | |
| X1*X1 | 1 | 0.0523 | 0.0523 | 5.5996 | 0.0356 | * |
| X1*X2 | 1 | 0.0140 | 0.0140 | 14.9940 | 0.0043 | ** |
| X1*X3 | 1 | 0.0000 | 0.0000 | 0.0018 | 0.9672 | |
| X1*X4 | 1 | 0.0254 | 0.0254 | 2.7222 | 0.1249 | |
| X2*X2 | 1 | 0.0314 | 0.0314 | 3.3624 | 0.0916 | |
| X2*X3 | 1 | 0.0349 | 0.0349 | 11.7395 | 0.0077 | ** |
| X2*X4 | 1 | 0.0023 | 0.0023 | 18.4836 | 0.0019 | ** |
| X3*X3 | 1 | 0.1441 | 0.1441 | 15.4258 | 0.0020 | ** |
| X3*X4 | 1 | 0.0400 | 0.0400 | 4.2831 | 0.0607 | |
| X4*X4 | 1 | 0.1502 | 0.1502 | 16.0780 | 0.0017 | ** |
| model | 14 | 0.0444 | 0.0032 | 8.3941 | 0.0042 | ** |
| linear | 4 | 0.0833 | 0.0208 | 2.2294 | 0.1268 | |
| quadratic | 4 | 0.0223 | 0.0557 | 5.9675 | 0.0070 | ** |
| cross-product | 6 | 0.0138 | 0.0229 | 6.4549 | 0.0575 | |
| error | 12 | 0.1121 | 0.0093 | | | |
| lack of fit | 10 | 0.0001 | 0.0001 | 0.2422 | 0.9712 | |
| pure error | 2 | 0.0000 | 0.0000 | | | |
| total | 26 | 0.5558 | | | | |

Note：*** P<0.001，extremely significant;** P<0.01，highly significant;* P<0.05，significant.

The first-order item of the equation is not high and the cross product has certain significance, it shows that the relationship between the response value and the test factors are a simple linear relationship. The cross product F value is large, which indicates that there is a certain interaction among various factors. However, lack of fit is not significant, indicating that the equations are well suited for adjust experiment and experimental error are small, therefore, this regression equation can be used for predicating survival rate during freeze-drying.

According to Figure 1, the response value of Y1 varies with the concentration of each factor. X3*X3 and X4*X4 are significant ($P<0.05$) effect for survival rate of bacteria, and the survival rate of freeze-dried Y1 is significant affected by X3 and X4. Maximum value are obtained at the turning point. The value of Y1 becomes higher with increase of X3 and X4, then reach the maximum value at the center point and then Y1 decreases gradually with the increase of concentration. Similarly, X1 and X2 also have the same impact on Y1, but the bending effect is not significant. This may be attributed to the initial addition of amino acids and salts too little cannot completely replace the loss of water molecules around the cell, when the amount is too high, which will produce osmotic pressure to cause rapid cell dehydration lead to damage to cell structure.

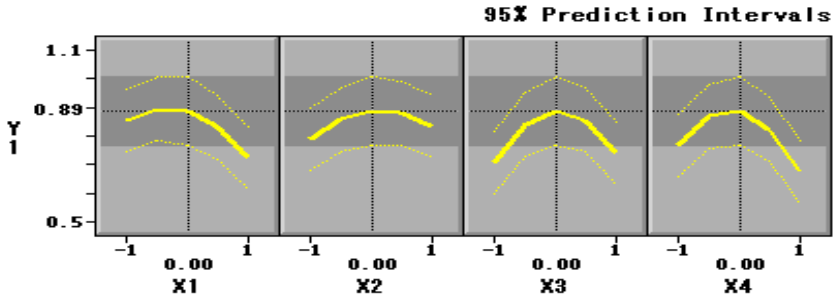


Figure 1. The trends of Y1 with factors

Based on the above results, response surface and contour are shown in Figures 2 - 7. From Figure 2 to 7 contour shapes, it can be known that the interaction of X1 and X3, X1 and X4, X2 and X4 are not obvious, but X1 and X2, X2 and X3, X2 and X4 have a significant cross effect. Based on the generated regression equation, we calculate partial derivative for X1, X2, X3 and X4 respectively, and the maximum point are also calculated. Under condition of glycine, 4.5% (W/V), arginine 5.5% (W/V), $NaHCO_3$ 0.8% (W/V) and ascorbic acid 2.3% (W/V), predicted freeze-dried survival rate of *B. bifidum* BB01 is 88.7%.

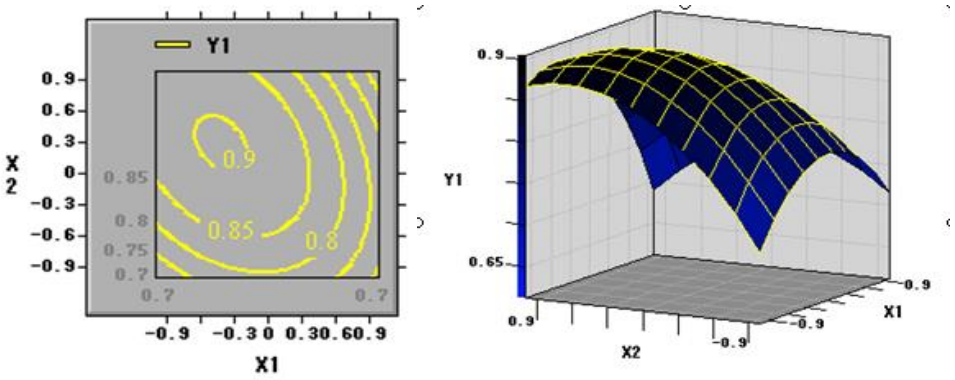


Figure 2. Response surface and contour plots of arginine (X1), glycine (X2) to survival rate of *B. bifidum* BB01 (Y1)

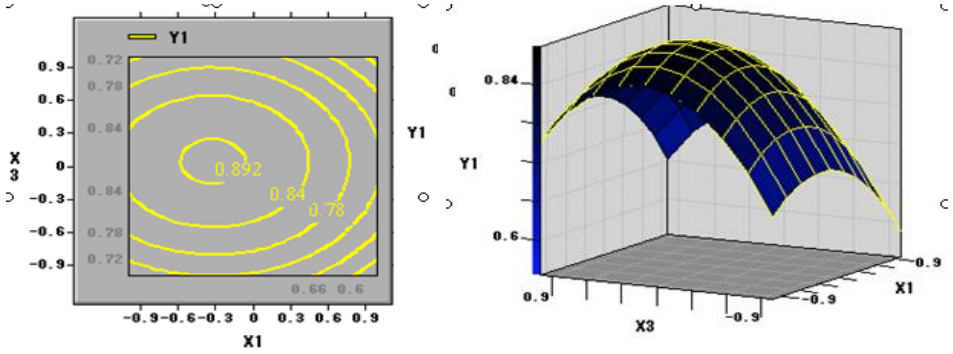


Figure 3. Response surface and contour plots of arginine (X1), $NaHCO_3$ (X3) to survival rate of *B. bifidum* BB01 (Y1)

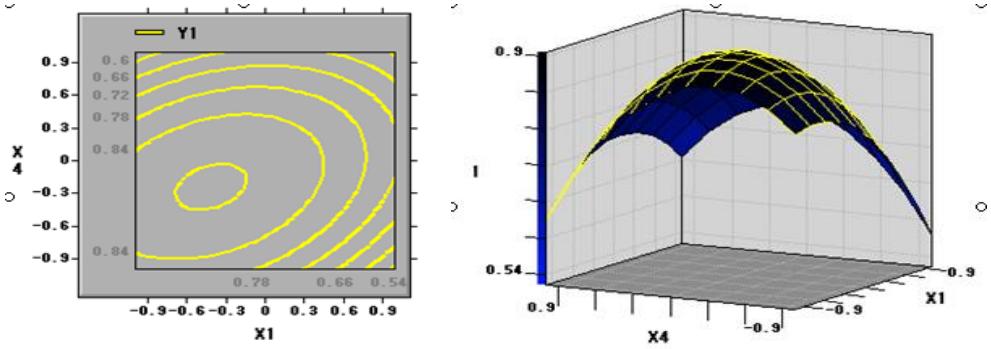


Figure 4. Response surface and contour plots of arginine (X1), ascorbic acid (X4) to survival rate of *B. bifidum* BB01 (Y1)

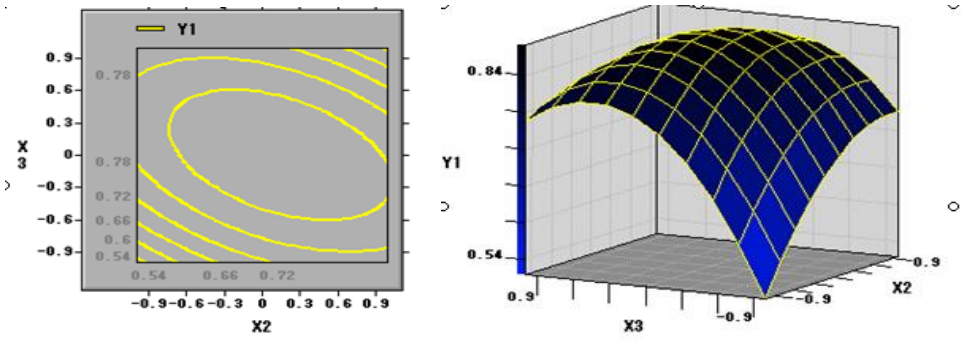


Figure 5. Response surface and contour plots of glycine (X2), $NaHCO_3$ (X3) to survival rate of *B. bifidum* BB01 (Y1)

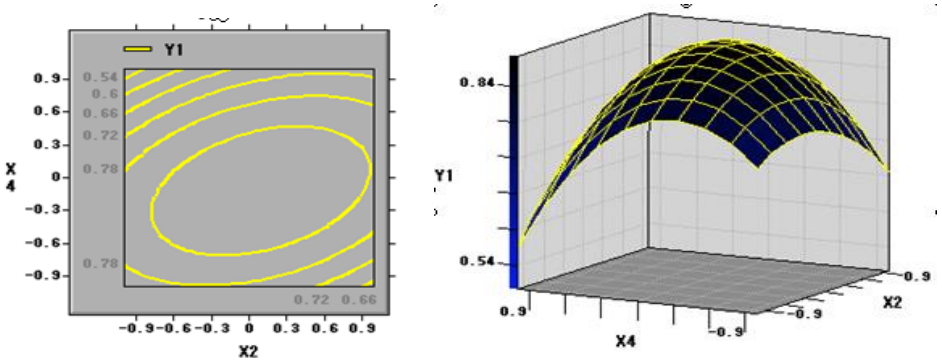


Figure 6. Response surface and contour plots of glycine (X2), ascorbic acid (X4) to survival rate of *B. bifidum* BB01 (Y1)

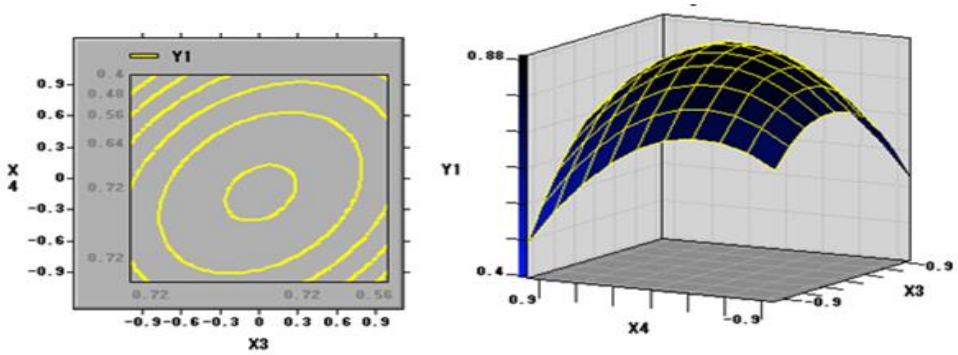


Figure 7. Response surface and contour plots of $NaHCO_3$ (X3), ascorbic acid (X4) to survival rate of *B. bifidum* BB01 ( Y1)

**Verification test:** In this work, control group without adding other substances and the experimental group with the lyoprotectants of amino acids and salts were conducted, the survival rate is calculated by 3 times repeated freeze drying tests, which repeated tests with 5% of inoculum size culture at 37 °C for 18 h , then sampling count. The control group and the experimental group are conducted three parallel and calcucate the average value. Finally experimental group survival rate of *B. bifidum* BB01 is 87.8±2.3% after freeze drying, which close to the prediction value (88.7%).

## CONCLUSIONS

Lyoprotectant contents for *B. bifidum* BB01 was optimized by Box–Behnken experiment design. The lyophilized survival rate of *B. bifidum* BB01 had the highest value when the concentrations of glycine, arginine, $NaHCO_3$ and ascorbic acid were 4.5% (W/V), 5.5% (W/V), 0.8% (W/V) and 2.3% (W/V), respectively. Meanwhile, survival rate of lyophilized powder of *B. bifidum* BB01 was significantly improved, which increased by 80.9% compared with the control group (6.9±0.62%). Experiments confirmed the predicted results, indicating that the optimized conditions and models used were reliable and effective, which can be propose for preservation and application of probiotics powder.

**Acknowledgements:** This project was supported by Shaanxi Innovation and Transformation Project of Agricultural Science and Technology (No.NYKJ-2015-004) and Shaanxi Innovation and Transformation Project of Agricultural Science and Technology [No.2016KTZDNY02-03].

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