OPTIMIZATION OF COMPATIBLE SOLUTES FOR IMPROVING SURVIVAL OF FREEZE-DRIED *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS* USING BOX-BEHNKEN DESIGN

– Short communication –

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Abstract: *Lactobacillus delbrueckii subsp. bulgaricus* is widely used in yogurt as a starter. The freeze-drying process may cause bacteria death. In the present work, the effect of three solutes (NaCl, sorbitol, and sodium glutamate) in MRS on viability of *L. bulgaricus* during freeze-drying was investigated. The optimal combination of adequate solutes was chosen by Box-Behnken Design. The survival rate and viable counts in freeze-dried powder, as well as the viable counts in broth, were used as responses. The results revealed that the optimum combination of solutes in MRS broth were 0.50% NaCl, 0.19% sorbitol, and 0.06% sodium glutamate. Under these optimal conditions, the survival rate was $53.2 \pm 0.14\%$, the viable counts in freeze-dried powder was $8.51 \pm 0.23 \times 10^{10}$ CFU/g, and the viable counts in broth was $6.05 \pm 0.19 \times 10^8$ CFU/mL, which were increased by 17.18%, 15.94%, and 17.31%, respectively, compared to the control. This research demonstrated the possibility of viability improvement of *L. bulgaricus*, which may provide a feasible reference for industrial development.

Keywords: *Lactobacillus bulgaricus*; compatible solutes; freeze-drying; response surface methodology

INTRODUCTION

L. bulgaricus is one of the widely applicable lactic acid bacteria (LAB) strains that is extensively used as a starter for the manufacture of a variety of fermented dairy products (Chen et al., 2015). The industrial development of yogurt starters needs to ensure that they provide viable and functional cultures for a long time. Freeze-drying is recognized as a suitable drying technique to preserve yogurt starters. However, this process may cause cell death and loss of fermentation function (Chen et al., 2015). *L. bulgaricus* is indeed sensitive to freeze-drying, which up to now can only be stabilized by freezing on an industrial scale. Finding ways to improve the survival of *L. bulgaricus* during freeze-drying is of great interest. Some research found that sorbitol and sodium glutamate have a positive effect on cell survival (Carvalho et al., 2003a; Carvalho et al., 2010; Santivarangkna et al., 2010). Besides, it has been suggested that the addition of NaCl to the growth medium, as well as certain components as

sources of compatible solutes, lead to an increase in the accumulation of compatible solutes, and therefore promote survival of LAB during storage in the powder (Carvalho et al., 2003b). There is clear evidence that an adequate increase of osmotic pressure of the medium by adding NaCl to the MRS broth has improved the survival of freeze-dried *L. bulgaricus* during storage (Carvalho et al., 2010). However, the intracellular accumulation and protection of compatible solutes in each lactic acid bacteria strain may be different under osmotic stress (Obis et al., 2010; Le et al., 2007). Thus, mechanisms of adaptation involving the accumulation of compatible solutes in each LAB strains are the current research focuses (Johannes et al., 2021).

In our work, the Plackett-Burman design was revealed that sorbitol, sodium glutamate, and NaCl in MRS medium could affect significantly the survival rate of *L. bulgaricus* during freeze-drying and demonstrated highly significant effects (Chen et al., 2017). In this study, Box-Behnken design (BBD) was adopted to further optimize the combination of the three solutes in MRS medium.

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MATERIALS AND METHODS

Microorganism and media

L.bulgaricus was obtained from the School of Food and Biological Engineering (Shaanxi University of Science & Technology) which was screened from Inner Mongolia traditional yogurt. The MRS broth and MRS agar medium were purchased from Shandong Tuopu Biological Engineering Co., Ltd. (Zhaoyuan City, China).

Preparation of antifreeze agent and freeze-dried *L.bulgaricus*

The impact of the main factors has been studied by Plackett-Burman design in previous research (Chen et al., 2017). Consequently, NaCl (Tian in Fuyu Fine Chemical Co., Ltd., Tianjin, China), sorbitol (Shanghai Shan Pu Chemical Co., Ltd., Shanghai, China), and sodium glutamate (Xi'an Rosen Bio-tech Co., Ltd., Xi'an, China) were selected as the best freeze-drying antifreeze agents for *L.bulgaricus*. NaCl, sorbitol, and sodium glutamate were formulated into various concentrations with distilled water and added into MRS broth. After sterilization at 121°C for 15 min, the *L.bulgaricus* was continuously activated for three generations, which inoculated at a level of 2% (v/v) into MRS medium, and then the medium was stored at 37°C for 22 h. The sediments of *L.bulgaricus* culture were centrifuged (8000 r/min, 15 minutes) at 25°C (Model GL21, Instrumentation, Hunan), washed with saline, and centrifuged. The procedure was repeated twice then an equal volume of phosphate buffer was added and prefrozen under -80°C for 4 h and then frozen using a vacuum freeze dryer (-50 °C, 6.95 Pa for 24 h).

$$\text{Survival rate (\%)} = \frac{\text{Viable counts after freeze - drying (CFU/mL)}}{\text{Viable counts before freeze - drying (CFU/mL)}} \times 100 \quad (1)$$

RESULTS AND DISCUSSION

Experimental results of Box–Behnken design

The results of the experimental design using BBD for antifreeze agents of *L.bulgaricus* are shown in Table 2.

Table 2. Experimental results of Box–Behnken design for antifreeze factor of *L.bulgaricus*

Runs	X ₁ (%)	X ₂ (%)	X ₃ (%)	Y ₁ (%)	Y ₂ (×10 ¹⁰ CFU/g)	Y ₃ (×10 ⁸ CFU/mL)
1	-1(0.40)	-1(0.17)	0(0.06)	32.6	5.3	5.30
2	-1	1(0.21)	0	29.3	5.1	5.19
3	1(0.60)	-1	0	33.5	5.4	5.17
4	1	1	0	28.9	4.1	5.25
5	0(0.50)	-1	-1(0.04)	35.4	5.1	5.75
6	0	-1	1(0.08)	31.2	5.6	5.77
7	0	1	-1	30.1	4.7	5.81
8	0	1	1	32.3	5.3	5.87
9	-1	0(0.19)	-1	32.5	5.4	5.32

Determination of Cell Counts

The number of *L.bulgaricus* was determined before and after freeze-drying. The freeze-dried powder of *L.bulgaricus* was added to sterile saline for serial dilution. Later, a sterile syringe has been used to draw 1 mL of bacterial suspension on the MRS agar plate and spread the evenly, then keep the plate at 37°C for 72 h. The viability of *L. bulgaricus* was detected by the standard counting method on MRS agar. The survival rate calculation method is those given in eq. (1).

Optimization of antifreeze agents by Box–Behnken design (BBD)

The Box–Behnken design (BBD) was used to optimize the antifreeze agents of *L.bulgaricus* (Table 1). In order to evaluate the fitness of the data, a quadratic polynomial equation was set.

Table 1. Factor levels coding of Box–Behnken for antifreeze agents of *L.bulgaricus*

Factors	Coded Levels		
	-1	0	1
NaCl (%)	0.40	0.50	0.60
Sorbitol (%)	0.17	0.19	0.21
Sodium glutamate (%)	0.04	0.06	0.08

Statistical analysis of the data

Design Expert (Version 8.0.6, Stat-Ease. Inc) used to perform variance analysis on the obtained experimental data, establish quadratic regression equations and three-dimensional response surface plots.

10	1	0	-1	44.3	6.3	5.58
11	-1	0	1	35.8	6.7	5.47
12	1	0	1	33.5	5.1	5.41
13	0	0	0	53.2	8.7	6.18
14	0	0	0	58.3	8.4	6.20
15	0	0	0	52.1	8.6	6.13

The survival rate, the viable counts in freeze-dried powder, and the viable counts in broth were represented by Y_1 (%), Y_2 ($\times 10^{10}$ CFU /g), and Y_3 ($\times 10^8$ CFU/mL), respectively.

Regression analysis and analysis of variance

The developed quadratic equations were obtained by using Design Expert as follows:

$$Y_1 = 54.53 + 1.25X_1 - 1.51X_2 - 1.19X_3 - 0.32X_1X_2 - 3.52X_1X_3 + 1.60X_2X_3 - 9.59X_1^2 - 13.87X_2^2 - 8.42X_3^2 \quad (3)$$

$$Y_2 = 8.57 - 0.20X_1 - 0.28X_2 + 0.15X_3 - 0.27X_1X_2 - 0.63X_1X_3 + 0.025X_2X_3 - 1.45X_1^2 - 2.15X_2^2 - 1.25X_3^2 \quad (4)$$

$$Y_3 = 6.17 + 0.016X_1 + 0.016X_2 + 0.0075X_3 + 0.047X_1X_2 - 0.08X_1X_3 + 0.01X_2X_3 - 0.65X_1^2 - 0.29X_2^2 \quad (5)$$

where Y_1 , Y_2 , and Y_3 correspond to predicted value of survival rate, the viable counts in freeze-dried powder, and the viable counts in broth of *L.bulgaricus*, respectively. X_1 , X_2 , and X_3 are the real values of the independent variables, NaCl, sorbitol, and sodium glutamate, respectively.

Analysis of variance results summarized in Table 3 indicated that the regression models were very significant ($p < 0.01$). The quadratic terms of all regression equations except X_3^2 of Y_3 were smaller than 0.05, which suggested X_1^2 , X_2^2 , and X_3^2 have a significant impact on the response value. The p -value of the interaction coefficient X_1X_3 was smaller than 0.05, which indicated the significant interaction between NaCl and sodium glutamate. However, the interactions between NaCl and sorbitol, sorbitol, and sodium glutamate were not significant ($p > 0.05$). The R^2_{adj} value for Y_1 of 0.9186 was close to the R^2 meaning that the model was significant. Besides, R^2_{adj} values for Y_2 and Y_3 were close to R^2 indicating that the developed models were highly significant with a good

relationship between predicted and measured values of viable counts in both freeze-dried powder (Y_2) and viable counts in broth (Y_3).

Response surface plot analysis

Design-Expert was applied to analyze the regression equation, and 3D plots conception (Figures 1-3). These response surface plots could explain the influence of independent variables and their interactions. When the addition of NaCl was 0.50%, the concentration of sorbitol was 0.19%, and the concentration of sodium glutamate was 0.06%, the values of survival rate (54.54%), the viable counts in freeze-dried powder (8.57×10^{10} CFU.g⁻¹), and viable counts in broth (6.17×10^8 CFU.mL⁻¹) of *L.bulgaricus* were obtained respectively, which reached the maximum responses.

Table 3. Experimental results of Box–Behnken design for antifreeze factor of *L.bulgaricus*

Source	Y_1			Y_2		Y_3	
	DF ^a	MS ^b	p -value ^c	MS ^b	p -value ^c	MS ^b	p -value ^c
model	9	139.11	0.0025	3.30	0.0001	0.20	0.0002
X_1	1	12.50	0.2532	0.32	0.0547	0.0021	0.4721
X_2	1	18.30	0.1790	0.61	0.0186	0.0021	0.4721
X_3	1	11.28	0.2747	0.18	0.1200	0.0004	0.7344
X_1X_2	1	0.42	0.8218	0.30	0.0596	0.0090	0.1690
X_1X_3	1	49.70	0.0498	1.56	0.0027	0.026	0.0425
X_2X_3	1	10.24	0.2953	0.0025	0.8341	0.0004	0.7489
X_1^2	1	339.69	0.0011	7.72	<0.0001	1.55	<0.0001
X_2^2	1	709.97	0.0002	17.00	<0.0001	0.32	0.0002
X_3^2	1	261.56	0.0020	5.73	0.0001	0.021	0.0560
Residual	5	7.50		0.051		0.0035	
Lack of fit	3	5.21	0.7313	0.070	0.2599	0.0049	0.2147
Pure error	2	10.94		0.023		0.0013	
R^2			0.9709		0.9914		0.9905
R^2_{adj}			0.9186		0.9760		0.9733

^a Degrees of freedom; ^b Mean square; ^c The F-test probability values; the survival rate, the viable counts in freeze-dried powder, and the viable counts in broth were represented by Y_1 (%), Y_2 ($\times 10^{10}$ CFU /g), Y_3 ($\times 10^8$ CFU/mL), respectively.; NaCl, sorbitol, and sodium glutamate were represented by X_1 , X_2 and X_3 , respectively.

The results of verification experiments, tested in triplicate, showed that the mean value of the survival rate, the viable counts in freeze-dried powder, and the viable counts in broth of *L.bulgaricus* were $53.2\pm0.14\%$, $8.51\pm0.23\times10^{10}$ CFU/g, and $6.05\pm0.19\times10^8$ CFU/mL. These results were statistically similar to the predicted values. The control had values of the survival rate as 45.4%,

the viable counts in freeze-dried powder as 5.2×10^{10} CFU/g, and the viable counts in broth as 5.13×10^8 CFU/mL. Comparing the control and optimized mediums, the viable counts, the viable counts in freeze-dried powder, and the survival rate in broth were improved by 17.18%, 63.65%, and 17.93%, respectively.

Figure 1. Response surface plots of survival rate showing interactions between (a) NaCl and sorbitol, (b) NaCl and sodium glutamate, (c) sorbitol and sodium glutamate

Figure 2. Response surface plots of the viable counts in freeze-dried powder showing interactions between (a) NaCl and sorbitol, (b) NaCl and sodium glutamate, (c) sorbitol and sodium glutamate

Figure 3. Response surface plots of the viable counts in broth showing interactions between (a) NaCl and sorbitol, (b) NaCl and sodium glutamate, (c) sorbitol and sodium glutamate

The activity of *L.bulgaricus* after freeze-drying was of great economic significance to the dairy industry, so there was a lot of research on how to improve its freeze-dried activity, and the measures taken include accumulation of compatible solutes such as betaine and carnitine (Kets & de Bont, 1994; Kets et al., 1996), sub-lethal treatments (heat, cold, and acid shocks) (Broadbent and Lin, 1999, Silva et al., 2005) and optimization of freeze-drying protectants (Romano et al., 2016). However, research on the accumulation of compatible solutes in lactic acid bacteria to improve their freeze-drying survival rate and stability has received little attention and its mechanism of action is still unclear. Kets & de Bont (1994) found that adding betaine in the presence of 0.6 M NaCl has a significant effect on the growth rate of *Lactobacillus plantarum* and can resist drying better than unstressed cells.

In this study, we optimized the best combination of NaCl, sorbitol, and sodium glutamate using BBD and found NaCl and sodium glutamate have a significant interactive effect on improving the survival rate, the viable counts in freeze-dried powder, and the viable counts in broth. It was

demonstrated that the addition of the optimal combination solutes to MRS broth increases significantly survival of *L.bulgaricus*.

In the process of continuous drying, when the moisture content decreases rapidly, the cells were prone to rupture due to the phase change in the drying or rehydration process. Therefore, the cell membrane was the key part of the loss of bacterial activity during the freeze-drying process. Besides, sorbitol can prevent lipid oxidation of cell membrane thus decreasing cell membrane damage. Relevant evidence shows the ability to stabilize protein structure and retain more residual water through the reaction between the amino group of protective agent and carboxyl group of microbial protein (de Valdéz et al., 1983), which was an explanation for the effect of sodium glutamate in the freeze-drying process. Our research showed that choosing a suitable protective agent as the additive of the drying medium was a necessary condition for the strong protection of lyophilized *L. bulgaricus*. The best combination of three substances added to the MRS broth can improve significantly viability of freeze-dried *L.bulgaricus*.

CONCLUSION

The optimal combination of three compounds on improving survival of *L.bulgaricus* was NaCl of 0.50%, sorbitol of 0.19%, and sodium glutamate of 0.06%. Under these conditions, the survival rate, the

viable counts in freeze-dried powder, and the viable counts in broth of freeze-dried *L.bulgaricus* increased by 53.2%, 8.51×10^{10} CFU/g, and 6.05×10^8 CFU/mL compared to control, respectively. Our study provided a good reference for industrial exploitation of *L.bulgaricus* starters.

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