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ORIGINAL PAPER

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Optimization of a protective medium for enhancing the viability of freeze-dried *Lactobacillus delbrueckii* subsp. *bulgaricus* based on response surface methodology

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Abstract Response surface methodology (RSM) was used to optimize a protective medium for enhancing the cell viability of *Lactobacillus delbrueckii* subsp. *bulgaricus* LB14 during freeze-drying. Using a previous Plackett–Burman design, it was found that sucrose, glycerol, sorbitol and skim milk were the most effective freeze-drying protective agents for *L. bulgaricus* LB14. A full factorial central composite design was applied to determine the optimum levels of these four protective agents. The experimental data allowed the development of an empirical model ($P < 0.0001$) describing the inter-relationships between the independent and dependent variables. By solving the regression equation, and analyzing the response surface contour and surface plots, the optimal concentrations of the agents were determined as: sucrose 66.40 g/L, glycerol 101.20 g/L, sorbitol 113.00 g/L, and skim milk 130.00 g/L. *L. bulgaricus* LB14 freeze-dried in this medium obtained a cell viability of up to 86.53%.

Keywords *Lactobacillus delbrueckii* subsp. *bulgaricus* · Freeze-drying · Protective mediums · Full factorial central composite design · Response surface methodology

Introduction

Starter cultures play an important role in the dairy industry in the manufacture of fermented milks and cheese. The industrial exploitation of lactic acid bacteria (LAB) as starter culture is dependent on concentration and preservation technologies that can guarantee the delivery of stable cultures in terms of viability and bacterial functions [19]. Therefore, freeze-dried lactic acid starter cultures, which are ready to use culture concentrates for direct inoculation of vat milk, are of considerable interest for research and industry since they have the advantages of high cell concentration, high functional activity and long storage life [22]. However, a number of factors, such as species [11], freeze-drying parameters [1], freeze-drying medium [6], physiological state of the cells [4], and rehydration conditions [17], influence the viability of freeze-dried starter culture.

The major causes of loss of cell viability during freeze-drying are probably ice formation, high osmolarity due to high concentration of internal solutes with membrane damage, macromolecule denaturation, and the removal of water, which affect the properties of many hydrophilic macromolecules in cells [23]. During attempts to reduce such adverse changes on the freeze-dried cells, several compounds have been examined as protective agents in freeze-drying; for example, skim milk, glycerol, mannitol, sorbitol, trehalose, sucrose, maltose, lactose, fructose, glucose, betaine, amino acids and their salts [1, 20, 24]. However, the influences of these protective agents on the viability of LAB during freeze-drying vary between the studies reported to date. The one-variable-at-a-time approach (OVAT), frequently used to study the effect of many protective agents on LAB [6, 1], consumes time and ignores the interactions between the agents, resulting in confusion and a lack of predictive ability. Response surface methodology (RSM) is an efficient statistical technique for the optimization of multiple variables in order to predict the best performance conditions with a minimum number of experiments [14]. Hence, RSM, which has already been successfully applied in other fields [3, 5, 10, 25], may be suited to studying the main and interaction

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effects of the factors on the viability of LAB during freeze-drying.

Lactobacillus delbrueckii subsp. *bulgaricus* is one of the most widespread strains used in the production of yoghurt and other dairy products [26]. Our previous Plackett–Burman design indicated that sucrose, glycerol, sorbitol and skim milk were the most effective freeze-drying protective agents for *L bulgaricus* LB14. The aim of the present work was to find the optimum levels of the important protective agents (sucrose, glycerol, sorbitol and skim milk) considered for *L bulgaricus* LB14 in order to maximize cell viability during freeze-drying, and to determine the mutual interactions between pairs of the selected factors simultaneously using RSM.

Materials and methods

Microorganism

Lactobacillus delbrueckii subsp. *bulgaricus* LB14 was isolated in our laboratory from Rosell yoghurt culture (Rosell Institute, Canada). The strain was maintained in 12% (w/v) skim milk supplemented with 5% $CaCO_3$ at 4 °C and subcultured every two months.

Culture conditions

The stock culture was reactivated by at least three successive transfers in 12% (w/v) sterilized skim milk. Then MRS broth [16] was inoculated from this pure culture and incubated at 37 °C under stable conditions overnight. This culture was subsequently inoculated, at the level of 5% (v/v), into a second MRS broth and incubated as before to obtain the organism needed for the assays.

Protective agents

The substances used to protect cells during freeze-drying were sucrose, glycerol, sorbitol and skim milk. The protective media were prepared by suspending these agents in distilled water according to the experimental plan shown in Tables 1 and 2, and were sterilized at 108 °C for 15 min.

Preparation procedure

Cells in the early stage of the stationary phase were harvested under aseptic conditions by centrifugation at 10,000 rpm for 10 min at 4 °C in a centrifuge (5804 R, Eppendorf, Germany). The growth medium was decanted and the harvested cells were washed twice in aseptic distilled water, and centrifuged again. Each pellet was resuspended in the experimental protective medium to make a cell suspension containing approximately 1.0×10^{10} CFU/ml. Aliquots (1 ml) of each resuspension were transferred into two sterilized vials (7 ml) and were frozen at −20 °C overnight. Then the samples were immediately freeze-dried for 24 h in a freeze-dryer (Alpha 1-2, Christ, Germany).

Determination of cell viability

The number of viable cells before and after freeze-drying was determined as colony forming units (CFU). Decimal dilutions were prepared from the suspension before freezing and suitable dilutions were plated on modified TJA agar [13], consisting of (per liter of tap water) yeast extract 5 g, beef extract 10 g, lactose 20 g, sucrose 2 g, sodium acetate 5 g, K_2HPO_4 2 g, Tween 80.1 g, tomato juice 50 ml, and agar 15 g (pH 6.8 ± 0.2), by the drop count technique. The freeze-dried samples were resuspended in skim milk by shaking, incubated at room temperature for 15 min and subsequently plated as described above. Plates were incubated at 37 °C for 48 hr before the colonies were counted.

The viability of cell suspension for each protective medium was calculated using the following equation:

$$\text{Viability}\,(\%) = \frac{\text{Viable cells after freeze - drying}\,(\text{CFU/ml})}{\text{Viable cells before freezing}\,(\text{CFU/ml})} \times 100$$

Experimental design and statistical analysis

A full factorial central composite design (CCD) was carried out to determine the optimum level of the major variables previously selected by Plackett–Burman design, including sucrose, glycerol, sorbitol and skim milk. The experimental design in the coded (x_i) and in the actual (X_i) levels of variables are shown in Table 1.

Table 1 Level and code of variables chosen for CCD

Variable	Symbol		Coded level				
	Uncoded	Coded	−2	−1	0	+1	+2
Sucrose(g/L)	X_1	x_1	20.00	50.00	80.00	110.00	140.00
Glycerol(g/L)	X_2	x_2	20.00	50.00	80.00	110.00	140.00
Sorbitol(g/L)	X_3	x_3	15.00	50.00	85.00	120.00	155.00
Skim milk (g/L)	X_4	x_4	10.00	50.00	90.00	130.00	170.00

When developing the regression equation, the test factors were coded according to the following equation:

$$xi = (X_i - X_0)/\Delta X_i i = 1, 2, 3, ..., k, \quad (1)$$

where x_i is the dimensionless value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of the independent variable at the center point, and ΔX_i is the step change value.

RSM was used to analyze the experimental data. We attempted to fit the response variable to a quadratic model in order to correlate the response variable to the independent variables. The behavior of the system was explained by the following quadratic polynomial equation:

$$Y = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_i^2 + \sum \beta_{ij} x_i x_j \quad (2)$$

where, Y is the predicted response, β_0 is the intercept term, β_i is the linear term, β_{ii} is the squared term, and β_{ij} is the interaction term.

In the central composite design, the total number of treatment combinations was $2^K + 2K + n_0$, where K is the number of independent variables and $n_0$is the number of repetitions of the experiments at the central point, which indicated that 30 experiments were required for this procedure [10]. All of the experiments were carried out in duplicate. Design Expert software (Version 6.0.5, Stat-Ease. Inc., Minneapolis, MN, USA) was used for regression and graphical analysis of the experimental data obtained. The optimum levels of the selected variables were obtained by solving the regression equation and also by analyzing the response surface contour and surface plots.

Results and discussion

Developing and checking the fitted model

The objective of the present study was to find the optimum combination of sucrose, glycerol, sorbitol and skim milk level to maximize the cell viability of *L. bulgaricus* LB14 during freeze-drying. The experiments were carried out according to the experimental plan given in Tables 1 and 2. The experimental and predicted responses for cell viability are illustrated in Table 2. Using multiple regression analysis on the experimental data, the following quadratic polynomial equation was found to express the cell viability of freeze-dried *L. bulgaricus* LB14:

$$\begin{aligned} Y = {} & 74.1043 - 3.8520x_1 + 7.480159x_2 + 4.7988x_3 \\ & + 10.5697x_4 - 12.6013x_1^2 - 12.1592x_2^2 - 10.9007x_3^2 \\ & - 10.3904x_4^2 - 2.04507x_1x_2 - 1.8325x_1x_3 \\ & - 4.6386x_1x_4 + 1.8155x_2x_3 + 7.3257x_2x_4 \\ & + 10.5145x_3x_4 \end{aligned} \quad (3)$$

Table 2 CCD experimental design matrix with experimental and predicted values of cell viability of *L. bulgaricus* LB14

Run no.	x_1	x_2	x_3	x_4	Viability (%)	
					Experimental	Predicted
1	−1	−1	−1	−1	13.33	20.20
2	1	−1	−1	−1	29.66	29.52
3	−1	1	−1	−1	25.71	20.96
4	1	1	−1	−1	16.94	22.11
5	−1	−1	1	−1	13.67	8.80
6	1	−1	1	−1	5.71	10.80
7	−1	1	1	−1	13.33	16.83
8	1	1	1	−1	12.79	10.65
9	−1	−1	−1	1	14.35	14.93
10	1	−1	−1	1	6.05	5.71
11	−1	1	−1	1	46.94	45.00
12	1	1	−1	1	24.29	27.60
13	−1	−1	1	1	47.62	45.59
14	1	−1	1	1	25.85	29.04
15	−1	1	1	1	84.35	82.93
16	1	1	1	1	61.90	58.19
17	−2	0	0	0	28.57	31.40
18	2	0	0	0	20.41	15.99
19	0	−2	0	0	13.88	10.51
20	0	2	0	0	38.64	40.43
21	0	0	−2	0	24.49	20.90
22	0	0	2	0	38.10	40.10
23	0	0	0	−2	14.97	11.40
24	0	0	0	2	51.70	53.68
25	0	0	0	0	77.55	74.10
26	0	0	0	0	76.87	74.10
27	0	0	0	0	72.11	74.10
28	0	0	0	0	70.07	74.10
29	0	0	0	0	75.92	74.10
30	0	0	0	0	72.11	74.10

where Y (%) is the cell viability of freeze-dried *L. bulgaricus* LB14, and x_1, x_2, x_3 and x_4 are the coded values of sucrose, glycerol, sorbitol and skim milk, respectively.

The statistical significance of the model was determined by F-test, and the analysis of variance (ANOVA) for the fitted quadratic polynomial model is summarized in Table 3. The value of $P_{\text{model}} > F$ was less than 0.0001, indicating that the model was significant at the probability level of $\alpha = 0.01$. However, the lack of fit was observed to be insignificant ($P_{\text{lack of fit}} > F = 0.1176$), implying that the obtained model was adequate to represent the experimental data.

The goodness of fit of the polynomial model was checked via the determination coefficient (R^2) and the correlation coefficient (R). In this case, the value of R^2 (0.9823) meant that only 2% of the variability in the responses was not explained by the model. A high value of the correlation coefficient ($R = 0.9911$) indicated a good agreement between the experimental and predicted values of cell viability, thus suggesting a high significance for the model.

The significance of each coefficient of the model was determined by Student's t-test and P-value (Table 4). The larger the magnitude of the t-value and smaller the P-value, the more significant the corresponding coefficient [2]. This meant that glycerol and skim milk were highly significant ($P_{x2} < 0.0001$, $P_{x4} < 0.0001$), and the quadratic main effects of sucrose and sorbitol ($P_{x1}{}^2 < 0.0001$; $P_{x3}{}^2 < 0.0001$) were more significant than their respective first-order effects ($P_{x1} = 0.0011$; $P_{x3} = 0.0002$). Among the interaction effects, the effects of sucrose × skim milk, glycerol × skim milk and sorbitol × skim milk were highly significant ($P_{x1x4} = 0.0013$, $P_{x2x4} < 0.0001$, $P_{x3x4} < 0.0001$), whereas those of sucrose × glycerol, sucrose × sorbitol and glycerol × sorbitol were insignificant ($P_{x1x2} = 0.1020$, $P_{x1x3} = 0.1394$, $P_{x2x3} = 0.1428$), indicating that skim milk may be the most important protective agent influencing the resistance of *L. bulgaricus* LB14 to freeze-drying.

Effect of the protective agents

The isoresponse contour and surface plots of RSM as a function of two factors at a time, holding all other factors at fixed level (zero, for instance), are helpful for understanding both the main and the interaction effects of these two factors [8]. The response values for the variables can be predicted from these plots. Figures 1–3 represent the isoresponse contour and surface plots for the cell viability of *L. bulgaricus* LB14 during freeze-drying.

The effect of varying concentration of sucrose and skim milk on the cell viability of *L. bulgaricus* LB14 during freeze-drying, while other two viariables (glycerol and sorbitol) were fixed at central concentration, is shown in Fig. 1. It was evident that the cell viability of freeze-dried *L. bulgricus* LB14 steadily increased with increasing skim milk concentration up to 110.00 g/L, but decreased slowly beyond this concentration at low sucrose concentration. While at high sucrose concentration, the increase in the response value was negligible with as the concentration of skim milk was increased. The cell viability of freeze-dried *L. bulgricus* LB14 decreased as the concentration of sucrose was increased in the range 70.00∼110.00 g/L. So a higher concentration of skim milk and lower concentration of sucrose enhance the freeze-drying tolerance of *L. bulgricus* LB14. A positive effect of sucrose was previously observed during the preservation of frozen and freeze-dried LAB [6, 28]. Abadias et al [1] claimed that sucrose produced one of the best results for the preservation of *Candida sake* cells during freeze-drying when combined with skim milk; however, sucrose could not increase cell viability markedly when used alone. The protection from disaccharides could be due to their capacity to hydrate biological structures, such as proteins and membranes, refered to as the "water replacement hypothesis" [9].

Skim milk is shown to be a most useful suspending medium for frozen or freeze-dried starter culture preparations due to its cryoprotective effects. *L. lactis* ssp. *lactis* CECT 5180 cells freeze-dried in skim milk remained 44.3% cell viable [6]. Zayed et al [28] found that skim milk powder offered a 22.4% survival rate for freeze-dried *L. salivarius* when used alone, and a survival rate of 83–85% was obtained when supplemented skim milk with trehalose and sucrose. The positive effect of skim milk on freeze-dried microorganisms can be explained by its capacity to stabilize the cell membrane constituents and to create a porous structure in the freeze-dried products that makes rehydration easier [21]. Moreover, skim milk contains proteins that provide a protective coating for the cells during freeze-drying [1].

Figure 2 depicts response surface and contour plots showing the effects of glycerol and skim milk on the cell viability of *L. bulgaricus* LB14 during freeze-drying at fixed sucrose (80.00 g/L) and sorbitol (85.00 g/L)

Table 3 ANOVA for the fitted quadratic polynomial model of the cell viability of *L. bulgaricus* LB14

Source	Sum of squares	DF	Mean square	F value	Prob > F
Model	18361.25	14	1311.52	59.48	< 0.0001
Lack of fit	283.65	10	28.37	3.01	0.1176
Pure error	47.08	5	9.42		
Total	18691.99	29			

$R = 0.9911$, $R^2 = 0.9823$ $R^2_{Adj} = 0.9658$

Table 4 Model coefficients, as estimated by multiple linear regression

Term	Estimate	Std error	*t* ratio	Prob > |t|
Intercept	74.1043	1.91695	38.66	< .0001
x_1	−3.8520	0.958475	−4.02	0.0011
x_2	7.4802	0.958475	7.80	< .0001
x_3	4.7988	0.958475	5.01	0.0002
x_4	10.5697	0.958475	11.03	< .0001
$x_1\ x_2$	−2.0451	1.173887	−1.74	0.1020
$x_1\ x_3$	−1.8325	1.173887	−1.56	0.1394
$x_2\ x_3$	1.8155	1.173887	1.55	0.1428
$x_1\ x_4$	−4.6386	1.173887	−3.95	0.0013
$x_2\ x_4$	7.3257	1.173887	6.24	< .0001
$x_3\ x_4$	10.5145	1.173887	8.96	< .0001
x_1^2	−12.6013	0.896571	−14.06	< .0001
x_2^2	−12.1592	0.896571	−13.56	< .0001
x_3^2	−10.9007	0.896571	−12.16	< .0001
x_3^2	−10.3904	0.896571	−11.59	< .0001

concentrations. The drastic interactions between glycerol and skim milk were apparent not only from the low probability value ($P < 0.0001$, Table 4), but also from the elliptical contour plot. A circular contour plot indicates that the interactions between the corresponding variables are negligible, while an elliptical contour plot indicates that the interactions between them are significant [18]. As can be seen from Fig. 2, an increase in skim milk concentration markedly increased the cell viability of freeze-dried *L. bulgricus* LB14 within the tested concentration range. Increasing the glycerol concentration resulted in a marked increase in cell viability at higher skim milk concentrations. So the interaction between the skim milk and glycerol was very significant ($P < 0.0001$, Table 4). Glycerol is a permeable compound that penetrates both the cell wall and the cytoplasmatic membrane. It makes the cell membrane more plastic and binds water, which suppresses excess dehydration, reduces salt toxicity and prevents the formation of ice crystals within the cell during freeze-drying [7]. The results indicate that cells freeze-dried in the presence of glycerol can achieve significantly higher viability. The positive effects of glycerol on freeze-dried LAB have also been described by Fonseca et al [12]. However, it was observed that glycerol showed hardly any protective effects for some microorganisms, such as *Lactococcus lactis* ssp. *lactis* [6].

Figure 3 shows similar plots at various values of sorbitol and skim milk concentrations and at fixed sucrose (80.00 g/L) and glycerol (80.00 g/L) concentrations. From Fig. 3, as the concentration of sorbitol was increased, the cell viability of freeze-dried *L. bulgricus* LB14 decreased at low skim milk concentrations, but drastically increased at higher concentrations of skim milk. The interaction effect of sorbitol and skim milk ($P < 0.0001$) was responsible for this behavior. These results show that sorbitol provides a protective effect for freeze-dried *L. bulgricus* LB14 during freeze-drying. This was in accordance with results previously reported by Carvalho et al [7], who found that for *L. plantarum* and *L. rhamnosus*, increased survival rate during freeze-dry-

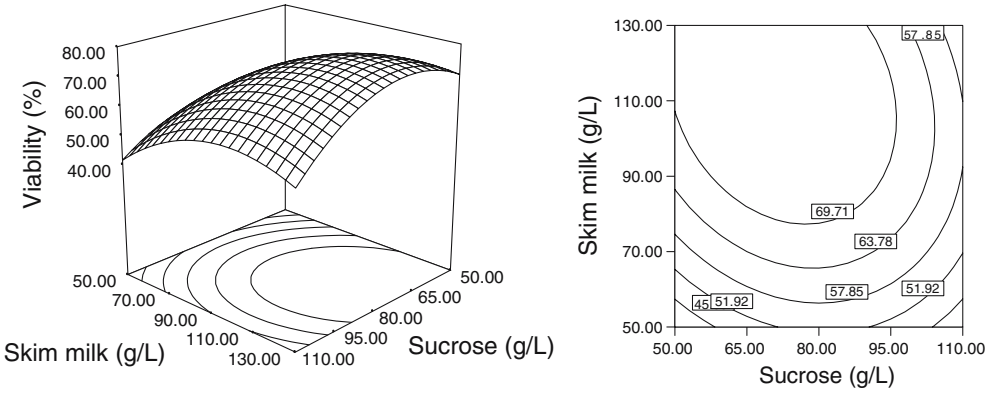

Fig. 1 Response surface and contour plot of skim milk versus sucrose on the cell viability of *L. bulgaricus* LB14

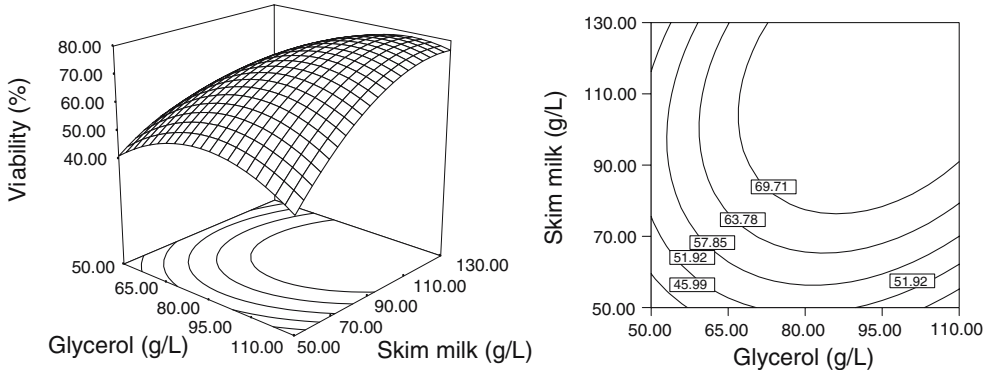

Fig. 2 Response surface and contour plot of glycerol versus skim milk on the cell viability of *L. bulgaricus* LB14

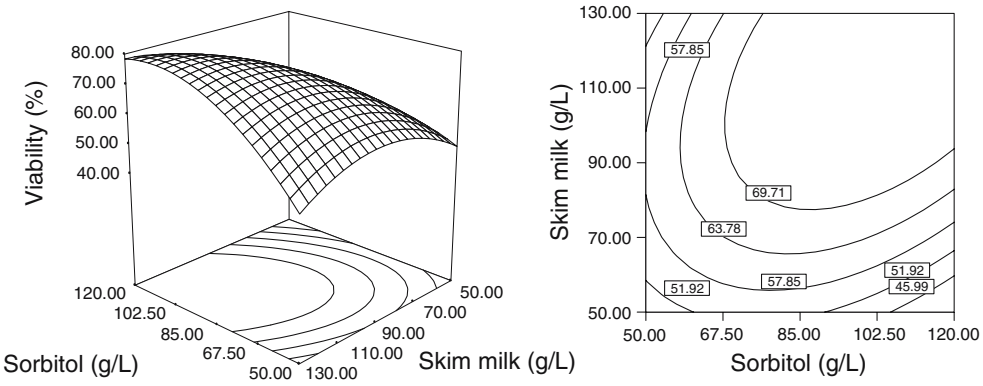

Fig. 3 Response surface and contour plot of sorbitol versus skim milk on the cell viability of *L. bulgaricus* LB14

ing and subsequent storage was obtained when the cells were freeze-dried in the presence of sorbitol. The protective effect of sorbitol on freeze-drying survival has been attributed to its capacity to prevent membrane damage by interacting with the membrane [15] and stabilizing protein functionality and structure [27].

From equations derived by differentiating Eq. 3, the optimum values for the independent variables investigated were sucrose 66.40 g/L, glycerol 101.20 g/L, sorbitol 113.00 g/L, and skim milk 130.00 g/L, with the corresponding $Y = 87.55\%$. To confirm the results, *L. bulgaricus* LB14 was freeze-dried in this optimum protective medium, and a viability of $(86.53 \pm 1.65)\%$ ($N = 3$) was obtained. The good correlation between these two results verified the goodness of fit of the model.

Conclusion

Since conventional studies on protective media optimization are usually time-consuming and expensive, a CCD and RSM were applied to determine the optimal levels of protective agents in order to enhance the viability of *L. bulgaricus* LB14 during freeze-drying. Only 30 experiments were necessary and the obtained model was adequate ($P < 0.0001$). By solving the regression equation, the optimum protective medium for *L. bulgaricus* LB14 was determined as followings: sucrose 66.40 g/L, glycerol 101.20 g/L, sorbitol 113.00 g/L, and skim milk 130.00 g/L. This protective media resulted in a viability of 86.53% for freeze-dried *L. bulgaricus* LB14. The research results indicated that RSM not only helped us locate the optimum concentrations of the protective agents in order to enhance the maximum viability of *L. bulgaricus* LB14 during freeze-drying, but also proved to be well-suited to evaluating the main and interaction effects of the protective agents on the cell viability.

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