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ORIGINAL RESEARCH PAPER

OPTIMIZATION OF *LACTOBACILLUS RHAMNOSUS* VIABILITY DURING FREEZE-DRYING BY BOX-BEHNKEN DESIGN

YANG ZHAO, XIAOPING HUO, YONGMIN ZHANG, ZONGCHENG MIAO*

Department of Applied Statistics and Science, Xijing University, Xi'an, Shaanxi 710123, China

*Corresponding author: mzongcheng@gmail.com

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Freeze drying is the most extensively used preparation and preservation technology for the cells. However, the freeze-dried cells may be injured during this process and can cause significant cell death. In this work, we aimed to optimize hydroxyproline, sucrose and isomaltooligosaccharide concentrations in order to maximize the survival rate of *Lactobacillus rhamnosus* during freeze drying, using response surface methodology (RSM). A Box-Behnken design was used to evaluate the effects of these cryoprotectants on the *Lactobacillus rhamnosus* cells in terms of viability. The optimal concentrations of the added compounds in the medium were experimentally studied and they were found to be (w/w): 12.11 % for hydroxyproline, 9.67 % for sucrose and 2.56 % for isomaltooligosaccharide. When *Lactobacillus rhamnosus* cells were dried by using the cryoprotectants found in the optimum concentration, a rate of 67.78±0.26 % cells survived and the cell viability reached the value of 1.79±0.05×1011 CFU/g.

Keywords: *Lactobacillus rhamnosus*, freeze drying, cryoprotectant, prebiotic, amino acids

Introduction

Lactobacillus rhamnosus was the overriding used probiotic for the health benefits on human beings. The functions of the bacteria were studied and identified as follows: prevention of cancer (Fang *et al.*, 2014), reduction in allergies (Canani *et al*., 2016), strength of resistance of the host against influenza virus infection (Song *et al*., 2016), prevention of antibiotic-associated diarrhea (Szajewska *et al*., 2016), suppression of pathogen colonization (Beltran et al., 2016). During the last few decades, probiotics have been widely used in developing dried formulations for nutraceutical applications (Siaterlis *et al.*, 2009). To manufacture dried formulations, freeze drying, which is of vital importance in actively maintaining biological products and for the long-term storage of cellular and tissue

biospecimens, was employed (Passot *et al*., 2015). However, not all the cells can survive under the stress conditions induced by the freeze drying process. Damage to the cells resulting from freeze-drying can be attributed to the following causes: (a) exposure to increasing solute concentrations during slow freezing; and (b) intracellular ice formation during rapid freezing (Mazur, 1977; Mazur, 1984; Passot *et al*., 2015). From an industrial point of view, drying microorganisms represents a very important process in order to maximize the survival cells rate (Stephan *et al*., 2016).

Additionally, the cryoprotective agents, namely cryoprotectants, can enhance the survival and viability of the cells after freeze drying process (Abadias *et al*., 2001). In food industry, the cryoprotectants can help probiotics to survive in the adverse condition, especially during the preparation and storage steps. Nevertheless, the viability of microorganisms may vary after freeze-drying process when different cryoprotectants are used (Carvalho *et al*., 2004). Thus, when involved in industrial manufacturing, it is necessary to develop suitable and efficient cryoprotectants for the probiotic strain.

In the present study, the survival rate and viability of the *L. rhamnosus* cells cultured in a matrix with three affecting factors such as hydroxyproline (Hyp), sucrose and isomaltooligosaccharide (IMO) were studied using response surface methodology (RSM).

Materials and methods

Microorganisms and culture media

The *Lactobacillus rhamnosus*, obtained from *Department of Applied Statistics and Science, Xijing University*, was used throughout the screening experimental designs. The microorganism cells were stored at -20 °C in freeze-dried De Man, Rogosa, Sharpe (MRS) broth (Aoboxing biological technology co., LTD, Beijing, China) supplemented with skim milk (20 %, w/w) (Anchor, New Zealand), and then cultured in MRS broth (sterilized at 118 °C for 15 min) with 3% (v/v) of inoculums, and 16h of growth at 37°C.

Freeze drying medium

Isomaltooligosaccharide (IMO) (LSbio, Xi'an, China), sucrose (LSbio, Xi'an, China) and hydroxyproline (Hyp) (LSbio, Xi'an, China) solutions were sterilized through filtration by 0.22 μm cellulose acetate membrane. The control of lyoprotectant consisted of double distilled water (sterilized at 121 °C for 20 min). All lyoprotectants were mixed with the *L. rhamnosus* cells before the freeze drying process.

Preparation of freeze-dried cell

All the cells were centrifuged at 10 000 × g for 15 min at 4 °C, and washed twice using 0.9 % NaCl solution. Before prefrozen at –80 °C for 12 h, lyoprotectants

were mixed with cells, and then freeze dried at –55 °C, 6.93 Pa for 18-24 h by a vacuum freeze dryer (Biocool, Beijing, China).

Viability assay

The population of the viable cells was determined as CFU (colony forming units) and evaluated by direct count of plate dilution method on MRS agar medium (carried out at 37°C for 48 h, in triplicates). After centrifugation, the cells were considered as "before freeze drying (N_0)" data. The "after freeze drying (N_t)" data were conducted by viability of the cells in powdered products. The survival rate was calculated using the equation:

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

Experimental design and statistical analysis

In the present experiment, the responses were the survival rate and the *L. rhamnosus* viable cells/g powdered cells (CFU/g). To efficiently explore the best condition of the three variables, a Box-Behnken design with 15 experimental trails was applied. The selected runs were randomly arranged to minimize the effect of unexplained variability on the method, and the coded and actual concentration (%, w/w) of all variables are listed in Table 1 and 2, low and high factors were coded as −1 and +1, the center point was coded as 0. Moreover, Design-expert (version 8.0.6, Stat-Ease, Inc., USA) was used to analyze the experimental designs, the analysis of variance (ANOVA) technique was then applied to determine which factors were statistically significant, the significances of all terms in the polynomial were judged statistically by the $p<0.05$. The response variables (Response $1^{\#}$ and $2^{\#}$, R1 and R2) were fitted with a multiquadratic regression model as follows (Tarek *et al*., 2013):

$$R = \beta_0 + \sum_{i=1} \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum_{i=1} \sum_{j=i+1} \beta_{ij} X_i X_j$$

where R is the predicted responses of the dependent variable, β_0 is the second-order reaction constant, X_i and X_j are independent variables, β_i is the linear regression coefficient, β_{ii} is the quadratic regression coefficient, and β_{ij} is the regression coefficient of interactions between two independent variables (Chen *et al*., 2015).

Table 1. Coding and actual levels of variables (%, w/w)

Factors	Symbol	Actual values of coded levels		
		+1	0	-1
Hyp	A	13	12	11
Sucrose	B	11	10	9
IMO	C	2.75	2.50	2.25

Results and Discussion

Fitting the model and response surface analysis

Following the experimental design, results of RSM experimental trials are presented in Table 2. The survival rate (R1, %) and Log_{10} population after freeze drying (R2, Log CFU/g) of *L. rhamnosus* cells were analyzed to get a regression model, a function of the independent variables, as follows (Equation 2 and 3):

$$R1 = 65.4367 - 0.6888\times A - 1.5138\times B + 1.3250\times C - 2.2750\times AB + 4.3525\times AC + 1.3325\times BC - 5.8733\times A^2 - 2.3083\times B^2 - 3.3558\times C^2 \quad (2)$$

$$R2 = 11.2426 - 0.0177\times A - 0.0340\times B + 0.0322\times C - 0.0484\times AB + 0.1018\ AC + 0.0419\times BC - 0.0963\times A^2 - 0.0410\times B^2 - 0.0468\times C^2 \quad (3)$$

In the models, A, B and C present Hyp (hydroxyproline) (%, w/w), sucrose (%, w/w) and IMO (isomaltooligosaccharide) (%, w/w), respectively.

Table 2. Box-Behnken design and responses of dependent variable

Run	A	B	C	R1	R2
1	0	1	-1	54.01	11.01
2	0	1	1	61.93	11.22
3	-1	1	0	59.52	11.16
4	-1	-1	0	57.42	11.11
5	-1	0	1	52.04	11.00
6	1	0	-1	51.67	10.99
7	1	1	0	52.54	11.00
8	1	0	1	60.42	11.20
9	-1	0	-1	60.7	11.20
10	0	0	0	62.33	11.22
11	0	-1	1	62.87	11.22
12	1	-1	0	59.54	11.15
13	0	0	0	66.52	11.24
14	0	0	0	67.46	11.27
15	0	-1	-1	60.28	11.18
16	Control			1.20	9.3

ANOVA was shown in Table 3 and 4. The lack of fit test of these two models were all not significant (p>0.05), whereas the models p-R1=0.038<0.05, p-R2=0.0257<0.05 implied that the models fitness was acceptable, meaning that the suitable fit of the model lead to a preferable and correct result from a fitted response surface. On the other hand, smaller values of the coefficient of determination R^2 indicates lower relevance of the dependent variables explained by the model (Lee *et al.*, 2006). For Eq. (Model) 1, R_1^2=0.9077 means more than 94.31% of variability in the response could be explained by the model, and *Adj*

R_1^2= 0.7416 is closer to the R^2 indicating that the model was highly significant. Therefore, the same result showed Eq. 2 (R_2^2=0.9223, *Adj* R_2^2= 0.7824). From the Table 3, a high F value of the interaction term AC, and quadratic terms A^2 and C^2 implied a higher contribution of these three terms on the R1, and *p*-value < 0.05 suggested that these three terms are significant effecters for R1. Similarly, terms B, AB, AC, and C^2 affect R2 significantly.

Table 3. ANOVA for the response surface model of R1

Source	Squares	df	Mean	F Value	*p*-value
Model	308.6	9	34.29	5.46	0.038*
A-Hyp	3.8	1	3.8	0.6	0.4719
B-Sucrose	18.33	1	18.33	2.92	0.1481
C-IMO	14.05	1	14.05	2.24	0.1949
AB	20.7	1	20.7	3.3	0.129
AC	75.78	1	75.78	12.08	0.0178*
BC	7.1	1	7.1	1.13	0.336
A^2	127.37	1	127.37	20.3	0.0064**
B^2	19.67	1	19.67	3.14	0.1368
C^2	41.58	1	41.58	6.63	0.0498*
Residual	31.37	5	6.27		
Lack of Fit	16.45	3	5.48	0.74	0.6202
Pure Error	14.92	2	7.46		
Cor Total	339.97	14			

Note: ** p<0.01, very significant; * p<0.05, significant.

Effects of independent variables on responses

The 3D-response surfaces and contour plots of survival rate (R1) of *L. rhamnosus* cells are shown in Figures 1–3 (response surfaces and contour plots of R2 have been omitted). As shown in Figures 1 and 3, the survival rates were changed following the change of Hyp concentration (%, w/w) and sucrose concentration (%, w/w) (or sucrose an IMO) to a certain value. Nevertheless, compared with the above effects, R1 changed consistently with the rapid increase and decrease of the concentration of Hyp and IMO to the limit extent (Figure 2). Obviously, interactions of these two factors have a significant effect on the survival. It is well documented the fact that addition of sucrose before freeze drying can enhance the survival rate of probiotics (Abadias *et al*., 2001; N'Guessan *et al*., 2016). Previous studies suggested that these cryoprotectants can raise the glass-phase transition temperature, and therefore, viable cells can reach the glassy phase without nucleating intracellular ice. Furthermore, it plays a key role in maintaining the cytoplasmic membrane properties stable during freeze drying (Carvalho *et al*., 2003a; Fowler & Toner, 2005; Passot *et al.*, 2015). Besides, previous work showed that carbohydrates and prebiotics were the vital factors for protecting the probiotic

cell membrane from damage of crystals formation and improved the survival of cells (Fowler, *et al*., 2005; Chen, *et al*., 2015). Furthermore, amino acids and amino acid derivatives such as phenylalanine, arginine, glycine, and betaine can stabilize the cell membrane during freeze drying process (Kets & deBont, 1994; Mattern *et al*., 1999; Carvalho *et al*., 2003b).

Table 4. ANOVA for the response surface model of R2

Source	Sum of Squares	df	Mean Square	F Value	*p*-value
Model	0.1215	9	0.0135	6.5919	0.0257*
A-Hyp	0.0025	1	0.0025	1.2250	0.3188
B-Sucrose	0.0093	1	0.0093	4.5269	0.0867
C-IMO	0.0083	1	0.0083	4.0613	0.1000
AB	0.0094	1	0.0094	4.5787	0.0853
AC	0.0415	1	0.0415	20.2507	0.0064**
BC	0.0070	1	0.0070	3.4257	0.1234
A^2	0.0342	1	0.0342	16.7248	0.0095**
B^2	0.0062	1	0.0062	3.0265	0.1424
C^2	0.0081	1	0.0081	3.9442	0.1038
Residual	0.0102	5	0.0020		
Lack of Fit	0.0092	3	0.0031	6.0247	0.1457
Pure Error	0.0010	2	0.0005		
Cor Total	0.1317	14			

Note: ** $p<0.01$, very significant; * $p<0.05$, significant.

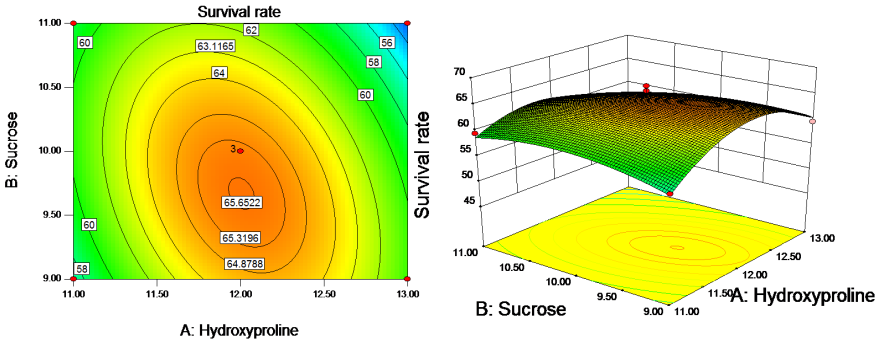

Figure 1. Effects of Hyp concentration (%, w/w) and sucrose concentration (%, w/w) on survival rate (%) of *L. rhamnosus* represented by contour plot (left) and 3D-response surface plot (right)

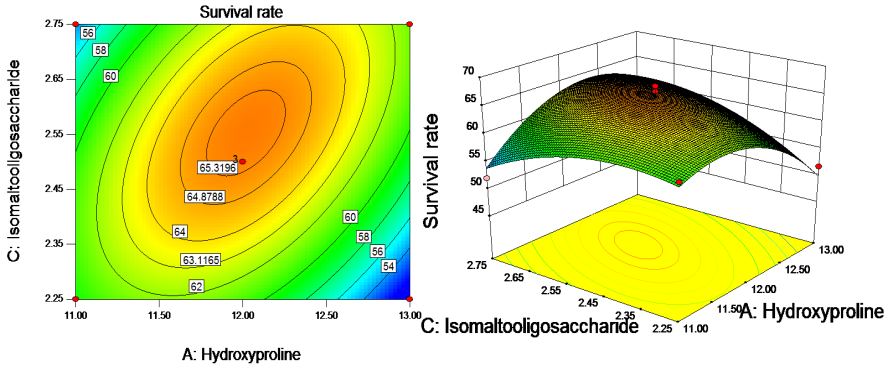

Figure 2. Effects of Hyp concentration (%, w/w) and isomaltooligosaccharide concentration (%, w/w) on survival rate (%) of *L. rhamnosus* represented by contour plot (left) and 3D-response surface plot (right)

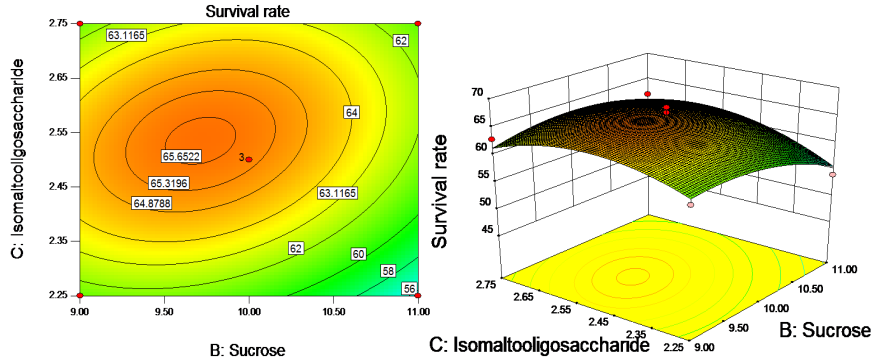

Figure 3. Effects of isomaltooligosaccharide concentration (%, w/w) and sucrose concentration (%, w/w) on survival rate (%) of *L. rhamnosus* represented by contour plot (left) and 3D-response surface plot (right)

Prediction and experimental validation

The predicted optimized levels of factors were 12.11 % hydroxyproline, 9.67 % sucrose and 2.56 % isomaltooligosaccharide; the suitability of the model equation for predicting the optimum response value was tested by verification through additional independent experiments under the previous operational conditions. Consequently, compared with the control (R1 = 1.13%, R2 = 2×10^9 CFU/g), the responses significantly increased the survival rate to the extent of 67.78 ± 0.26 % of cells and the cell viability to the extent of 1.79 ± 0.05 × 10^{11} CFU/g of population after freeze drying process.

Conclusions

Our study focused on freeze drying preservation of *Lactobacillus rhamnosus* cells, and on the concentrations of a potential cryoprotectant complex in order to ensure

the bacteria survival. Three materials, which can be used in commercial and industrial process, were investigated as protective agents for the cells. The results showed that the optimal cryoprotectant complex could significantly improve the survival of *L. rhamnosus*. When optimal concentrations of the three materials were used in freeze-drying process, the present probiotic cells had a survival rate of at least 67.78 % and reached 1.79×10^{11} CFU/g of freeze-dried product.

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