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10.1515/aucft-2016-0009

ASSESSMENT AND DETERMINATION OF LYOPROTECTANT FOR SURVIVAL OF FREEZE-DRIED *LACTOBACILLUS RHAMNOSUS*

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Abstract: Currently research of lactic acid bacteria focus primarily on the functional probiotics, which are major beneficial biota in the gastrointestinal tract, have been industrial manufactured. Probiotics confer health benefits on the host need adequate amounts. However, the absence of data makes it difficult to ensure the maintenance biological activities and population of probiotic. In this research, a fractional factorial design and steepest ascent experiment were used to analyze the influence of lyoprotectant as carbohydrates, prebiotics and amino acids on the survival of the probiotic *Lactobacillus rhamnosus*. The results indicated a maximum survival rate and population of viable bacteria of *L. rhamnosus* to be 55.84 % and 1.60 $\times 10^{11}$ CFU/g after freeze-dried by using a combination of 10 g/100mL Sucrose, 2.5 g/100mL Isomaltooligosaccharide, 12 g/100mL Hydroxyproline. To a large extent, the survival and viability were dependent on the cryoprotectant used and make probiotics more attractive from a practical application in industrial viewpoint.

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

INTRODUCTION

The probiotic *Lactobacillus rhamnosus* (*L. rhamnosus*) was widely used for the health benefis on hunman beings. The role of this bacteria in host health can be summarized as prevention of cancer (Fang et al., 2014), reduction in allergies (Canani et al., 2016), increase resistance of the host to against influenza virus infection (Song et al., 2016), antibiotic-associated diarrhoea (Szajewska et al., 2016), inhibit colonization of pathogen (Beltran et al., 2016)

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etc. Recent studies in *L. rhamnosus* focus chiefly on the function of thallus or theirs products, and rarely pay the attention on an industrial practical application. The food probiotics, which developed in dairy and non-dairy probiotic products, occupies a great deal of probiotics application. Commercially dairy and non-dairy probiotics have many different forms of products, such as yoghurt, cheese, ice cream, non-fermented milk beverage, fruit and vegetable juices, bread and bakery products, puddings, sausages, soy yoghurt and curd (Kumar et al., 2015). The above fermented foods and fermented dairy foods pay an important role in carrier of probiotics (Heller, 2001).

In the probiotic foods, the protective matrix can help probiotics to survive from the adverse condition, especially in the preparation and storage process. Freeze drying is the major method to produce the powdered products and maintain probiotic properties (Morgan, C. & Vesey, G., 2009). During freeze drying, cell viability loss may caused by ice crystal formation and desiccation(Telang et al., 2003). Thus, it's necessary to provide efficient desiccation protectants. Carbohydrates were well documented to have protective effects for probiotic bacteria during freeze-drying (Chen et al., 2015). Furthermore, milk proteins, the protective agents, can act effectively in protecting the probiotic cells till they reach the site of action in small intestines (Ritter et al. 2009).

In the present study, the combination of fractional factorial design and steepest ascent experiment was used to evaluate the potential protective action for *L. rhamnosus* during freeze drying. Multiple carbohydrates, prebiotics and amino acids were selected and the concentrations and survival rate of *L. rhamnosus* were monitored in these experiences.

MATERIALS AND METHODS

Preparation of microorganisms and media: The probiotic *Lactobacillus rhamnosus*, which obtained from *Department of Applied Statistics and Science, Xijing University*, was used throughout the screening experimental designs. The strain was 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).

Three successive microorganisms (with 3% (v/v) of inoculum, 16h of growth at 37°C) were transferred into MRS broth that sterilized at 118 °C in 15 min.

Preparation of freeze drying medium: All lyoprotectant candidates, carbohydrates (sucrose, lactose, glucose, maltose, trehalose) (LSbio, Xi'an, China), prebiotics(oligofructose, chitosan, xylooligosaccharide, inulin, isomaltooligosaccharide, galactooligosaccharide)(LSbio, Xi'an, China) and

amino acids(LSbio, Xi'an, China)(glutamate, aspartate, serine, arginine, tyrosine and hydroxyproline) (LSbio, Xi'an, China) solutions were filtration sterilized by with 0.22 μm cellulose acetate membrane. The control of lyoprotectant candidates, double distilled water, were sterilized at 121 °C for 20 min. The additional lyoprotectant, skim milk, were sterilized at 110°C for 15 min. The lyoprotectant candidates were mixed with the *Lactobacillus rhamnosus* cells before freeze drying.

Preparation of freeze-dried cell: Each culture (cultured under the foregoing conditions) was centrifuges at 4 °C (10 000 ×g, 15 min), then baptized the harvested cells twice using saline solution. Before pre-frozen at –80°C for 12 h, lyoprotectant candidates were added and mixed with the 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 viability of the cells were 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). Population (viable number) after centrifugation were considered as "before freeze drying (N_0)" data. The "after freeze drying (N_t)" data was conducted by number of viable cells powdered products, which reconstituted to their original volumes and counted as above. Percentage survival was calculated using the formula: Survival rate (%) = $N_0/ N_t \times 100$

Experimental design and statistical analysis: In the present experiment, the responses were survival rate and population of viable cells/g powered *L. rhamnosus* cells (CFU/g). To determine the key protctive agents in the candidates that significantly affect suvival of *L. rhamnosus* cells in freeze drying process, the $2^{(6-3)}$ fractional factorial design(FFD) was employed and all candidates were randomly arranged to minimise the effect of unexplained variability on the responses, real levels of all selected candidates used in the $2^{(6-3)}$ FFD design are listed in Table 1.

Table 1. Values (%, w/w) and results of survival rate (Y1,%) and the population of freeze-dried products (Y2, $\times 10^{10}$ CFU/g) as response variables obtained by $2^{(6-3)}$ fractional factorial design

Run	Ser	Hyp	Trehalose	Sucrose	Inulin	IMO	Y1	Y2
1	12	12	10	20	2.5	2.5	38.7	4.1
2	12	6	10	10	2.5	5	4.68	0.76
3	12	6	20	10	5	2.5	18.56	1.63
4	6	6	10	20	5	5	19.93	2.04
5	6	12	10	10	5	2.5	26.3	2.52
6	6	6	20	20	2.5	2.5	19.74	2.11
7	12	12	20	20	5	5	26.88	2.49
8	6	12	20	10	2.5	5	24.43	2.26
9	Control						1.17	0.185

Moreover, Design-expert (version 8.0.6, Stat-Ease,Inc., USA) was used to analysis of the experimental designs, and single treatments were compared by one-factor test, and further optimal of the key agents were performed by steepest ascent experiment.

RESULTS AND DISCUSSIONS

Finding primary factors in candidates

The limited amounts of carbohydrates (20%, w/w), prebiotics (5%, w/w) and amino acids (12%, w/w) were mixed with cells before freeze drying, respectively. Survival rate and population (viable cell count) varied according to the different combinations of lyoprotectant candidates from 2.97% (control) to 43.38% and 0.343×10^{10} CFU/g (control) to 3.95×10^{10} CFU/g, respectively. As Figure 1 shows, all candidates have a higher survival and population than control, showing that the candidates have positive effect on the viability of the freeze-dried cells. Within carbohydrates, sucrose and trehalose lead to a higher viability, represented by survival and population than other carbohydrates candidates mentioned above ($p<0.05$). In comparison of the protective effects of a series of disaccharides on *L. rhamnosus* GG, survival during freeze-drying indicated that trehalose, trehalose/lactose are the most efficacious disaccharides during freeze-drying (Meng et al., 2008).

An earlier reseach have demonstrated that trehalose and sucrose have evident protective capability both on gram-negative and gram-positive bacteria (Leslie *et al*., 1995), the significant protective effect is likely to be due to the ability of carbohydrates to lower the phase transition temperature and to maintain stability of protein structure during drying process (Siaterlis et al., 2009). On the other hand, among the six prebiotics tested, inulin and isomaltooligosaccharide, which at a concentration of 5% (w/w), offer best protection during freeze drying compared with control and other candidates, for the *L. rhamnosus* strains. The cells demonstrated survival of 35.88% and 33.77%, respectively. Moreover, in the case of amino acids, the cell survival was when supplied serine and hydroxyproline (28.32°% and 40.83%, respectively) significantly higher than control.

Assessing effects of the factors on cells

Following the findings of primary factors, six significant candidates, serine (Ser), hydroxyproline (Hyp), trehalose, sucrose, inulin and isomaltooligosaccharide (IMO), were estimated by conducting eight runs $2^{(6-3)}$ fractional factorial design(FFD). Individual effects of three most significant factors (%, w/w) on survival rate(Y1,%) and the population of freeze-dried products (Y2, $\times10^{10}$ CFU/g) as the response variables of *L. rhamnosus* were studied (Table 1). It can be seen in Table 1 that the highest Y1 and Y2,

38.7% and 4.1×10^{10} CFU/g, are detected in the presence of chosen lyoprotectant candidates. These data are significantly higher (p<0.05) than the count achieved without supplements (control).

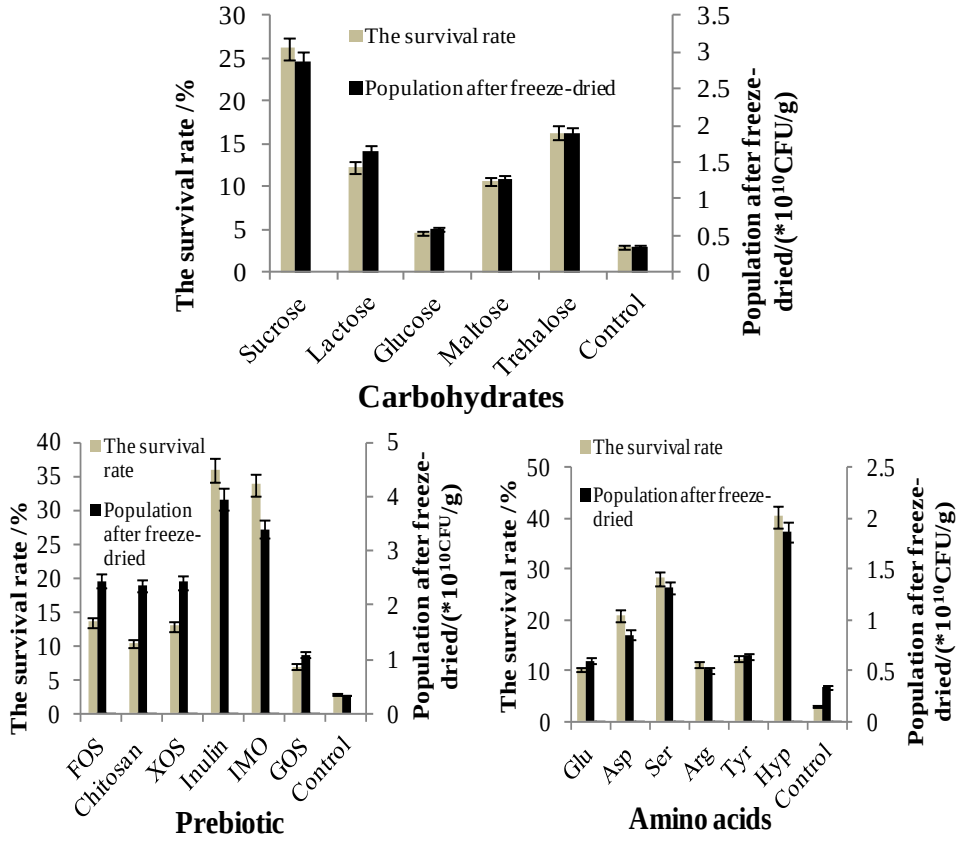

Figure 1. Tests and results of one-factor selection of protective candidates during freeze drying. Black bar represent the population (viable cell count) of freeze-dried products, and grey bar showed the survival rate of *L. rhamnosus* cells after freeze-dried. In which, Control = double distilled water, FOS= Oligofructose, XOS= Xylooligosaccharide, IMO= Isomaltooligosaccharide, GOS= Galactooligosaccharide.

The main effects of the factors on Y1 and Y2 after lyophilization are shown in Table 2 and 3, respectively. It turned out that Hyp is statistically significant factor (p<0.05) for survival of *L. rhamnosus* cells during freeze drying in regression analysis. In addition, according to principles of statistics which p-value between 0.10 and 0.05 implied that the factors have effect on the responses and be as main factors (Bulatovic et al., 2014), sucrose and IMO take important contribution on cells survival when served as the ingredients of drying media before freeze-drying. Impact and the 95% confidence interval of above three factors on Y1 and Y2 are pictured as Figure 2.

Table 2. Main effects regression analysis for *L. rhamnosus* on Y1 from the FFD after freeze drying.

Source	SS*	df**	MS***	F	*p*	
Model	572.458	3	190.819	10.238	0.0239	significant
Hyp	356.445	1	356.445	19.124	0.0119	
Surcose	122.305	1	122.305	6.562	0.0625	
IMO	93.708	1	93.708	5.028	0.0884	
Residual	74.554	4	18.639			
Cor Total	647.012	7				

*Sum of squares, ** Degrees of freedom, ***Mean square

Table 3. Main effects regression analysis for *L. rhamnosus* on Y2 from the FFD after freeze drying.

Source	SS*	df**	MS***	F	*p*	
Model	5.496	3	1.832	10.121	0.0244	significant
Hyp	2.916	1	2.916	16.110	0.0159	
Surcose	1.593	1	1.593	8.801	0.0413	
IMO	0.987	1	0.987	5.453	0.0798	
Residual	0.724	4	0.181			
Cor Total	6.220	7				

* Sum of squares, ** Degrees of freedom, ***Mean square

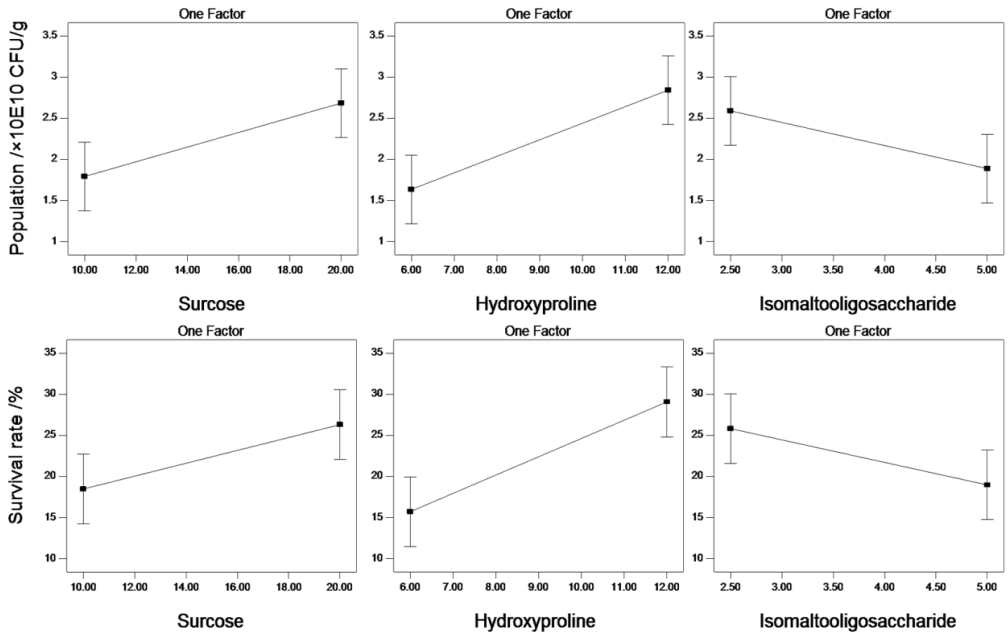

Figure 2. Impact of three factors on Y1 and Y2. The positive trends of 95% confidence interval implied positive effect on the responses

Determinning the optimum piont of the drying media

The preliminary tests showed that Hyp, Sucrose and IMO had evident effect than the other agents on cell survival and Hyp and sucrose showed positive effects expect for IMO (see Figure 2). To provide an efficient formulation for protecting the cells from loss during freeze drying and maximise viable cells of *L. rhamnosu*, the steepest ascent experiment was used. In this test, step orientation were chosen according to effect described above and the results listed in Table 4. In the 5 steps(trials), Y1 ranged from 26.97% to 55.84% , and Y2 was declined following increase of factors' concentration range between 7.67×10^{10} CFU/g and 16×10^{10} CFU/g. In addition, the maximum levels of Y1 and Y2 were reached when 10% sucrose, 2.5% isomaltooligosaccharide and 12% hydroxyproline were mixed in drying medium. This result indicates that the concentration of chosen lyoprotectants affected the survival of *L. rhamnosus* significantly and sucrose provides a effectively protection for the cell.

Table 4. Optimization of the concentrations of the factors (%, w/v) selected by previous works. Lyoprotectants were performed on drying media according to a steepest ascent test.

Trial	Hyp	Surcose	IMO	Y1	Y2
1	12	10	2.5	55.84	16
2	14	12	3	41.51	13.2
3	16	14	3.5	36.85	8.51
4	18	16	4	32.63	8.09
5	20	18	4.5	26.97	7.67

Siaterlis et *al*. (2009) showed a similar observations in *L. rhamnosu* with at concentration of 5% and 10%, if the carbohydrates can be taken up and present on both sides of the membrane of the cells; increase of the T_m (the membrane phase transition temperature) lead to an improved cell tolerance to drying (Leslie *et al*., 1995; Santivarangkna *et al*., 2008). Some works showed that carbohydrates and prebiotics were the vital factors for protect the probiotic cell membrane from damage of the crystals formation and improve the viability of the cells (Fowler, *et al*., 2005; Chen, *et al*., 2015). However, matrix based on compatible solutes such as amino acids and amino derivatives can also help to stabilize the cell membrane during drying processes (Carvalho *et al*., 2003; Kets & deBont, 1994).

CONCLUSIONS

Carbohydrates, prebiotics and amino acids, especially Hyp, Sucrose and IMO had significant effects on survival and viability of probiotic *Lactobacillus*

rhamnosus when freeze drying is used. To a large extent, the effectiveness of these lyoprotectants depends on the kinds and doses of lyoprotectant candidates. A lower concentration of the three lyoprotectants (10%(w/v, g/100mL) of sucrose, 2.5% (w/v, g/100mL of isomaltooligosaccharide and 12%(w/v, g/100mL of hydroxyproline) results a higher survival rate (55.84 %) and number of viable bacteria(1.60×10^{11} CFU/g) of *Lactobacillus rhamnosus* after freeze-dried than the other lyoprotectant candidates selected in present study. Furthermore, the method of lyoprotectants preparation was simpler and more efficient for research and practical application.

ACKNOWLEDGEMENTS

The work was partly supported by the Scholastic Science Research Foundation of Xijing University (Grant No. XJ140222).

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