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EFFECTS OF SUGAR ALCOHOL AND PROTEINS ON THE SURVIVAL OF *LACTOBACILLUS BULGARICUS LB6* DURING FREEZE DRYING

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ABSTRACT

Background. *Lactobacillus bulgaricus LB*6 is a bacterium which was selected in the commercial yoghurt with high angiotensin converting enzyme (ACE) inhibitory activity. Preparation of concentrated starter cultures via freeze drying is of practical importance to dairy and food industries.
Material and methods. We optimized the optimal sugar alcohol and proteins for *Lactobacillus bulgaricus LB*6 during the process of freeze drying using a Plackett-Burman design. In our initial tests survival rate and the number of viable cells were associated with the type of lyoprotectant used and so our optimization protocol focused on increasing survival rate. Substances that had previously had a protective effect during freeze drying were investigated, for example: mannitol, sorbitol, xylitol, meso-erythritol, lactitol, whey protein isolate 90, bovine serum albumin, and whey protein concentrate 80 and soy protein isolate 70.
Results. We found that the optimum sugar alcohol and proteins for survival of *Lactobacillus bulgaricus LB*6 were whey protein concentrate ($p = 0.0040$ for survival rate), xylitol ($p = 0.0067$ for survival rate) and sorbitol ($p = 0.0073$ for survival rate), they showed positive effect (whey protein concentrate and sorbitol) or negative effect (xylitol).
Discussion. The effectiveness of three chosen sugar alcohols and protein implied that they could be used as lyoprotectant for *Lactobacillus bulgaricus LB*6 in the further research, the optimal composition of sugar alcohol and protein for the lyoprotectant use must be established.

Key words: *Lactobacillus bulgaricus*, freeze drying, lyoprotectant, bacteria survival rate, sugar alcohol

INTRODUCTION

Lactobacillus bulgaricus is employed worldwide because it is able to produce lactic acid in the production of yoghurt, cheese and other fermented products (Guillouard et al., 2004; De Urraza and De Antoni, 1997) and is of vital importance to manufacture of fermented foods in combination with *Streptococcus thermophilus*. Although specific numbers are not mentioned in the definition, high levels of viable microorganisms are recommended in probiotic foods for efficacy (Knorr, 1998). During the preparation of the starter cultures, freeze drying become the most convenient and successful method of maximizing storage stability, viability, and activity of the bacterial cells (Berny and Hennebert, 1991; Lim et al., 2009) and it has been widely used in microbiology for many decades to stabilize and store cultures (Morgan and Vesey, 2009). However, not all strains survived during this process and quantitative viability rates as low as 0.1% have been reported (Abadias et al., 2001). The major causes of cell viability loss during freeze drying are related

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to ice crystal formation, membrane damage from high osmolarity due to high concentrations of internal solutes, macromolecule denaturation, and the removal of water, which affects properties of many hydrophilic macromolecules in cells (Allison et al., 1999; Chitra et al., 2003; De Paz et al., 2002; Thammavongs et al., 1996). For this reason, a variety of protective agents have been added to the drying media before freeze drying to protect the viability of probiotics during dehydration (Hubalek, 2003).

It is well documented that sugars have protective effects on lactic acid bacteria during freeze drying. For example, the trehalose has a high glass transition temperature (T_g), it can rise the glass-phase transition temperature of cells and therefore viable cells can be protected by reaching the glassy phase without nucleating intracellular ice (Fowler and Toner, 2005). Apart from this, sorbitol (Carvalho et al., 2002), mannitol (Efiuvwevwere et al., 1999), sucrose (Carvalho et al., 2003), lactose (Higl et al., 2007), mannose (Carvalho et al., 2004) were reported to have the same impact. Amino acids may have the same protective effects as carbohydrates. A study by Mattern et al. (1999) showed that phenylalanine, arginine, and glycine can prevent denaturation during protein freeze drying. Several studies have suggested that some salt buffers, such as NaCl or KCl (Carvalho et al., 2003), sodium citrate (Kets et al., 2004; Kurtmann et al., 2009) and phosphate (Ohtake, 2004) can help to protect cells during freeze drying combined with other protectants. Besides, Buitink et al. (2000) found that proteins had a higher T_g than sugar and suggested that proteins play an important role in glass formation. Hence, proteins, including skimmed milk, whey protein, blood serum, serum albumin, sodium caseinate and peptone are efficient desiccation protectants (Abadias et al., 2001; Hubalek, 2003; Sharma et al., 2014). All the above results showed that there was a lack of data on sugar alcohol and proteins was used as protectants for *Lactobacillus bulgaricus* during freeze drying, thus, there will be further insights into the studies of the effect of these various when applied in freeze drying.

In previous work, we screened 4 *Lactobacillus* strains that have high angiotensin converting enzyme (ACE) inhibitory activity from 28 probiotic strains. The *Lactobacillus reuteri*, *Lactobacillus bulgaricus* (*LB*6), *Lactobacillus rhamnosus* and *Lactobacillus helveticus* showed high ACE inhibitory activity with 95.92%, 84.61%, 82.79% and 78.57%, respectively (Chen et al., 2012). The aim of the present study was to investigate sugar alcohol and proteins that can potentially improve survival rate and the number of viable cells of *Lactobacillus bulgaricus LB*6 when freeze drying was employed.

EXPERIMENTAL SECTION

Microorganism and media preparation

*Lactobacillus bulgaricus LB*6 (*LB*6) were obtained from College of Life Science and Engineering, Shaanxi University of Science and Technology and inoculated three successive times with the MRS medium at 37°C for 24 h until the viability of bacteria stays stable. 3% active culture was added to the basal LAB growth media (500 mL) that containing substance that can promote cell proliferation, incubated at 37°C, and then viable counts at optional incubation time. All the media were autoclaved at 121°C for 15 min. The basal LAB growth medium contains 21 g of lactose, 4 g yeast extract powder, 10 g soya peptone, 9 g trehalose, 12 mg leucine and 1000 mL water.

Freeze drying medium

The combinations of freeze drying medium (lyoprotectants) were the selected sugar alcohol and proteins added at their designed levels. Sugar alcohol: D--mannitol (min. 98.0% LSbio, USA), D-sorbitol (min 98.0%, LSbio, USA), xylitol (min. 99.0%, LSbio, USA), meso-erythritol (min. 99.0%, LSbio, China) and lactitol (min. 98.0%, Food and feed additives, LSbio, China). All the sugar alcohols were autoclaved at 108°C for 15 min with 5% (w/w). The proteins: whey protein isolate 90 (WPI 90, Hilmar 9410 WPI90, USA), whey protein concentrate 80 (WPC 80, Hilmar 8010 WPC80, USA) and soy protein isolate 70 (SPI 70, Shanghai Yancui Import and Export Co., Ltd., China) were sterilized using 0.45 μm membrane filtration with 5% (w/w), and the bovine serum albumin V (BSA V, LSbio, USA) in solution of 2.5% (w/w). These protectants were mixed with *LB*6 cells at the ratio of 1:1 (w/w) (The shortage was supplemented by phosphate buffer) in freezed vials.

Vacuum freeze drying

After incubation, *LB*6 cell counts were determined immediately by the plate dilution method using MRS agar medium. The cells were harvested by centrifugation during the stationary phase of growth at 10 000 × g for 15 min at 4°C and washed twice by centrifugation and resuspended in the same volume of each medium with 0.9% NaCl solution. The cells were prefrozen at –40°C for 12 h after protective agents (the selected sugar alcohol and proteins) were added, and then freeze dried at –55°C, 6.93 Pa for 24 h using a vacuum freeze dryer.

Determination of cell counts

Before centrifugation, the samples were taken from each suspension and the number of CFU/mL (Colony Forming Units) was determined by the plate dilution method using MRS agar medium, and the plates were carried out at 37°C for 48 h, the viable cells of *LB*6 were conducted in triplicates by plating on the plate, and the results obtained were considered as "before freeze drying" data. The freeze-dried powder were reconstituted to their original pre-freeze dried volumes by adding sterile saline solution and number of viable cells counted as above, these results mean "after freeze drying" data.

Calculation of survival

Survival rate (%) = (CFU/mL after freeze drying / CFU/mL before freeze drying) × 100%

Screening of sugar alcohol and proteins using Plackett-Burman design

The Plackett-Burman design was employed to determine the effect of the selected sugar alcohol (mannitol, sorbitol, xylitol, meso-erythritol, and lactitol) and proteins (WPI 90, BSA V, WPC 80 and SPI 70) on survival rate and viability of *LB6* after freeze drying at a lower and a higher level coded as (+1) and (–1) (Table 1), respectively. The design matrix is shown in Table 2 where the effect can be seen of 11 variables (including two error terms: X6 and X11, in order to estimate the standard deviation) was investigated in 12 independent experimental runs.

Table 1. Sugar alcohol and proteins at different levels in Plackett-Burman design

Variables	Medium components	Lower level %	Higher level %
X1	mannitol	5	10
X2	sorbitol	5	10
X3	xylitol	5	10
X4	meso-erythritol	5	10
X5	lactitol	5	10
X7	WPI 90	5	10
X8	BSA V	2.5	5
X9	WPC 80	5	10
X10	SPI 70	5	10

Statistical analysis

The statistical analysis was performed by the Design-Expert (Version, 8.0.6) to identify the significant variables and their corresponding coefficients, so that the various levels managed to obtain a desired output. Hence, F-value, sum of squares, p-value and confidence interval (CI) was analysed using the experimental results of survival rate and viability. The experimental results (response function, Y) were fitted to first order multiple regression equations (eq. (1)) using coded level (–1 or +1) of the variables (X_i):

$$Y = b_o + \sum_{i=1}^{k} b_i x_i + \varepsilon \quad (1)$$

RESULTS

The experimental design and results

The relation between protective agents and survival rate of *L. bulgaricus LB*6 is shown in Table 2. The survival rate and viability of freeze-dried *LB*6 cells were represented by Y1 (%) and Y2 ($\times 10^{10}$ CFU/g), respectively. The survival rate was calculated by an equation containing the factor of viable cells, where the influence of protective agents was measured by the survival rate. All of the protective agents had different effects on the cells so that when the agents were changed, the survival rate and viability of *LB*6 also changed.

Table 2. Experimental design and results of the Plackett-Burman tests

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Y1, %	Y2, $\times 10^{10}$ CFU/g
1	1	–1	1	–1	–1	–1	1	1	1	–1	1	5.3	0.424
2	1	1	–1	1	–1	–1	–1	1	1	1	–1	65.67	4.29
3	–1	1	1	–1	1	–1	–1	–1	1	1	1	55.97	3.75
4	1	–1	1	1	–1	1	–1	–1	–1	1	1	10.01	0.915
5	1	1	–1	1	1	–1	1	–1	–1	–1	1	41.79	2.8
6	1	1	1	–1	1	1	–1	1	–1	–1	–1	4.88	0.331
7	–1	1	1	1	–1	1	1	–1	1	–1	–1	43.28	3.64
8	–1	–1	1	1	1	–1	1	1	–1	1	–1	19.4	1.26
9	–1	–1	–1	1	1	1	–1	1	1	–1	1	44.78	3.31
10	1	–1	–1	–1	1	1	1	–1	1	1	–1	50	3.37
11	–1	1	–1	–1	–1	1	1	1	–1	1	1	34.33	2.61
12	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	–1	11.63	1.07

Effect of the various on survival of *L. bulgaricus LB6*

Analysis of variance (ANOVA) was performed to estimate the effect on growth of each factor (Table 3). In the ANOVA, "p-value" less than 0.1000 indicate that the terms are significant. Thus, the variables WPC 80 (X9) ($p = 0.0040$), xylitol (X3) ($p = 0.0067$) and sorbitol (X2) ($p = 0.0073$) had the most significant impact on the survival rate and they can protect the cell better than the other agents tested in this study. Furthermore, the positive or negative of coefficients in Final Equation in Terms of actual factors means that all the selected variables have positive or negative effect on viable counts (Y1), and the trends of the 95%

Table 3. Result of ANOVA of variables for Survival rate (Y1)

Source	Sum of squares	df	Mean square	F-value	p-value
X1 – mannitol	83.9523	1	83.9523	12.3662	0.0722
X2 – sorbitol	915.2533	1	915.2533	134.8168	0.0073
X3 – xylitol	996.6341	1	996.6341	146.8042	0.0067
X4 – meso-erythritol	328.8627	1	328.8627	48.4415	0.0200
X5 – lactitol	180.9633	1	180.9633	26.6559	0.0355
X7 – WPI 90	0.1121	1	0.1121	0.0165	0.9095
X8 – BSA V	122.3685	1	122.3685	18.0249	0.0513
X9 – WPC 80	1 703.1301	1	1 703.1301	250.8711	0.0040
X10 – SPI 70	584.0865	1	584.0865	86.0359	0.0114

confidence interval of the variables (Fig. 1, 2 and 3) can also demonstrated this, the equation have been shown as follow (determination coefficient $R^2 = 0.9972$):

$$Y1 = 32.25 - 2.64X1 + 8.73X2 - 9.11X3 + 5.23X4 + 3.88X5 + 0.097X7 - 3.19X8 + 11.91X9 + 6.98X10$$

Effect of the various on viability of *L. bulgaricus LB6*

The Table 4 showed the ANOVA of the variables for viability of freeze-dried *LB6*. As the Table 4 showed, the relative importance of the variables was as follows: X9 > X3 > X2 > X4 > X10 > X1 > X8 > X5 > X7. Out of

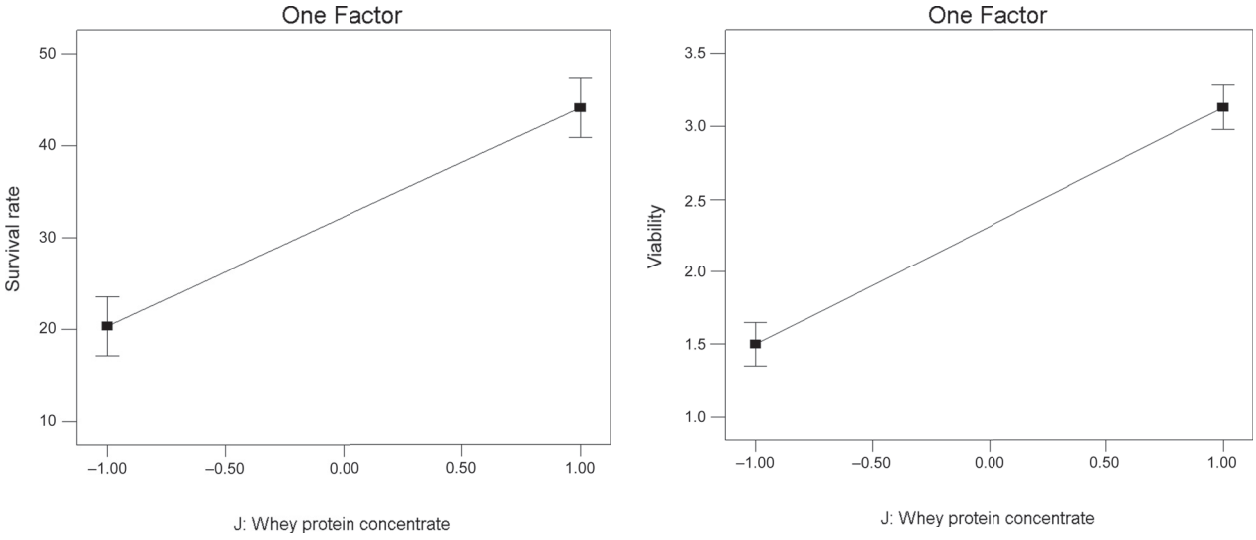

Fig. 1. 95% confidence interval of WPC 80 (X9)

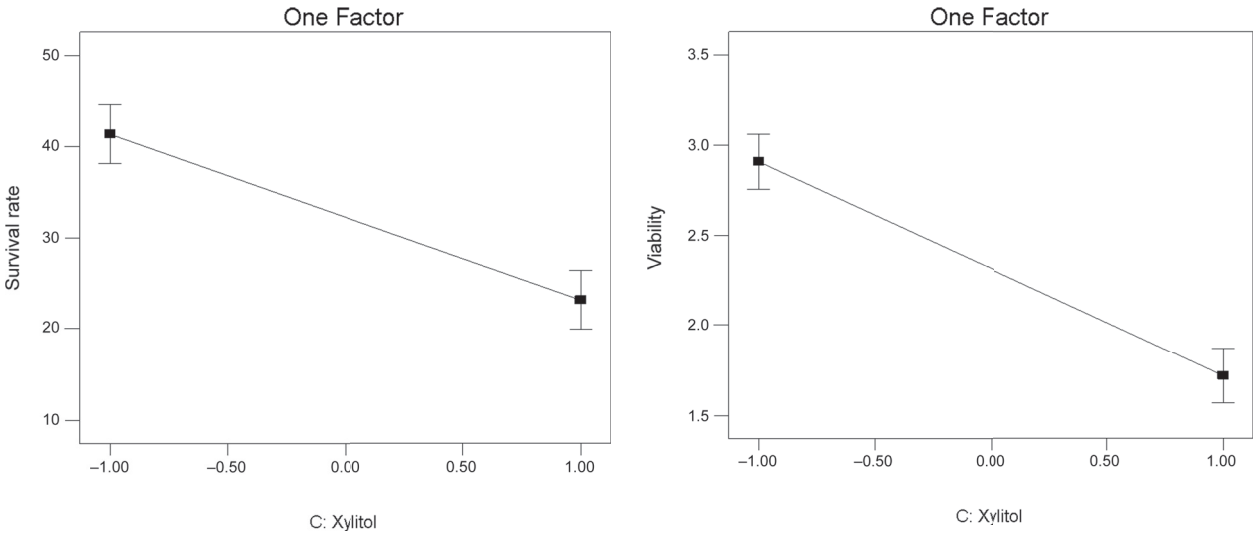

Fig. 2. 95% confidence interval of xylitol (X3)

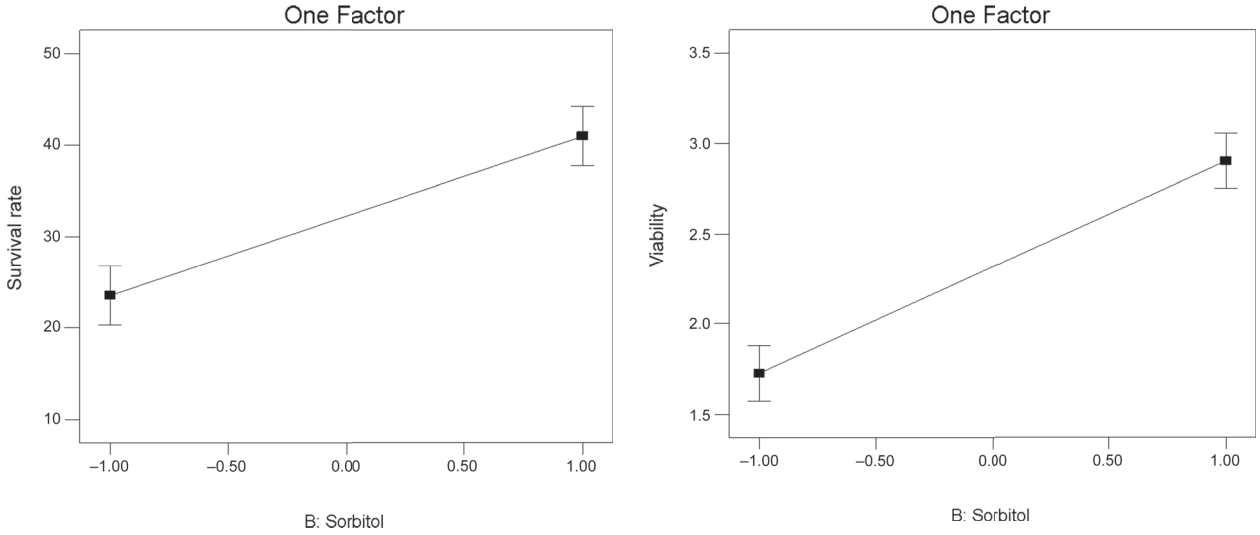

Fig. 3. 95% confidence interval of sorbitol (X2)

Table 4. Result of ANOVA of variables for viability of freeze-dried *LB*6 (Y2)

Source	Sum of squares	df	Mean square	F-value	p-value
x1 – mannitol	1.0267	1	1.0267	68.0992	0.0144
x2 – sorbitol	4.1678	1	4.1678	276.4473	0.0036
x3 – xylitol	4.2364	1	4.2364	281.0004	0.0035
x4 – meso-erythritol	1.8096	1	1.8096	120.0327	0.0082
x5 – lactitol	0.2920	1	0.2920	19.3704	0.0479
X7 – WPI 90	0.0160	1	0.0160	1.0604	0.4114
X8 – BSA V	0.9185	1	0.9185	60.9262	0.0160
X9 – WPC 80	8.0001	1	8.0001	530.6433	0.0019
X10 – SPI 70	1.7787	1	1.7787	117.9809	0.0084

the above factors, WPC 80 (X9) (p = 0.0019), xylitol (X3) (p = 0.0035) and sorbitol (X2) (p = 0.0036) can affect the survival rate of *LB*6 significantly. The 95% confidence interval of the variables showed as Figures 1, 2 and 3. The WPC 80 and sorbitol have positive effect on the viability of freeze-dried *LB*6 when added in the designed amount, but xylitol negative.

DISCUSSION

Freeze drying has been used to manufacture lactic acid bacteria powders for decades and is based upon sublimation. Typically, cells are first frozen and then dried by sublimation under high vacuum (Santivarangkna et al., 2007). It has been shown that cellular inactivation occurs mostly at the freezing step (Tsvetkov and Brankova, 1983). Therefore, many studies have focused on

approaches to minimize damage by protective agents. Sugars, including some sugars alcohol were reported to help maintain the tertiary protein structure in the absence of water by forming hydrogen bonds with proteins during drying (Leslie et al., 1995). In this case, the selected xylitol and sorbitol showed very excellent results on protect the *LB*6 from injuring by the absence of water. In addition, proteins can also decrease the injury of cells by preventing cellular damage by stabilizing the cell membrane constituents (Castro et al., 1995). This indicates that the proteins were valid and suitable to be protecting agents for the *L. bulgaricus*.

CONCLUSIONS

In this study mannitol, sorbitol, xylitol, meso-erythritol, lactitol, WPI 90, BSA V, WPC 80 and SPI 70 were investigated as protective agents for freeze-dried *Lactobacillus bulgaricus LB*6. Studying the protect ability for *LB*6 used a Plackett-Burman design and demonstrated that WPC 80, xylitol and sorbitol have a significant impact on the survival and viability of *LB*6 during freeze drying and all of the above protective agents were demonstrated to have a positive effect, except xylitol. These results provide the basis for further optimization of the lyoprotectant mixture during freeze drying of *Lactobacillus bulgaricus*.

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