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# EFFECTS OF SIX SUBSTANCES ON THE GROWTH AND FREEZE--DRYING OF *LACTOBACILLUS DELBRUECKII* SUBSP. *BULGARICUS**

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### ABSTRACT

**Background.** The efficacy of *Lactobacillus delbrueckii* subsp. *bulgaricus* as starter cultures for the dairy industry depends largely on the number of viable and active cells. Freeze-drying is the most convenient and successful method to preserve the bacterial cells. However, not all strains survived during freeze-drying.
**Methods.** The effects of six substances including NaCl, sorbitol, mannitol, mannose, sodium glutamate, betaine added to the MRS medium on the growth and freeze-drying survival rate and viable counts of *Lb. delbrueckii* subsp. *bulgaricus* were studied through a single-factor test and Plackett-Burman design. Subsequently, the optimum freeze-drying conditions of *Lb. delbrueckii* subsp. *bulgaricus* were determined.
**Results.** *Lb. delbrueckii* subsp. *bulgaricus* survival rates were up to the maximum of 42.7%, 45.4%, 23.6%, while the concentrations of NaCl, sorbitol, sodium glutamate were 0.6%, 0.15%, 0.09%, respectively. In the optimum concentration, the viable counts in broth is 6.1, 6.9, 5.13 ($\times 10^8$ CFU/mL), respectively; the viable counts in freeze-drying power are 3.09, 5.2, 2.7 ($\times 10^{10}$ CFU/g), respectively.
**Conclusion.** Three antifreeze factors including NaCl, sorbitol, sodium glutamate have a positive effect on the growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus.* The results are beneficial for developing *Lb. delbrueckii* subsp. *bulgaricus*.

**Keywords:** *Lactobacillus delbrueckii* subsp. *bulgaricus*, antifreeze factors, freeze-drying, single-factor test, PB screening test

## INTRODUCTION

Dairy starter cultures are of industrial significance and commercial importance for fermented foods and have been well acknowledged worldwide (Dhewa et al., 2014; Santivarangkna et al., 2008). Gram-positive bacterium *Lactobacillus delbrueckii* subsp. *bulgaricus* has been widely used as a probiotic culture (Guchte et al., 2006), because it is able to produce lactic acid in the production of cheese, yogurt and other fermented products (Guillouard et al., 2004), and is of crucial importance to the fermented food in combination with *Streptococcus thermophilus*.

The efficacy of *Lb. delbrueckii* subsp. *bulgaricus* as starter cultures for the dairy industry is largely dependent on the number of viable and active cells.

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Freeze-drying has become the most convenient and successful way to preserve bacterial cells (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 this process and viability rates as low as 0.1% have been reported (Abadias et al., 2001). The main reasons for bacteria viability loss during freeze-drying are ice crystal formation, membrane damage from high osmolarity due to high concentrations of internal solutes, macromolecule denaturation, and the removal of water, which affects the properties of many hydrophilic macromolecules in cells (Allison et al., 1999; Chitra et al., 2003; De Paz et al., 2002; Thammavongs et al., 1996). In the meantime, bacteria have developed adaptive strategies to face the challenges of changing environments and to survive under conditions of stress.

Since the freeze-drying process subjects cells to low water stress conditions, mechanisms of adaptation involving accumulation of osmotic stress compounds may enhance survival during those processes. Compatible solutes are small organic molecules and can be accumulated to high levels in the cytoplasm of osmotically stressed cells. They do not change enzyme activity, and may even prevent enzymes from denaturation brought about by salts, as well as protect them against freezing and drying. Compounds such as betaine have been proven to protect lactic acid bacteria during freeze-drying (Kets and de Bont, 1994; Kets et al., 1996; Louesdon et al., 2014). Evidence has been presented (Li et al., 2015) that raising the medium osmolarity through addition of NaCl has distinct consequences for *Lb. delbrueckii* subsp. *bulgaricus* survival during storage in a dried form. Higher survival rates during storage in a dried state were only observed when these bacteria were previously grown in MRS supplemented with NaCl. Studies have suggested (Herrera et al., 2017; Hofvendahl et al., 2000; Srinivasan et al., 2017) that the positive effect of mannitol, sorbitol and glutamate as compatible solutes on microorganisms subjected to low water activity may lead to the enhanced viability of dried starter cultures. The cryoprotectant effect of four carbohydrates (glucose, fructose, mannose and maltose) on para-dodecanoylca-lix arene-based SLNs has been investigated. These four carbohydrates have been shown to act as good cryoprotectants, allowing reconstitution of the suspensions after the freeze-drying process (Shahgaldian et al., 2003). Thus, these six substances including NaCl, sorbitol, mannitol, mannose, sodium glutamate and betaine have a protective effect on bacteria during freeze-drying. However, there are still insufficient studies on the effects of six substances as an antifreeze factor for *Lb. delbrueckii* subsp. *bulgaricus* during freeze drying.

Thus, there will be further insights into the effect of these various substances as an antifreeze factor added to the MRS medium on the growth and freeze-drying survival rate and viable counts of *Lb. delbrueckii* subsp. *bulgaricus*.

## MATERIAL AND METHOD

### Microorganism and growth medium

*Lb. delbrueckii* subsp. *bulgaricus* were obtained from the School of Food and Biological Engineering, Shaanxi University of Science & Technology (Xi'an, China) and inoculated three successive times with the MRS medium at 37°C for 24 h until the viability of bacteria remained stable. The MRS medium contains 10 g peptone, 8 g beef extract, 4 g yeast extract powder, 20 g glucose, 2 g di-ammonium citrate, 5 g sodium acetate, 0.2 g magnesium sulfate, 0.04 g manganese sulfate, 1 mL polysorbate-80 and 1000 mL water. All the mediums were autoclaved at 121°C for 15 min. MRS medium was used for the activation and cultivation of *Lb. delbrueckii* subsp. *bulgaricus*. The viability of cells was determined on an MRS agar medium.

### Centrifugation and collection

Different concentrations of NaCl, betaine, sodium glutamate, sorbitol, mannitol and mannose as antifreeze factors were added to the activated *Lb. delbrueckii* subsp. *bulgaricus.* These were then inoculated with 5% (v/v) inoculum in MRS medium and incubated at 37°C for 18 h. After incubation, the culture was centrifuged at 8000 rpm for 15 min to harvest the *Lb. delbrueckii* subsp. *bulgaricus* cells.

### Vacuum freeze-drying

The *Lb. delbrueckii* subsp. *bulgaricus* cells were prefrozen at –40°C for 12–24 h and then frozen at –51°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 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. delbrueckii* subsp. *bulgaricus* 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 a sterile saline solution and a 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 antifreeze factors using the Plackett-Burman design

The Plackett-Burman design was used to identify the effect of the selected six substances (NaCl, sorbitol, mannitol, mannose, sodium glutamate and betaine) on *Lb. delbrueckii* subsp. *bulgaricus*. According to the Plackett-Burman design, all 6 factors were tested at a lower and a higher level coded as (+1) and (–1), respectively (shown in Table 1). The design matrix is shown in Table 2, which presents the effect of 8 variables (including two error terms: X7, X8, in order to estimate the standard deviation) investigated in 12 independent experimental runs.

### Statistical analysis

Statistical analysis was performed in SAS9.1 to identify the significant variables and their corresponding coefficients.

## RESULTS

### Study of antifreeze factors of *Lb. delbrueckii* subsp. *bulgaricus*

Effects of NaCl on growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*. The NaCl was added to MRS broth at 0%, 0.2%, 0.4%, 0.6% and 1.0%. After being cultured, the bacteria were centrifuged and collected, and then lyophilized. The results are presented in Figure 1.

It can be seen from Figure 1 that adding NaCl to MRS broth can effectively improve the survival rate and viable counts of *Lb. delbrueckii* subsp. *bulgaricus* during freeze-drying. When the NaCl was not added, the survival rate was 11.7%, the viable count in freeze--drying powder was $0.882\times10^{10}$ CFU/g, and in broth $4.25\times10^{8}$ CFU/mL. With the increase in NaCl concentration, the viable count of *Lb. delbrueckii* subsp. *bulgaricus* first increased and then decreased. When the concentration of NaCl was 0.6%, the viable count in freeze-drying was highest at $3.09\times10^{10}$ CFU/g and the survival rate was the highest at 42.7%, as well as the viable count in broth reaching a maximum of $6.1\times10^{8}$ CFU/mL. These data indicated that a low concentration of NaCl could promote the activity the growth of *Lb. delbrueckii* subsp. *bulgaricus*, but a high concentration of NaCl has a passive effect on bacteria. This result is in accord with that obtained in a previous study, namely that *Lb. delbrueckii* subsp. *bulgaricus* can tolerate the highest concentration of 1.0 mol/L, and the appropriate growth range of 0.2 mol/L to 0.8 mol/L. Research proves that NaCl has a positive effect on growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus.*

Effects of sorbitol, mannitol and mannose on the growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus.* The sorbitol, mannitol and mannose were added to MRS broth at 0%, 0.05%, 0.1%, 0.15%, 0.2% and 0.25%, respectively. After being cultured, the bacteria were centrifuged and collected before being lyophilized. The results are shown in Figure 2–4.

It can be seen from Figure 2–4 that adding sorbitol, mannitol and mannose to MRS broth can greatly enhance the survival rate and viable counts of *Lb. delbrueckii* subsp. *bulgaricus* during freeze-drying. The viable count of *Lb. delbrueckii* subsp. *bulgaricus* first increased and then decreased as the concentration increased. When the sorbitol was not added, the survival rate was 36.4%, the viable count in freeze-drying powder was $3.6\times10^{10}$ CFU/g, and in broth $5.3\times10^{8}$ CFU/mL. The viable count in freeze-drying powder was highest at $5.2\times10^{10}$ CFU/g when the concentration of sorbitol was 0.15%, and the survival rate was 45.4%, as well as the maximum of viable counts in broth being $6.9\times10^{8}$ CFU/mL. When the mannitol

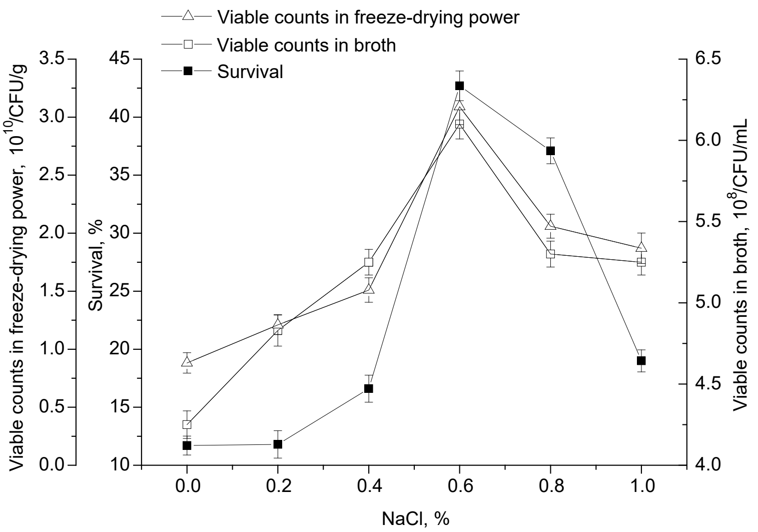


**Fig. 1.** Effects of NaCl on growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*

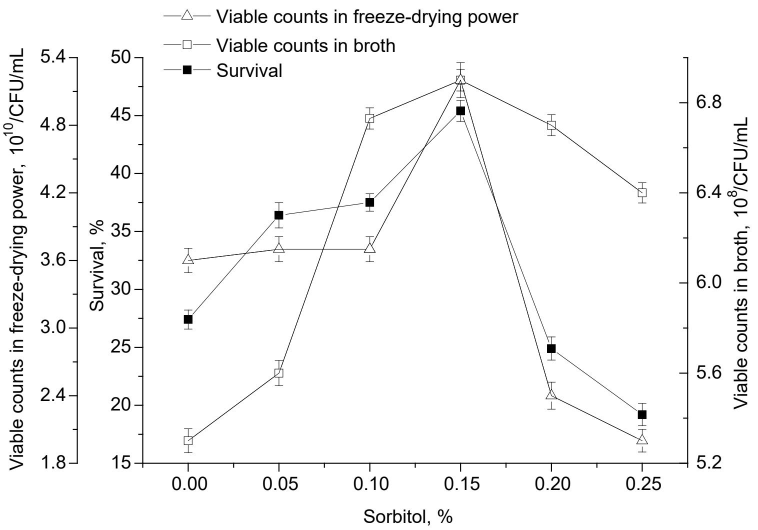


**Fig. 2.** Effects of sorbitol on growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*

was not added, the survival rate was 26.9%, the viable counts in freeze-drying powder were $1.6\times10^{10}$ CFU/g, and viable counts in broth $2.23\times10^{8}$ CFU/mL. The viable count in freeze-drying powder was highest at $2.5\times10^{10}$ CFU/g when the concentration of sorbitol was 0.1%, and the survival rate was 28.1%, as well as the maximum of viable counts in broth being $3.8\times10^{8}$ CFU/mL. When the mannose was not added, the survival rate was 14.5%, the viable count in freeze-drying powder was $1.2\times10^{10}$ CFU/g, and the viable count in broth was $3.8\times10^{8}$ CFU/mL. The viable count in freeze-drying powder was highest at $3.4\times10^{10}$ CFU/g

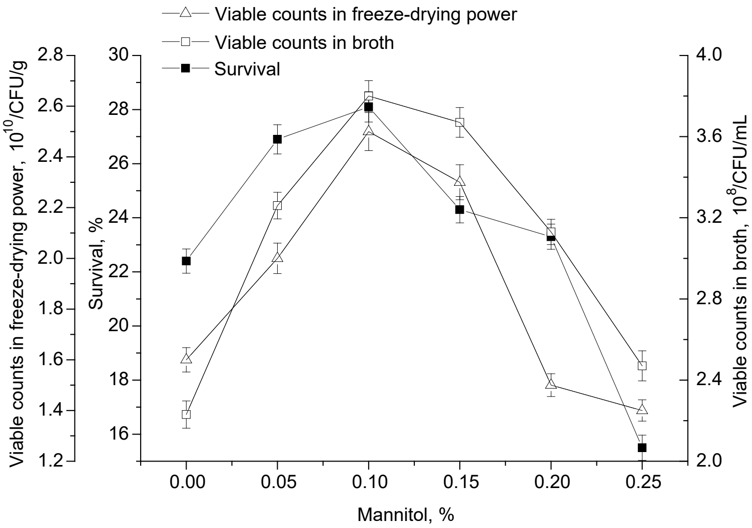


**Fig. 3.** Effects of mannitol on growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*

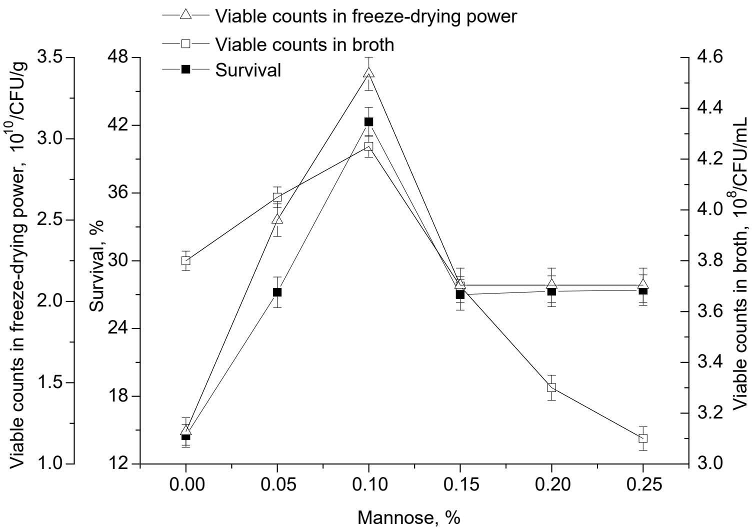


**Fig. 4.** Effects of mannose on growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*

when the concentration of mannose was 0.1%, and the survival rate was 42.3%, as well as the maximum of viable counts in broth being $4.25\times10^{8}$ CFU/mL. These indicated that a low concentration of sorbitol, mannitol and mannose could increase the growth of *Lb. delbrueckii* subsp. *bulgaricus*, but a high concentration of sorbitol, mannitol and mannose have a negative effect on bacteria. This research shows that three substances have a positive effect on the growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus.*

Effects of betaine on the growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*. Betaine was added to MRS broth at 0%, 0.02%, 0.04%, 0.06%, 0.08% and 1.0%. After culturing, the bacteria were centrifuged and collected, then lyophilized. The results are shown in Figure 5.

It can be seen from Figure 5 that with the increase of betaine concentration, survival rate, viable count in freeze-drying power and the viable count in broth first increased and then decreased. When the betaine was not added, the survival rate was 15.3%, viable count in freeze-drying powder was $1.1\times10^{10}$ CFU/g, and viable counts in broth was $2.95\times10^{8}$ CFU/mL. When the betaine concentration was 0.04%, the

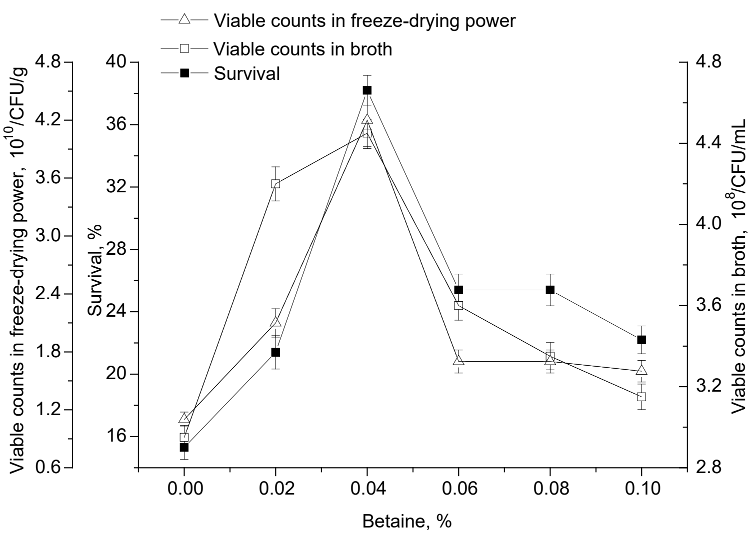


**Fig. 5.** Effects of betaine on growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*

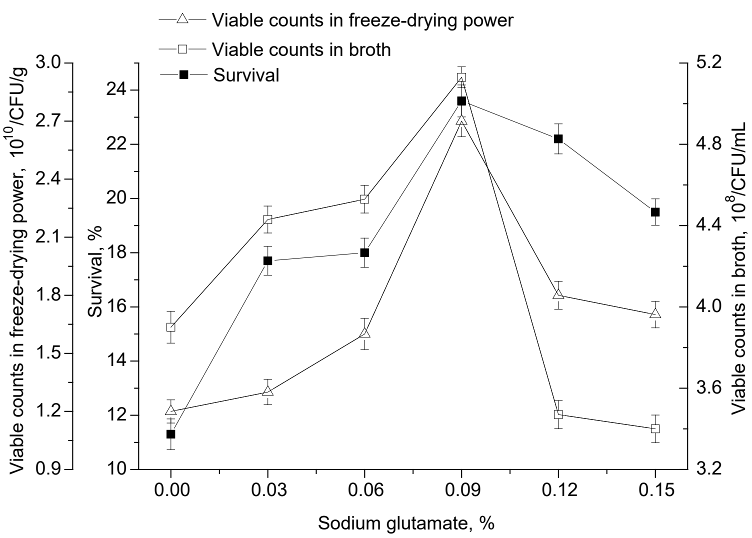


**Fig. 6.** Effects of sodium glutamate on growthand freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*

survival rate of *Lb. delbrueckii* subsp. *bulgaricus* reached the highest value of 38.2%, the viable count in freeze-drying power reached $4.2\times10^{10}$ CFU/g, and the viable count in broth reached the highest concentration of $4.45\times10^{8}$ CFU/mL, indicating that low concentrations of betaine can promote the growth of *Lb. delbrueckii* subsp. *bulgaricus* in the culture medium, but a high concentration of betaine inhibits the growth of bacteria. These results show that betaine has a great impact on the growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*.

Effects of sodium glutamate on the growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*. The sodium glutamate was added to MRS broth at 0%, 0.03%, 0.06%, 0.09%, 0.12% and 0.15%. After being cultured, the bacteria were were centrifuged and collected, then lyophilized. The results are shown in Figure 6.

It can be seen from Figure 6 that with an increase in sodium glutamate concentration, the viable count in freeze-drying power, viable count in broth and the survival rate first increased and then decreased. When the sodium glutamate was not added, the survival rate was 11.3%, the viable count in freeze-drying powder was $1.2\times10^{10}$ CFU/g, and viable count in broth was $4.43\times10^{8}$ CFU/mL. When the sodium glutamate concentration was 0.09%, the viable count in freeze-drying power reached its highest value of $2.7\times10^{10}$ CFU/g, and the viable count in broth reached the highest concentration of $5.13\times10^{8}$ CFU/mL. Meanwhile, the survival rate of *Lb. delbrueckii* subsp. *bulgaricus* reached the highest value of 23.6%. It can be seen from the above that the sodium glutamate has a positive impact on the growth and freeze-drying of *Lb. delbrueckii* subsp. *bulgaricus*.

## Plackett-Burman screening of antifreeze factors for *Lb. delbrueckii* subsp. *bulgaricus*

The Plackett-Burman screening test is based on the principle of non-fully balanced block. The test tries to minimize the number of trials to assess the importance of factors by designing high and low levels, and identifies the main factors from among many. Hence, the main factors can produce accurate estimates and provide a reliable basis for further research.

Based on the single factor, this paper used the SAS9.1 software to design a Plackett-Burman screening test that included 8 factors (six actual factors and two virtual items) and 2 levels. Significant factors were then analyzed and screened. The variety of level design is shown in Table 1.

The relationship between protective agents and the survival rate of *Lb. delbrueckii* subsp. *bulgaricus* is shown in Table 2. The values Y1 and Y2 represent the viable count in freeze-drying power and viable count in broth (the unit $10^{10}$ CFU/mL and $10^{8}$ CFU/g), respectively, while Y3 (%) stands for the survival rate after freeze-drying. All of these antifreeze factors had different effects on the cells, hence when the factors were changed, the survival rate of *Lb. delbrueckii* subsp. *bulgaricus* also changed.

Three substances, namely NaCl, sorbitol, sodium glutamate accounted for a large proportion of the percentage sum of squares on the Pareto chart (Fig. 7). This result indicates that these three variables had a significant impact on cell viability and they protect the cells better than the other substances tested in this study. Moreover, the trend lines of the 95% confidence interval of these factors (Fig. 8) suggested that these three variables had a positive effect. When the concentrations of these three substances were increased, the survival rate of *Lb. delbrueckii* subsp. *bulgaricus* also gradually increased.

**Table 1.** Plackett-Burman test coding table of antifreeze factors, %

| Level of factor | X1 | X2 | X3 | X4 | X5 | X6 |
|---|---|---|---|---|---|---|
| | NaCl | Sorbitol | Mannitol | Sodium glutamate | Betaine | Mannose |
| 1 | 0.75 | 0.15 | 0.13 | 0.11 | 0.04 | 0.10 |
| –1 | 0.60 | 0.12 | 0.10 | 0.09 | 0.03 | 0.08 |

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

| RUN | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | Y1, % | Y2, $\times 10^{10}$ CFU/g | Y3, $\times 10^{8}$ CFU/mL |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | –1 | 1 | –1 | –1 | –1 | 1 | 1 | 8.30 | 0.67 | 2.4 |
| 2 | 1 | 1 | –1 | 1 | –1 | –1 | –1 | 1 | 34.4 | 1.5 | 1.6 |
| 3 | –1 | 1 | 1 | –1 | 1 | –1 | –1 | –1 | 24.1 | 2.0 | 2.7 |
| 4 | 1 | –1 | 1 | 1 | –1 | 1 | –1 | –1 | 17.1 | 2.1 | 4.1 |
| 5 | 1 | 1 | –1 | 1 | 1 | –1 | 1 | –1 | 31.4 | 3.3 | 3.5 |
| 6 | 1 | 1 | 1 | –1 | 1 | 1 | –1 | 1 | 13.4 | 1.4 | 3.4 |
| 7 | –1 | 1 | 1 | 1 | –1 | 1 | 1 | –1 | 38.3 | 3.5 | 3.0 |
| 8 | –1 | –1 | 1 | 1 | 1 | –1 | 1 | 1 | 35.8 | 1.2 | 3.4 |
| 9 | –1 | –1 | –1 | 1 | 1 | 1 | –1 | 1 | 28.0 | 1.9 | 2.5 |
| 10 | 1 | –1 | –1 | –1 | 1 | 1 | 1 | –1 | 11.9 | 1.0 | 3.4 |
| 11 | –1 | 1 | –1 | –1 | –1 | 1 | 1 | 1 | 57.8 | 7.8 | 4.5 |
| 12 | –1 | –1 | –1 | –1 | –1 | –1 | –1 | –1 | 13.3 | 1.3 | 3.0 |

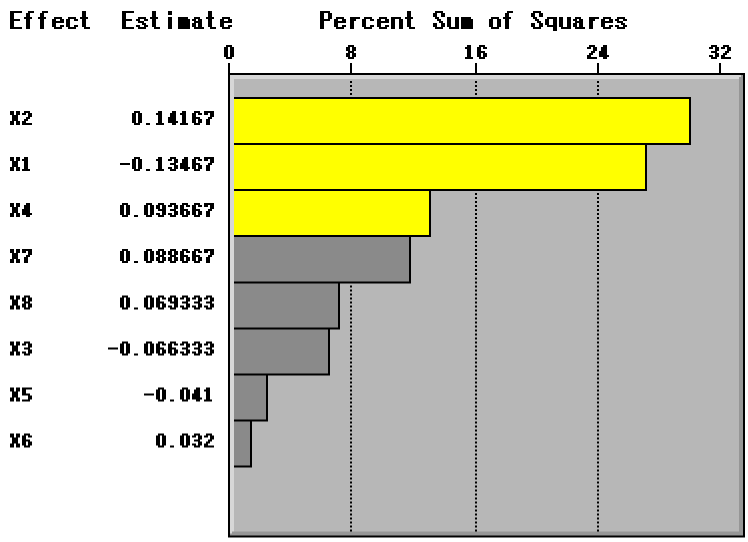


**Fig. 7.** The importance of antifreeze factor to survival of *Lb. delbrueckii* subsp. *bulgaricus*

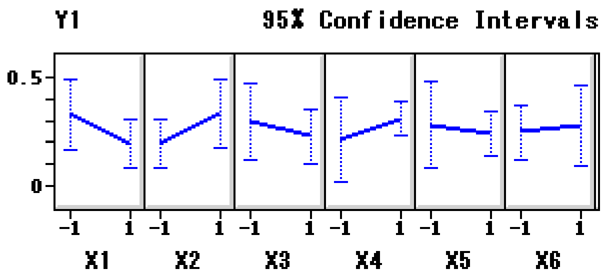


**Fig. 8.** The 95% confidence interval chart of antifreeze factors

## DISCUSSION

Although lactic acid bacteria have been an object of research for a long time, studies on the accumulation of compatible solutes in lactic acid bacteria have received little attention. Consequently, the effects of the use of compatible solutes in processes for the preservation of starter cultures are still obscure. During freeze-drying, the addition of high concentrations of polyols such as adonitol had a positive effect on the survival of lactic acid bacteria (Valdez et al., 1985). In the present study, the use of mannose, mannitol and sorbitol can enhance the cell viability of *Lb. delbrueckii* subsp. *bulgaricus.* Meanwhile, the substance sorbitol had a significant impact on *Lb. delbrueckii* subsp. *bulgaricus* during the Plackett-Burman tests. Hutkins et al. (1987) studied the uptake of betaine present in the medium by *Lactobacillus acidophilus*. The growth of *L. acidophilus* under osmotic stress of 1 M NaCl resulted in an increased uptake of betaine. Kets and de Bont (1994) showed that there was a clear effect on the growth rate of *Lactobacillus plantarum* when betaine was added in the presence of 0.6 M NaCl. Their study also revealed that cells grown under osmotic stress in the presence of betaine are better protected against drying than unstressed cells. In this study betaine and NaCl were used as a compatible solute protecting *Lb. delbrueckii* subsp. *bulgaricus* during drying. This study suggested that NaCl as an antifreeze factor had a significant impact on *Lb. delbrueckii* subsp. *bulgaricus.* The effects of sorbitol and monosodium glutamate upon survival during the storage of freeze-dried *Lb. delbrueckii* subsp. *bulgaricus*, *Lactobacillus plantarum*, *Lactobacillus rhamnosus*, *Enterococcus durans* and *Enterococcus faecalis* were examined by Carvalho et al. (2003). There were no significant differences in survival during freeze-drying after the addition of sorbitol or sodium glutamate. However, these compounds were found to increase the stability of most strains during long-term storage. The present study also proves that the addition of sorbitol or sodium glutamate can offer protective effects for freeze-dried *Lb. delbrueckii* subsp. *bulgaricus.*

In this study, NaCl, sorbitol, mannitol, mannose, sodium glutamate and betaine were used as a compatible solute protecting *Lb. delbrueckii* subsp. *bulgaricus* during drying. It is interesting to note that NaCl, sorbitol and sodium glutamate have a positive effect on the survival of dried lactic acid bacteria during the Plackett-Burman test. Moreover, it is proven that *Lb. delbrueckii* subsp. *bulgaricus* could be able to grow at salinities with a higher concentration of NaCl. Presumably, the salt tolerant strains can accumulate compatible solutes more efficiently and hence will be better protected against drying.

## CONCLUSIONS

In the present study, the effect of six substances including NaCl, betaine, sodium glutamate, sorbitol, mannitol and mannosel as antifreeze factors for freeze-dried *Lb. delbrueckii* subsp. *bulgaricus* was investigated. The results showed that sorbitol, NaCl and sodium glutamate had a significant impact on the survival of *Lb. delbrueckii* subsp. *bulgaricus* during freeze-drying. The optimum concentrations of sorbitol, NaCl and sodium glutamate were 0.15%, 0.6% and 0.09%, respectively, and the cell vaility of bacteria reached the maximum level. These results provide a reference for suitable selection of the composition of the drying medium to afford protection during subsequent storage.

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