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**ORIGINAL RESEARCH PAPER**

# APPLICATION OF PLACKETT-BURMAN DESIGN IN SCREENING FREEZE DRYING CRYOPROTECTANTS FOR *Lactobacillus bulgaricus*

[1]GUOWEI SHU*, [1]YIXIN HUI, [1]SHIWEI CHEN, [2] HONGCHANG WAN, [1]HE CHEN
[1]School of Food and Biological Engineering, Shaanxi University of Science & Technology, Xi'an, 710021, China
[2]*Shaanxi Yatai Dairy Co., Ltd., Xianyang 713701, China*
***** Corresponding author: shuguowei@gmail.com

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*Lactobacillus bulgaricus* is the bacteria commonly used in probiotic dairy product, including yogurt and cheese. The bacteria may be stored for long periods of time if it is freeze-dried. The cryoprotectant mixture for *L. bulgaricus* was optimized during the process of freeze-drying using a Plackett-Burman design and the steepest ascent test. In our initial tests, the cell survival rate and the number of viable cells were associated with the type of cyroprotectant used. Therefore, our optimization protocol focused on increasing survival rate. Substances that previously had a protective effect during freeze-drying were investigated, for example: sucrose, lactose, skim milk powder, sodium bicarbonate, sodium glutamate, magnesium sulfate, sodium ascorbate, yeast extract, vitamin $B_2$, and phosphate buffer. We determined that the optimum cryoprotectant composition for *L. bulgaricus* consists of 28.0 g/100 mL skim milk powder, 24.0 g/100 mL lactose and 4.8 g/100 mL sodium ascorbate. The optimized cryoprotectant provides a 63.25% cell survival rate.

**Keywords**: *Lactobacillus bulgaricus*, freeze-drying, cryoprotectant, bacteria survival rate, Plackett-Burman design

## Introduction

The Gram-positive bacterium *Lactobacillus bulgaricus* is a member of the acidophilus group of lactobacilli and is widely used in probiotic cultures (van de Guchte *et al*., 2006). *L. bulgaricus* is employed worldwide because it is able to produce lactic acid in the production of yogurt, cheese and other fermented products (Guillouard *et al*., 2004; De Urraza *et al*., 1997) and is of vital importance to the food industry, in combination with *Streptococcus thermophilus*.

During the preparation of the starter cultures, the production and maintenance techniques that maximize the storage stability, viability and activity of the bacterial cells must be established (Passot *et al*., 2012). Freeze-drying is the most convenient and successful method of preserving bacteria, yeasts and sporulating fungi (Berny *et al*., 1991) and it has been widely used in microbiology for many decades to stabilize and store cultures (Morgan *et al*., 2009). However, not all bacterial strains survive the freeze-drying process and quantitative viability rates as low as 0.1% have been reported (Abadias *et al*., 2001). The major causes of cell death during freeze-drying are related 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 (Thammavongs *et al*., 1996; Chitra *et al*., 2003; De Paz *et al*., 2002; Allison *et al*., 1999). 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).

The stability of probiotic microorganisms during freeze-drying and storage may be enhanced by the addition of protective agents (Zayed and Roos, 2004). It is well documented that carbohydrates have protective effects for probiotic bacteria during freeze-drying. For example, the remarkably high glass transition temperature ($T_g$) of trehalose can raise the glass-phase transition temperature of cells and therefore viable cells can be protected by reaching the glassy phase without nucleating intracellular ice (Fowler *et al*., 2005). Apart from this, sorbitol (Linders *et al*., 1997; Carvalho *et al*., 2002), mannitol (Efiuvwevwere *et al*., 1999), sucrose (Carvalho *et al*., 2003), lactose (Higl *et al*., 2007), mannose (Carvalho *et al*., 2004a), inulin, and fructo-oligosaccharides (Schwab *et al*., 2007) 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 vacuum-drying. Sodium glutamate can also protect the cell (de Valdez *et al*., 1983; Teixeira *et al*., 1995). 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), phosphate (Ohtake,2004), calcium carbonate and manganese sulfate can help to protect cells during freeze-drying if they are used with other protectants. 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 skim milk, whey protein, blood serum, serum albumin and peptone are efficient desiccation protectants (Hubalek *et al*., 2003; Abadias *et al*., 2001).

The classic method of cryoprotectant optimization involves changing one ingredient at a time, keeping the other substrate components at fixed levels. This laborious and time-consuming method does not guarantee that the optimal mixture will be determined. The Plackett–Burman design is a statistical screening method where $n$ variables are studied in $n+1$ experimental runs and since there are fewer experimental replicates, time and resources are saved (Srinivas *et al*., 1994; Carvalho *et al*., 1997). Moreover, the design is orthogonal in nature, implying that the effect of each variable is pure in nature and not confounded with interaction between variables. Software to plan the experimental design and to run the data analysis makes the analysis easier than other approaches (Naveena *et al*., 2005). Furthermore, the steepest ascent test can determine the correct dosage of the significant factors.

In our previous work, we studied the effects of sucrose, lactose, skim milk, yeast, vitamin B2, $NaHCO_3$, $MgSO_4$, sodium ascorbate, sodium glutamate, and phosphate buffer on the survival of *Lactobacillus bulgaricus* during freeze-drying in single factor experiments. We found that the optimum concentration of single protective agents for *L. bulgaricus* during freeze-drying was 25% (W/V) sucrose, 20% (W/V) lactose, 25% (W/V) skim milk, 20% (W/V) yeast, 25% (W/V) vitamin B2, and 4.5% (W/V) sodium ascorbate. The survival rate with these concentrations were 24.5% with sucrose, 35.6% with lactose, 64.4% with skim milk, 62.2% with yeast, 16.3% with vitamin B2, and 84.7% with sodium ascorbate (Chen *et al.*, 2013a; Chen *et al.*, 2013b). In this paper, we chose several protective agents based on previous studies to screen and optimize a mixture of cryoprotectant substances when freeze-drying *L. bulgaricus* and to measure the survival and viability of cells after freeze-drying.

## Materials and Methods

### Microorganism and growth medium

*Lactobacillus bulgaricus* was obtained from the School of Food and Biological Engineering, Shaanxi University of Science & Technology (Xi'an, China). The strain was isolated from commercial yogurt and has the ability to ferment goat milk when inoculated with 3% culture starter of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (at a ratio of 1:1.5) and added to 8% sugar, then incubated at 43$^{o}$C for 4h. The total number of viable cells can reach up to $1.90\times10^{9}$CFU/mL, with pH 4.16 and an optimal incubation time of 18h (Wang, 2011). MRS medium was used for activation and cultivation of *L. bulgaricus*. Viability of cells was determined on MRS agar medium.

### Lactobacillus bulgaricus culture and microorganism collection

Activated *L. bulgaricus* was inoculated with 4% (v/v) inoculum in MRS medium, then incubated at 37$^{o}$C for 18h. The culture was centrifuged at 6000×g for 10 min to harvest the *L. bulgaricus* cells.

### Preparation of protective agents

The protective agent solutes used in the experiment were made with distilled water and formulated into various concentrations. The sugars were sterilized at 115$^{o}$C for 15 min. Because amino acids and vitamin $B_2$ cannot tolerate high temperatures, the concentrated solution of these compounds were sterilized by filtration using a membrane with pore size of 0.22 μm, which was preliminary sterilized at 121$^{o}$C for 20 min. The other protective agents were sterilized at 108$^{o}$C for 15 min.

### Freeze-drying

The *L. bulgaricus* cells were frozen at -40$^{o}$C for 12-24h after protective agents were added and then frozen at -51$^{o}$C, 6.93 Pa for 24h using a vacuum freeze dryer.

### Viable count

The diluted bacterial suspension was aliquoted into 0.1 mL doses with a syringe and dropped into a count plate, then spread uniformly. The undercut plate was incubated at 37°C for 36-48h and then the viable *L. bulgaricus* cells were counted.

### Calculation of survival

Survival rate was calculated as the number of viable cells after drying/number of viable cells before drying×100%

### Plackett–Burman screening of protective agents and the steepest ascent test

The Plackett–Burman design used 10 factors spanning 12 runs, with each factor fixed at two levels (namely a lower level and a higher level, represented by +1 and -1) based on optimizations in previous studies (Chen *et al*., 2013a; Chen *et al*., 2013b). The protective agents tested are listed in Table 1, along with the amount used. These agents included sucrose, lactose, skim milk powder, sodium bicarbonate, sodium glutamate, magnesium sulfate, sodium ascorbate, yeast extract, vitamin B2 and phosphate buffer, which were screened for their impact on viable counts and survival rates of *L. bulgaricus* cells.

**Table 1**. Protective agents tested in a Plackett-Burman survey for their efficacy in increasing the cell survival of *Lactobacillus bulgaricus* during freeze-drying

| Variables | Protective agents | Lower level (g/100 mL) | Higher level (g/100 mL) |
|---|---|---|---|
| X1 | Sucrose | 20.00 | 25.00 |
| X2 | Lactose | 16.00 | 20.00 |
| X3 | Skim milk | 20.00 | 25.00 |
| X4 | Sodium bicarbonate | 0.64 | 0.80 |
| X5 | Sodium glutamate | 0.24 | 0.30 |
| X6 | Magnesium sulfate | 0.40 | 0.50 |
| X7 | Sodium ascorbate | 3.60 | 4.50 |
| X8 | Yeast extract | 4.80 | 6.00 |
| X9 | Vitamin B2 | 0.80 | 1.00 |
| X10 | Phosphate buffer | 0.20:1.00 | 0.25:1.00 |

### Statistical analysis

A statistical analysis was performed in SAS (Version, 12.0, SAS Institute Inc., Cary, NC, USA) to identify the significant variables and their corresponding coefficients. The coefficient, sum of squares (SS %), and confidence intervals (CI) were evaluated to analyze the number of viable bacteria and the survival rate from each of the trials.

## Results

### *Plackett–Burman screening of protective agents for Lactobacillus bulgaricus*

The relationship between protective agents and the survival rate of *L. bulgaricus* is shown in Table 2. The survival rate of freeze-dried *L. bulgaricus* cells is represented by Y1 (%) and the number of viable cells of freeze-dried powder is represented by Y2 (×$10^{11}$ CFU/g). The survival rate was calculated by a formula containing a 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 of *L. bulgaricus* also changed.

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

| Run | X1 | X2 | X3 | X4 | X5 | X6 | X7 | X8 | X9 | X10 | Y1/% | Y2/$10^{11}$ CFU/g) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 75.66 | 2.30 |
| 2 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 67.11 | 2.14 |
| 3 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 60.04 | 2.08 |
| 4 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 52.35 | 1.63 |
| 5 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 76.26 | 2.32 |
| 6 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | 66.76 | 2.13 |
| 7 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | 88.45 | 2.72 |
| 8 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | 63.74 | 2.03 |
| 9 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 48.25 | 1.39 |
| 10 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | 48.64 | 1.47 |
| 11 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 67.00 | 2.19 |
| 12 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 36.00 | 1.14 |

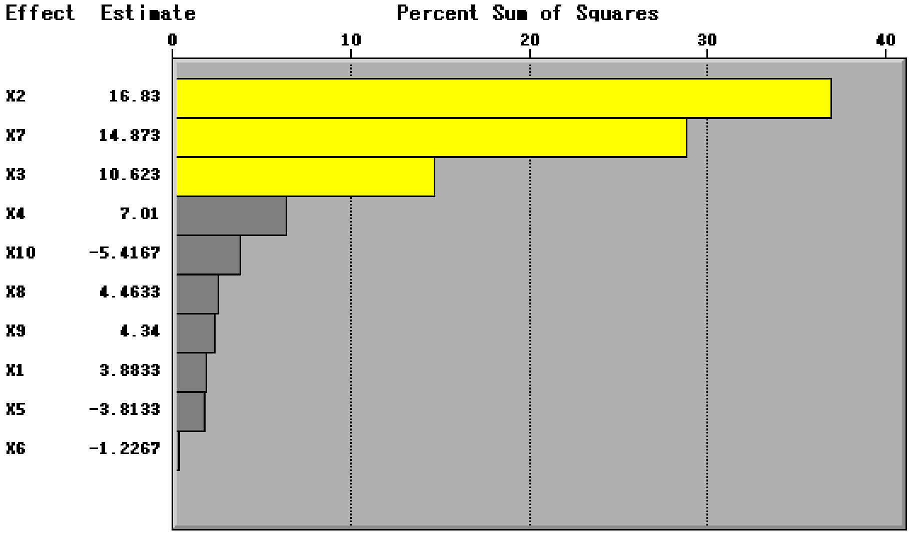

**Figure 1**. Comparison of protective agents in a Plackett-Burman survey of their effect on cell survival in *Lactobacillus bulgaricus* during freeze-drying

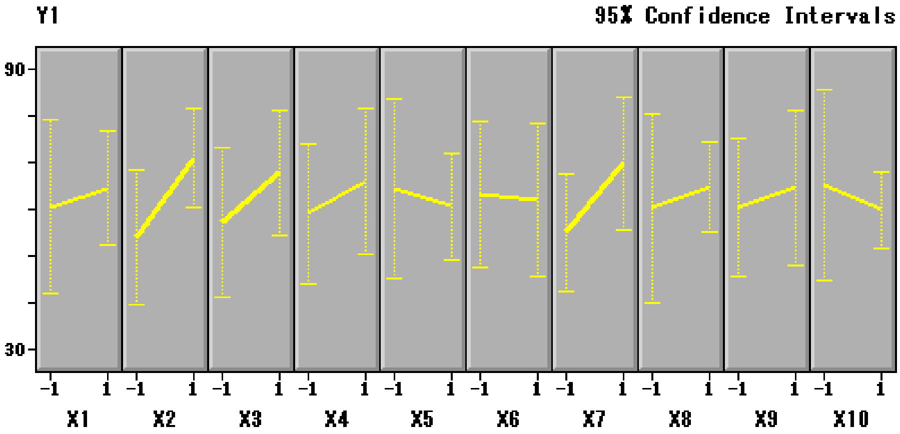

**Figure 2.** The confidence intervals for each protective agent

Three variables namely lactose, sodium ascorbate and skim milk powder accounted for a large proportion of the percent sum of squares on the Pareto chart (Fig. 1). This indicates that these three variables had a significant impact on the survival rate and they protect the cells better than the other agents tested in this study. Furthermore, the trend lines of the 95% confidence interval of these factors (Fig. 2) suggested that these three variables had a positive effect. When the concentrations of these three variables were increased, the survival rate of *L. bulgaricus* also gradually increased.

### *The path of steepest ascent*

The results of the Plackett–Burman design suggests that lactose, sodium ascorbate and skim milk powder can protect the cell and significantly affect the survival rate of *L. bulgaricus* (Fig. 1). We increased or decreased concentrations of the significant factors, according to the signs of their main effects. The path of steepest ascent design and results are shown in Table 3. The value of Y1 and Y2 represent the survival rate of freeze-dried *L. bulgaricus* cells and the number of viable cells in the freeze-dried powder. The number of viable cells and the cell survival rate in the freeze-dried powder was higher in group 3 than the other groups. The optimized concentrations of the three factors in group 3 were 28.0 g/100 mL of lactose, 24.0 g/100 mL of sodium ascorbate, and 4.8 g/100 mL of skim milk powder.

**Table 3**. Experimental design and results of the steepest ascent test for three most effective protective agents

| Run | Skim milk powder % | Lactose % | Sodium ascorbate % | Y1 /% | Y2 /$10^{11}$ CFU/g |
|---|---|---|---|---|---|
| 1 | 24 | 20 | 4.4 | 58.85 | 2.87 |
| 2 | 26 | 22 | 4.6 | 60.36 | 2.95 |
| 3 | 28 | 24 | 4.8 | 63.25 | 3.12 |
| 4 | 30 | 26 | 5.0 | 61.57 | 3.01 |
| 5 | 32 | 28 | 5.2 | 59.64 | 2.90 |

## 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 *et al*., 1983). During freezing, the material is gradually dehydrated and ice slowly forms outside the cell, leading to extensive cellular damage. In addition, water bound in each cell plays an important role in stabilizing the structure and function of biological macromolecules, including those present on the cell wall and cell membrane. Consequently, water removal during freeze-drying can lead to destabilization of the structural integrity of these cellular components, resulting in loss or impairment of function (Brennan *et al*., 1986).

Therefore, in order to protect cells during freeze-drying, many studies have focused on approaches to minimize damage by protective agents. Sugars were reported to exhibit enhanced desiccation tolerance by forming hydrogen bonds with proteins during drying, which help maintaining the tertiary protein structure in the absence of water (Leslie *et al*., 1995). Proteins can also decrease the injury to cells. Skim milk has been used as a drying medium because it can prevent cellular damage by stabilizing the cell membrane constituents (Castro *et al*., 1995). Additionally, skim milk can protect microbial cells from damage caused by the formation of ice crystals during freezing, because proteins in skim milk can form a viscous layer on the surface of the cells, which can increase the solution's viscosity and maintain amorphous ice crystals near the cell (Carvalho *et al*., 2004b). This indicates that skim milk is a suitable cryoprotectant for *L. bulgaricus*.

The loss of activity of the freeze-dried cultures can also be a consequence of cell damage and membrane lipid oxidation (Castro *et al*., 1997). The addition of ascorbic acid to the drying medium has already been demonstrated to have a protective effect on *L. bulgaricus* during storage (Teixeira *et al*., 1995). Kurtmann *et al*. (2009) observed that the detrimental effects of atmospheric oxygen was reduced by including ascorbate in the freeze drying medium for *L. acidophilus*. This may explain why ascorbic acid protected the cells during freeze-drying in our experiments.

## Conclusions

In this study sucrose, lactose, skim milk powder, sodium bicarbonate, sodium glutamate, magnesium sulfate, sodium ascorbate, yeast extract, vitamin $B_2$, and phosphate buffer were investigated as protective agents for freeze-dried *Lactobacillus bulgaricus*. Screening of cryooprotectant contents for *L. bulgaricus* used a Plackett–Burman design and demonstrated that skim milk powder, sodium ascorbate and lactose have a significant impact on the survival of *L. bulgaricus* during freeze-drying and all of the above protective agents were demonstrated to have a positive effect. We found that the optimum concentration of these three agents was 28.0 g/100 mL skim milk, 24.0 g/100 mL sodium ascorbate, and 4.8 g/100 mL lactose. These results provide the basis for the safe and effective long-term storage of *Lactobacillus bulgaricus* and will provide economic benefits to the yogurt and cheese industry.

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