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ORIGINAL RESEARCH PAPER

SCREENING FREEZE-DRYING CRYOPROTECTANTS FOR SACCHAROMYCES BOULARDII BY PLACKETT-BURMAN DESIGN

[1]HE CHEN*, [1]ZHANGTENG LEI, [1]HAIPING HONG, [2]YALING ZHAI, [2]DAN HUANG

[1]School of Food and Biological Engineering, Shaanxi University of Science & Technology, Xi'an, 710021, China
[2]Shaanxi Pucheng Shiyang Feed Co., Ltd., 715501, Xi'an, China
*Corresponding author: chenhe419@gmail.com

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As a lyophilized product, *Saccharomyces boulardii* has been commonly used to treat diarrhea in adults. However, there are few studies focusing on the preparation of its freeze-drying powder. This paper investigated the effect of lyoprotectants on the freeze-drying survival rate and the number of viable cells for vacuum freeze-dried powder of *S. boulardii*. Single factor experiment and Plackett-Burman design were conducted to obtain the optimal compound lyoprotectant formulations. The result showed that lactose, trehalose and sodium glutamate could significantly enhance the freeze-drying survival rate of *S. boulardii*. Meanwhile, all these three lyoprotectants showed positive effect on the freeze-drying survival rate of the yeast, and the optimal lyoprotectant composition for *S. boulardii* was as follows: 18g/100mL lactose, 18g/100mL trehalose and 3g/100mL sodium glutamate.

Keywords: *Saccharomyces boulardii*, freeze-drying, lyoprotectants, Plackett-Burman design

Introduction

Saccharomyces boulardii is a non-pathogenic yeast which has been used for many years as probiotic to prevent or treat a variety of human gastrointestinal disorders, including antibiotics associated diarrhea and recurrent *Clostridium difficile* disease (McFarland and Bernasconi, 1993; Elmer *et al*., 1996). Although *S. boulardii* is a subspecies of *S. cerevisiae*, this strain differs from *S. cerevisiae* in several taxonomic, metabolic and genetic properties (Buts *et al*., 2002; Nomoto, 2005). *S. boulardii* is resistant to acidity, and to all antibacterial antibiotics (Sazawal *et al*., 2006; Guslandi *et al*., 2003). The lyophilized preparations of *S. boulardii* have been commonly used throughout the world, besides, some empirical evidence has confirmed its efficacy as an adjuvant agent to treat diarrhea and prevent antibiotic - associated complications (Czerucka and Rampal, 2002; Szajewska *et al*., 2007).

Vacuum freeze-drying, as a preservation method which is based upon sublimation, has been used to manufacture probiotic powders for decades (Benny and Hennebert, 1991; Fonseca *et al*., 2000). In contrast to other drying methods, the advantages of vacuum freeze-drying are more prominent in the aspect of microorganism cultures preservation: the denaturalization level of various components at low temperature operation is lower; the physiology of the cells could be maintained for longer time (Riveros *et al.*, 2009); the rehydration capability would be better; freeze-dried powder would be preserved and transported more easily (Corcoran *et al.*, 2004; Gardiner, *et al*., 2000; Mauriello *et al*., 1999). Many microorganisms, including bacteria, yeasts, filamentous fungi and others (Miyamoto-Shinohara *et al.*, 2000) have been successfully conserved by freeze-drying method. However, there are some undesirable effects during the freeze-drying process, such as denaturation of sensitive proteins and viability decreasement of many cell types.

Therefore, in order to protect cells during freeze-drying, many studies have been focused on the approaches to minimize damage by using the protective agents (Abadias *et al.*, 2001; Costa *et al*., 2000). Selecting the appropriate protective agents could effectively reduce the mortality rate of the cells during the process of freeze-drying (Carvalho *et al*., 2003; Fonseca *et al*., 2000). Protective agents have two functions (Chu-Ky *et al*., 2013; Diniz-Mendes *et al*., 1999; Hounsa *et al.*, 1998): as holder and receptor, providing a skeleton structure for dry matter in the process of rehydration and protecting viable cells during freeze-drying (Hubalek, 2003). The protective agents, including polyols, carbohydrates, amino acids, proteins, polymers and others, can make the microorganism retain a small amount of water in the process of freeze-drying to adapt to the ultralow temperature, low pH, high alcohol and other poor environment that can inhibit or destroy the microorganism (Toledo *et al*., 2010). When carbohydrates and alcohols are used as protective agents, their hydroxyl groups can replace water molecules, and then blind to some components of the cell, thus protect the cell membrane and intracellular proteins during freeze-drying (Blanquet *et al*., 2005; Chu-Ky *et al*., 2013). As far as the protection system is concerned, the ratio of the biomass and the protective agent will affect the survival rate of cells after freeze-drying. If the amount of protective agent is too small, there will be a large amount of cell exposed in the media, which may change the permeability of the cell and can't afford to protect the cells (Khayata *et al*., 2012; Nakagawa *et al*., 2011). Conversely, if the amount of protective agent is too large, the osmotic pressure and permeability of the cell will be affected. In addition, the protective effect of compound freeze-drying protective agents is better than that of single protective agent. For instance, Li *et al.* (2008) reported that using the skimmed milk, trehalose and manganese sulfate as the composite protective agents could make the survival rate of *Lactobacillus plantarum* reach 96.8%±1.3%. Chen *et al.* (2008) found that in the process of freeze-drying of *Lactobacillus LC15* strain, the composite protective agent can make its survival rate reach 82.2%.

The classic method of lyoprotectant optimization involves changing one ingredient at a time, keeping the other substrate components at fixed levels. This time-

consuming method does not guarantee the optimal result (Shu *et al*., 2015). 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 (Carvalho *et al*., 1997), time and resources are saved (Srinivas *et al*., 1994). 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. A software to plan the experimental design and to run the data analysis makes the analysis easier than other approaches (Naveena *et al*., 2005).

S. boulardii, as commercial product, is generally used as lyophilized powder. However, its preparation process has not been widely reported (Martins *et al*., 2009; de Andrade *et al*., 2000). In this study, we investigated the influence of carbohydrates, prebiotics and salts on the freeze-drying survival rate and viability of *S. boulardii* in the vacuum freeze dried powder using single factor variability and Plackett-Burman design of experiments. It would provide a basis for the research and application of freeze-dried powder of *S. boulardii*.

Materials and methods

Microorganisms and chemicals

The strain used in this study is *Saccharomycess boulardii*, which was provided by the School of Food and Biological Engineering, Shaanxi University of Science & Technology (Xi'an, China). It was preserved at 4°C.

The main reagents used in this study are as follows: glucose (Tianjin Zhiyuan Chemical Reagent Co., Ltd), sucrose (Guangdong Guanghua Science & Technology Co., Ltd), maltose, fructose and lactose (Beijing Aobox Biotechnology Co., Ltd), mannose (Sinopharm Chemical Reagent Co., Ltd), trehalose (Xi'an Luosenbo Technology Co., Ltd), fructo-oligosaccharide, xylooligosaccharide, isomalto-oligosaccharide, galacto-oligosaccharide and inulin (Xi'an Luosenbo Technology Co., Ltd), stachyose and raffinose (Xi'an Dapeng Biological & Technology Co., Ltd), $NaHCO_3$ (Zhengzhou Had Appointed Chemical Reagent Factory), $MgSO_4$ and sodium ascorbate (Tianjin Fuchen Chemical Reagent Factory), sodium glutamate (Xi'an Luosenbo Technology Co., Ltd), KH_2PO_4 and K_2HPO_4 (Tianjin Tianli Chemical Reagents Co., Ltd). In this study, the pH of phosphate buffer was 6.5. All the protective agents were dissolved in distilled water and formulated into various concentrations, and sterilized at 118°C for 15min. All the chemicals used in this experiment were of analytical grade, except for maltose and stachyose, which were of food grade.

Culture conditions

The YPD broth (pH=5.6±0.1) was used for the activation and cultivation of *S. boulardii*. YPD agar medium was obtained by supplementation of YPD broth with 20g agar and then sterilization at 118°C for 15min. The viability of cells was determined after cultivation on YPD agar medium. *S. boulardii* was inoculated with 2% (v/v) inoculum in 250 mL flask containing 35mL YPD broth, and then

incubated at 37°C for 36h in the shaker (Changzhou Runhua Electric Appliance Co., Ltd., Changzhou, China) at 180rpm.

Vacuum freeze-drying

After incubation, the culture was centrifuged at 6000×g for 15min by high-speed centrifuge TG16A-WS (Hunan Shaite Xiangyi Co., Ltd, Hunan, China) and the supernatant was discarded to harvest *S. boulardii* wet biomass. The cells supplemented with protective agents were prefrozen at -45°C for 2h, and then were frozen according to Table 1 data by a vacuum freeze dryer LGJ-22D (Beijing Four-Ring Science Instrument Plant Co., Ltd., Beijing, China).

Table 1. The parameters for freeze-drying processing

Temperature(°C)	Vacuum degree(Pa)	Time(h)
-35	8	2
-25	8	4
-15	8	4
-5	8	6
5	8	4
15	8	3
25	8	1

Plackett-Burman design for screening the protective agents

In order to evaluate the effects of different protective agents on the survival rates of yeast cells, the Plackett-Burman design was used. The range of variables was first determined by single factor experiment. The studied protective agents were lactose (X1), trehalose (X2), xylo-oligosaccharide (X3), inulin (X4), $NaHCO_3$ (X6), $MgSO_4$ (X7), sodium glutamate (X8), sodium ascorbate (X10) and phosphate buffer (X11). Each factor was tested at two levels, coded as lower level (-1) and a higher level (+1), in terms of concentrations (Table 2).

Table 2. The factors and their variation levels of Plackett-Burman design

Variables	**Protective agents**	**Lower level (g/100mL)**	**Higher level (g/100mL)**
X1	Lactose	12	18
X2	Trehalose	12	18
X3	Xylo-oligosaccharide (XOS)	0.4	0.6
X4	Inulin	0.4	0.6
X6	$NaHCO_3$	0.3	0.45
X7	$MgSO_4$	0.5	0.75
X8	Sodium glutamate	2	3
X10	Sodium ascorbate	10	15
X11	Phosphate buffer	80	120

Determination of cell counts

Starter cultures were diluted to suitable concentration with NaCl (0.9% w/v) and inoculated in YPD agar medium. The inoculated medium was incubated at 37°C for 48h and then viable *S. boulardii* cells were counted. The freeze-dried powders were reconstituted to their original pre-freeze dried volume by adding sterile saline solution and the number of viable cells counted as above. Finally, the viable count per milliliter (CFU/mL) was determined.

Calculation of survival

$$\text{Survival rate (\%)} = \frac{(\text{CFU/mL after freeze-drying})}{(\text{CFU/mL before freeze-drying})} \times 100\% \quad (1)$$

Calculation of the number of cells

$$\text{The number of cells (CFU/g)} = \frac{N \times V}{M} \quad (2)$$

where N (CFU/mL) is the number of viable cells after freeze-drying, V (mL) is the volume of the original yeast suspension, M (g) is the weight of the freeze-dried powder.

Statistical analysis

The Design-Expert (Version 8.0.6) software (Stat-Ease Inc., America) was used for the experiment design and analysis of the experiment data to identify the significant factors and their corresponding coefficients. Therefore, sum of squares, F-value, p-value and confidence intervals (CI) were evaluated to analyze the number of viable count and the survival rate from each of the trials.

Results and discussion

The effects of carbohydrates on the viability of S. boulardii strain

The selected carbohydrates, including glucose, sucrose, maltose, fructose, lactose, mannose and trehalose, were dissolved in distilled water, the concentration of these seven carbohydrates was set at 12%, and then the carbohydrates solution was sterilized at 118 °C for 15 min. As protective agents, these seven sterilized carbohydrates were added to the collected *S. boulardii* wet biomass, respectively, and then were proceeded to vacuum freeze-drying. The results were shown in Figure 1.

As shown in Figure 1, when the protective agent was not added, the survival rate of the freeze-dried powder was low, only 3.99%, and the number of viable cells was 0.36×10^{10}CFU/g. Meanwhile, when adding protective agent, different carbohydrates had different influence on the survival rate of freeze-dried *S. boulardii* cells.

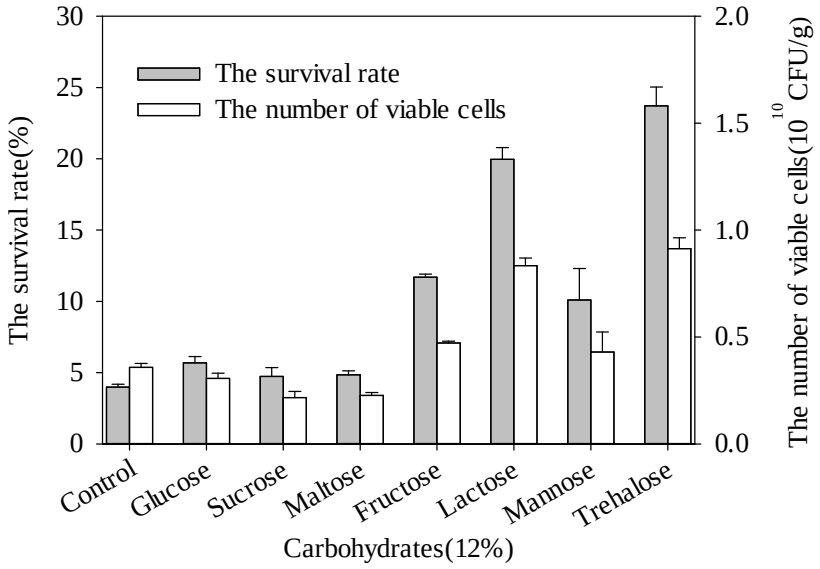

Figure 1. Effects of carbohydrates on the survival of freeze-dried *S. boulardii* strain

In Figure 1, it is shown that lactose and trehalose had significant influence on *S. boulardii* strain. In these cases, the survival rates of freeze-dried *S. boulardii* cells were 19.95% and 23.72%, respectively, and the numbers of viable cells of freeze-dried powder were 0.83×10^{10}CFU/g and 0.91×10^{10}CFU/g. The protective effect of the tested carbohydrates on the freeze-dried *S. boulardii* strain decreased in the following order: trehalose > lactose > fructose > mannose > glucose > maltose > sucrose. Therefore, trehalose and lactose were selected for further study. The protective agents mainly have two functions in preserving viability of freeze-dried cells, as a support material they provide the dry residue with definite physical structure and they work as a receptor in the process of rehydration (Corcoran *et al*., 2004; Gardiner *et al*., 2000; Riveros *et al*., 2009). In addition they are able to protect the living cells against biochemical damage during the freeze-drying process (Blanquet *et al*., 2005; Chu-Ky *et al*., 2013). For instance, sugars such as trehalose and sucrose are commonly used as cryoprotectants or lyoprotectants. Trehalose, as a disaccharide, is a natural cryoprotectant and has been successfully used to preserve different cell types during freeze-drying including bacterial and yeast cells (Abadias *et al*., 2001; Chen *et al*., 2012). Adding a certain amount of trehalose may either form a thicker glass layer surrounding the dehydrated spores (Prakash and Raj, 2010), or enhance the amount of hydrogen bonding in phospholipid head groups (Nakamura *et al*., 2008). What's more, it has been proved that the trehalose would protect proteins against denaturation (Hatley, 1990). Choi *et al*. (2008) observed that the highest survival rate of *Saccharomyces cerevisiae* occurred when saccharose and trehalose were used as protective agents.

Viability preservation of S. boulardii strain in the presence of prebiotics

The prebiotics, including raffinose, inulin, isomalto-oligosaccharide (IMO), stachyose, xylo-oligosaccharide (XOS), galacto-oligosaccharide (GOS) and fructo-oligosaccharide (FOS), were dissolved in distilled water and the concentration of these seven prebiotics was set as 0.4%. Then the prebiotics solution was sterilized at 118°C for 15min. As protective agents, these seven sterilized prebiotics were added to the collected *S. boulardii* wet biomass, which was then subjected to vacuum freeze-drying. The results concerning the survival of *S. bulardii* cells are shown in Figure 2.

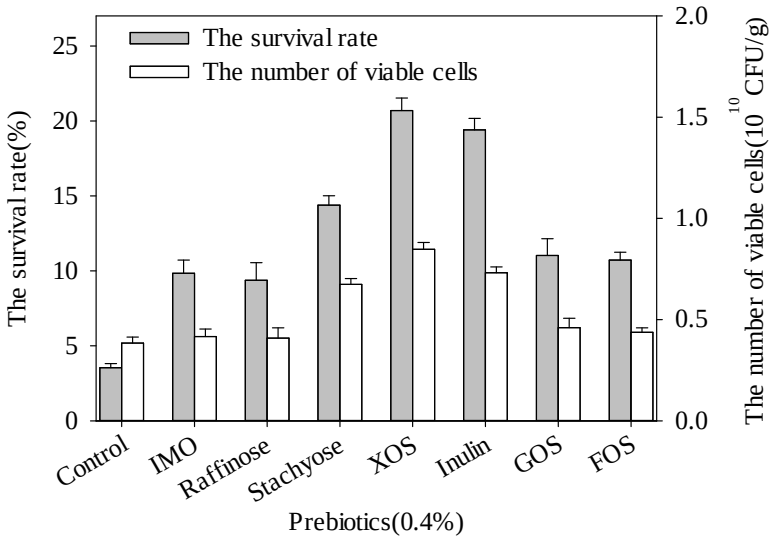

Figure 2. Effects of some prebiotics on the survival of freeze-dried *S. boulardii* yeast

As Figure 2 shows, when the protective agent was not added, the survival rate of the *S. boulardii* cells in the freeze-dried powder and the number of viable cells were 3.54% and 0.38×10^{10}CFU/g, respectively. When adding the protective agent, the effect of these seven prebiotics on the survival rate of freeze-dried *S. boulardii* cells was different. Especially XOS and inulin had better protective effect on *S. boulardii* strain, the survival rate was 20.70% and 19.41%, respectively, and the number of viable cells reached 0.85×10^{10}CFU/g and 0.73×10^{10}CFU/g. Therefore, XOS and inulin were selected for further study.

The effects of salts on S. boulardii yeast

The selected salts were sodium glutamate, sodium ascorbate, phosphate buffer, $MgSO_4$ and $NaHCO_3$, each of these salts was dissolved in distilled water and was then sterilized at 118°C for 15min. As protective agents, these five salts were added to the collected *S. boulardii* wet biomass, respectively, and then were proceeded to vacuum freeze-drying. The results are showed in Figure 3.

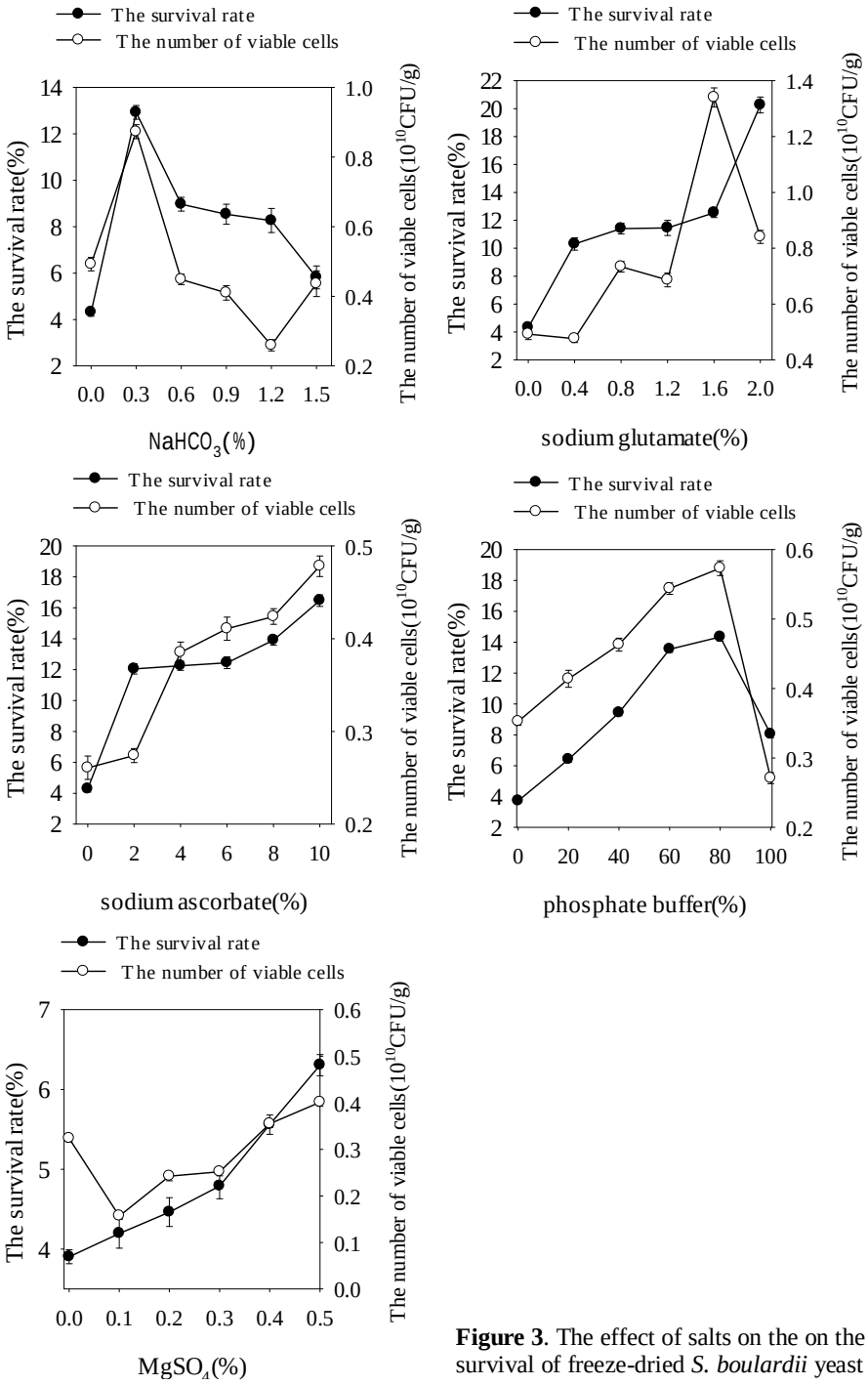

Figure 3. The effect of salts on the on the survival of freeze-dried *S. boulardii* yeast

According to the results shown in Figure 3, as the concentration of $NaHCO_3$ was increasing, the survival rate of freeze-dried biomass increased rapidly and then decreased. And the optimal concentration of $NaHCO_3$ was 0.3%, where the survival rate reached 12.92%, which increased 3 times compared to the control sample. However, the survival rate increased with the increase of the concentration of sodium glutamate. When the additive amount of sodium glutamate was 2%, the survival rate was 20.26%, which was 4.7 times bigger compared to the control group. In addition, the survival rate and the number of viable cells of freeze-dried powder increased with the increase of the concentration of sodium ascorbate and $MgSO_4$. The presence of sodium ascorbate and $MgSO_4$ is beneficial to improve the stability of the protein and enzyme in the cell membrane and cytoplasm. The addition of 0.1 % $MgSO_4$ caused the decrease of the number of viable cells, followed by increase at concentration of 0.2 % $MgSO_4$. The possible reason is that, when adding magnesium sulfate and then proceeding to vacuum freeze-drying, the weight of the freeze-dried powder is heavier than that of the control sample. According to equation (2), the number of viable cells decreased at a concentration of 0.1 %. While the concentration of phosphate buffer was increasing, the survival rate and the number of viable cells increased and then decreased. And the optimal concentrations of sodium ascorbate, phosphate buffer and $MgSO_4$ were 10%, 80% and 0.5%, respectively; the survival rate reached 16.47%, 14.34% and 6.3%, respectively.

In addition, according to the study of Kandylis *et al*. (2014) sodium glutamate allowed the best viability of *S. cerevisiae* AXAZ-1 cells after freeze-drying when immobilized on potato pieces. Shu *et al.* (2015) reported that phosphate buffer had a protective effect on the viability of *Lactobacillus bulgaricus*. Furthermore, using skim milk as the support material in combination with two compounds from honey, sodium glutamate, trehalose or raffinose, the viability of *Saccharomyces cerevisiae* cells was enhanced from 30% to 96–98% (Abadias *et al.*, 2001; Hubalek *et al.*, 2003). Obviously, skim milk has a significant effect on the viability. However, the survival rate of *S. boulardii* cells was more than 100% in our previous work when using skim milk as protective agent. The possible reason is that *S. boulardii* could proliferate remarkably in the nutrient-rich skim milk, and the number of viable cells after freeze-drying is much more than that of viable cells before freeze-drying. Thus, skim milk was selected for further research.

Plackett-Burman design for screening of protective agents

According to the previously results, Plackett-Burman design was used to screen significant factors affecting the freeze-drying of *S. boulardii* strain. The matrix of independent variable variation and the results of Plackett-Burman design are shown in Table 3, where X5 and X9 are virtual items, which were used to estimate error set. The survival rate of freeze-dried *S. boulardii* cells represented by Y1 (%) and the number of viable cells of freeze-dried powder represented by Y2 ($\times 10^{10}$CFU/g) were used as responses.

In Table 4, the analysis of variance (ANOVA) was performed to estimate the effect of each factor on the survival rate of freeze-dried *S. boulardii* strain. According to

the Table 4 data analysis of variance, the model p-value of 0.0301 implied the model was significant. In the ANOVA, the p-value less than 0.0500 indicated that the terms are significant. In this case, lactose (X1) (p=0.0143), trehalose (X2) (p=0.0297) and sodium glutamate (X8) (p=0.0078) are the most significant factors, which showed the most important effect on the yeast viability preservation during freeze-drying.

Table 3. The experimental design matrix and the results of Plackett-Burman design of experiments

Run	X1	X2	X3	X4	X5	X6	X7	X8	X9	X10	X11	Y1(%)	Y2(×10^{10}CFU/g)
1	-1	1	1	-1	1	1	1	-1	-1	-1	1	20.11	0.3254
2	1	-1	1	1	1	-1	-1	-1	1	-1	1	21.98	0.3872
3	-1	-1	-1	1	-1	1	1	-1	1	1	1	21.12	0.3614
4	-1	-1	1	-1	1	1	-1	1	1	1	-1	20.13	0.3267
5	1	1	1	-1	-1	-1	1	-1	1	1	-1	24.31	0.3662
6	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	18.22	0.2516
7	1	1	-1	-1	-1	1	-1	1	1	-1	1	27.41	0.5213
8	1	1	-1	1	1	1	-1	-1	-1	1	-1	24.81	0.4311
9	1	-1	-1	-1	1	-1	1	1	-1	1	1	29.81	0.5671
10	-1	1	1	1	-1	-1	-1	1	-1	1	1	27.21	0.4638
11	1	-1	1	1	-1	1	1	1	-1	-1	-1	26.72	0.4136
12	-1	1	-1	1	1	-1	1	1	1	-1	-1	27.88	0.4316

Furthermore, lactose (X1), trehalose (X2) and sodium glutamate (X8) showed positive effect on the survival rate of freeze-dried *S. boulardii* cells (Y1). Namely, when the concentration of these three variable factors increased, the response value also increased. Thus, the optimal concentrations of lactose, trehalose and sodium glutamate were 18g/100mL, 18g/100mL and 3g/100mL, respectively.

Table 4. Result of ANOVA statistical analysis for response Y1 (survival rate)

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1.62	9	0.180	32.56	0.0301*
A-X1	0.38	1	0.380	68.58	0.0143*
B-X2	0.18	1	0.180	32.13	0.0297*
C-X3	0.06	1	0.060	10.77	0.0816
D-X4	0.095	1	0.095	17.11	0.0538*
F-X6	0.066	1	0.066	11.98	0.0743
G-X7	0.091	1	0.091	16.42	0.0558
H-X8	0.7	1	0.700	126.75	0.0078*
K-X10	0.025	1	0.025	4.46	0.169
L-X11	0.027	1	0.027	4.8	0.1599
Residual	0.011	2	0.006		
Cor Total	1.63	11			

* $p<0.05$, significant

Table 5. Result of ANOVA statistical analysis for response Y2 (viable cells)

Source	Sum of Squares	DF	Mean Square	F Value	p-value Prob > F
Model	0.0520	9	0.0058	21.04	0.0462*
A-X1	0.0150	1	0.0150	53.20	0.0183*
B-X2	0.0033	1	0.0033	12.02	0.0740
C-X3	0.0033	1	0.0033	11.83	0.0751
D-X4	0.0016	1	0.0016	5.70	0.1396
F-X6	0.0003	1	0.0003	0.92	0.4387
G-X7	0.0005	1	0.0005	1.69	0.3228
H-X8	0.0180	1	0.0180	66.97	0.0146*
K-X10	0.0019	1	0.0019	7.00	0.1180
L-X11	0.0083	1	0.0083	30.00	0.0318*
Residual	0.0006	2	0.0003		

* $p < 0.05$, significant

The p-value of 0.0462 implied that the model was significant (Table 5). The established relative importance of variables was as follows: X8> X1> X11> X2> X3> X10> X4> X7> X6. Out of the above factors, sodium glutamate (X8) (p=0.0146), lactose (X1) (p=0.0183) and phosphate buffer (X11) (p=0.0318) were considered significant factors, which showed the most important effect on the yeast viability preservation during the freeze-dry treatment. Therefore, these three factors were determined as: 3g/100mL sodium glutamate, 18g/100mL lactose and 120g/100mL phosphate buffer.

According to the ANOVA statistical analysis data, sodium glutamate (X8) and lactose (X1) showed a significant effect on the yeast cells viability. In addition, the trehalose (X2) can obviously affect the survival rate of the cells; the phosphate buffer (X11) had remarkable impact on the number of viable cells of the freeze-dried powder. Obviously, the cell survival rate and the number of viable cells were associated with the type of lyoprotectant used. In this study, our protocol focused on increasing survival rate. Therefore, according to the result of ANOVA statistical analysis for response Y1 (Survival rate) and Y2 (viable cells), the concentration of phosphate buffer was set as 120g/100mL, and lactose (X1), trehalose (X2) and sodium glutamate (X8) were selected for further research.

Lyophilized cells of *S. boulardii* strain have been widely used to treat several types of diarrhea (Pothoulakis, 2009). The use of lyophilized yeast cells has several advantages over fresh ones: the samples can be easily transported or stored and variations of their metabolomic profiles do not occur during transport or storage (Martins *et al*., 2009). Thus, it is necessary to optimize the protective agents of lyophilized cells of *S. boulardii*. In present study, the result showed that lactose, trehalose and sodium glutamate could improve the survival rate of *S. boulardii* cells.

Conclusions

In this study, the effects of carbohydrates, prebiotics and salts (as protective agents) on the freeze-dry treatment of *Saccharomyces boulardii* strain were investigated. Using single factor analysis and Plackett-Burman design experiment to screen the lyoprotectant contents for *S. boulardii*, the result showed that lactose, trehalose and sodium glutamate had a significant influence on the survival rate of *S. boulardii* during the freeze-drying process. All of the above protective agents were demonstrated to have a positive effect. The results showed that the optimal concentrations of these three protective agents were as follows: 18g/100mL lactose, 18g/100mL trehalose and 3g/100mL sodium glutamate.

Acknowledgements

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