sciendo

10.2478/aucft-2018-0013

EFFECTS OF CARBOHYDRATES, PREBIOTICS AND SALTS ON SURVIVAL OF *SACCHAROMYCES BOULARDII* DURING FREEZE-DRYING

– Research paper –

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Abstract: *Saccharomyces boulardii*, as a probiotic yeast, had been commonly used in food, medicine and feed to treat diarrhea in humans or livestock. However, there are few researches focusing on the preparation of its freeze-drying *S.boulardii* powder. In this study, the effect of carbohydrates (glucose, sucrose, maltose, fructose, lactose, mannose and trehalose), prebiotics (isomalto-oligosaccharide, xylo-oligosaccharide, raffinose, stachyose, inulin, galacto-oligosaccharide and fructo-oligosaccharide) and salts ($NaHCO_3$, $MgSO_4$, sodium glutamate, sodium ascorbate, and phosphate buffer) on the freeze-dried survival of *S. boulardii* were investigated to screen the cryoprotectant by using single factor experiments. As the result, trehalose and XOS had better protective effect, the survival rate was 23.72% and 20.70% respectively, the number of viable cells reached 0.91×10^{10} CFU/g and 0.85×10^{10} CFU/g respectively; the addition amount of $NaHCO_3$ was 0.3%, the freeze-dried survival rate reached the maximum value of 12.92%. The phosphate buffer additive amount and the bacterial sludge weight were 0.8:1, the freeze-dried survival rate reached a maximum of 14.14%, the freeze-dried survival rate of sodium glutamate, sodium ascorbate and $MgSO_4$ groups was increasing, reaching a maximum of 20.26%, 16.47% and 6.29% when the addition amount was 2%, 10%, 0.5%.

Keywords: *Saccharomyces boulardii*, freeze-drying, protective agents, carbohydrates, prebiotics, salts

INTRODUCTION

Probiotics are defined as live micro-organisms that give a health benefit to the host. Most probiotics are bacteria, but one strain of yeast, *Saccharomyces boulardii*, has been found to be an effective probiotic in double-blind clinical studies (Czerucka D et al., 2010). *S. boulardii* is a nonpathogenic yeast isolated from the lychee fruit in Indochina by Henri Boulard in 1923 (Li P D et al., 2012). *S. boulardii* acts as a shuttle that releases effective enzymes, trophic factors and proteins during tis interstinal transit that improves host's immune defenses, absoption and digestion of nutrient. Moreover, *S.boulardii* secretes polyamines during its intestinal transit, mainly spermine and spermidine that regulate protein synthesis and gene expression (Buts J P, Keyser, 2006). Clinical studies have confirmed its efficacy in treatment or prevention of intestinal diseases, including traveller's diarrhoea (Elmer & McFarland, 2001), antibiotic associated diarrhoea (Kotowska, Albrecht, & Szajewska, 2005), and recurrent Clostridium difficile disease. In addition, as the "probiotics" used in animal husbandry, *S.boulardii* also has been proved to be effective for lowering pathogens and the corresponding toxins concentration, stimulating the immune system and enhancing the microbial balance.

Cryopreservation of intact yeast cells is not only an important aspect of scientific research but also an important industrial tool (Dinizmendes L et al., 2015). Vacuum freeze-drying is the most successful and convenient method for preserving yeast, bacteria and sporulation fungi. It can protect from contamination, ease strain distribution and increase viability (Abadias et al., 2001). 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 less; the physiology of the cells could be maintained for longer time (Riveros et al., 2009). Vacuum freeze-drying will be a commonly applied method of industrial production of bacterium powder and at the same time, it will become the main development of powder preparation (Jia Fang-fang et al., 2017). However, freezing and subsequent sublimation of frozen water can be attributed to cellular damage, including damage

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to the cell membrane and DNA (Tripathi and Giri, 2014). In addition, the changes in the physical state of membrane lipids during storage may lead to severe loss of bacterial viability during storage (Fonseca et al., 2015). Therefore, the use of protective agents is the most effective way to reduce the freezing injury.

Protective agents play an important role in maintaining viability. Adding a cryoprotectant can change the environment of the sample during freeze-drying and increase cell stability to reduce cell damage (Jagannath A et al., 2010). A good protectant should provide cryoprotection for cells in the process of freezing, be easily dried, and provide a good matrix to make them stable and ease of rehydration (G. Zhao and G. Zhang, 2010). Cryoprotectants which are widely used and have significant effects mainly include skim milk, trehalose, glycerin, peptone, mannitol, sodium glutamate, sorbitol, sucrose, glucose, lactose, wort, polyethylene glycol and other polymer (Chen H et al., 2017). These substances slow down the growth process of the crystal nucleus by reducing the content of free water, reduce the phase transition temperature, increase the fluidity of the membrane, prevent the protein from denaturation due to lyophilization, reduce the area of cells exposed to oxygen and medium, a protective layer was formed on the surface of the bacteria, to improve the survival rate of the bacteria (Hubálek Z, 2003).

S. boulardi is generally used as lyophilized powder (Martins et al., 2009). However, the preparation process has not been widely reported (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*. It would provide a basis for the research and application of freeze-dried *S. boulardii* powder.

MATERIALS AND METHODS

Microorganisms and Chemicals

The strain used in this study is *Saccharomycess boulardii*, which was provided by School of Food and Biological Engineering, Shaanxi University of Science & Technology (Xi'an, China). The main reagents used in this study are shown in Table 1. In this study, the pH of phosphate buffer was 6.5. All the protective agents were dissolved in distilled water and prepared at different concentrations, and sterilized at 118°C for 15min. All the chemicals used in this experiment were analytic grade, except maltose and stachyose (they were both food grade).

Table 1. The main reagents and their source

Reagent	manufacturer	Reagent	manufacturer
glucose	Tianjin Zhiyuan Chemical Reagent Co., Ltd	galacto-oligosaccharide	Xi'an Luosenbo Technology Co., Ltd
sucrose	Guangdong Guanghua Science & Technology Co., Ltd	fructo-oligosaccharide	Xi'an Luosenbo Technology Co., Ltd
lactose	Beijing Aobox Biotechnology Co., Ltd	xylooligosaccharide	Xi'an Luosenbo Technology Co., Ltd
fructose	Beijing Aobox Biotechnology Co., Ltd	isomalto-oligosaccharide	Xi'an Luosenbo Technology Co., Ltd
maltose	Beijing Aobox Biotechnology Co., Ltd	inulin	Xi'an Luosenbo Technology Co., Ltd
trehalose	Xi'an Luosenbo Technology Co., Ltd	$NaHCO_3$	Zhengzhou Had Appointed Chemical Reagent Factory
mannose	Sinopharm Chemical Reagent Co., Ltd	$MgSO_4$	Tianjin Fuchen Chemical Reagent Factory
stachyose	Xi'an Dapeng Biological & Technology Co., Ltd	sodium ascorbate	Tianjin Fuchen Chemical Reagent Factory
raffinose	Xi'an Dapeng Biological & Technology Co., Ltd	sodium glutamate	Xi'an Luosenbo Technology Co., Ltd
KH_2PO_4	Tianjin Tianli Chemical Reagents Co., Ltd	K_2HPO_4	Tianjin Tianli Chemical Reagents Co., Ltd

Solution preparation

The phosphate buffer was prepared by dissolving 76g of dipotassium hydrogen phosphate, 40.8g of potassium dihydrogen phosphate to 1000 mL, and sterilizing at 118°C for 15min. The preparation of the carbohydrate

and prebiotic solution was prepared by weighing an appropriate amount of sugar or prebiotic dissolved in distilled water to prepare a solution of the desired concentration, and sterilizing at 108°C for 15min for the addition of the lyophilized protective agent.

Culture conditions

YPD medium was obtained by 20g glucose, 20g peptone, 10g yeast extract, dissolved in 1000mL of distilled water, sterilized at 118°C for 15min. Used for the activation and culture of *S. boulardii*. YPD agar medium was obtained by supplementation of YPD broth with 20g agar and then sterilization at 121°C for 15min. The cells were cultured on YPD agar medium and the cell viability was determined. *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 the cultivation, the culture was centrifuged at 6000×g for 15 min using high-speed centrifuge TG16A-WS (Hunan Shaite Xiangyi Co., Ltd, Changshi, China), then the supernatant was discarded and harvest *S. boulardii* wet biomass. YPD medium without added protective agents was as a control sample. YPD medium supplemented with protective agent was the test group. Then the cells were prefrozen at -45°C for 2h and then frozen by a vacuum freeze dryer LGJ-22D (Beijing Four-Ring Science Instrument Plant Co., Ltd., Beijing, China) according to Table 2 data.

Table 2. 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

Determination of cell counts

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

Calculation of survival

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.

RESULTS AND DISCUSSIONS

Effects of carbohydrates on the survival of *S. boulardii* during freeze-drying

The *S. boulardii* cells were collected by centrifugation for 15 minutes at 6000 r/min using a high speed refrigerated centrifuge. The selected carbohydrates, including glucose, sucrose, maltose, fructose, lactose, mannose and trehalose were added to the precipitation of *S. boulardii* as freeze-dried protective agent, and the addition amount of carbohydrates were 12% (w/w), then phosphate buffer was added, so that the total amount of the phosphate buffer and the protective agent was 1:1 with the precipitation of *S. boulardii*. The mixtures were uniformly mixed and then were preceded to freeze-drying. The results are shown in Figure 1. The survival rate of freeze-dried *S.boulardii* was low when the freeze-dried protective agent was not added, only 3.99%, and the number of viable cells of *S.boulardii* was 0.36×10^{10} CFU/g, which is lower than that of the added protective agent. The test group, in which lactose and trehalose had a better protective effect on the *S. boulardii*, the survival rate of freeze-dried.

S.boulardii power was 19.95% and 23.72%, respectively, the number of viable cells of *S.boulardii* reached 0.83×10^{10} CFU/g and 0.91×10^{10} CFU/g, respectively. In addition, in the order of the effect on the frozen-dried *S. boulardii* from the largest to the smallest, it goes: trehalose > lactose> fructose> mannose> glucose> maltose> sucrose. Carbohydrate has a significant protective effect on the lyophilization of microbial cells. The protective effect of carbohydrate protectants is to inhibit

the phase change of the plasma membrane, that is, when the microbial cells are dehydrated, the "water replacement" is carried out.

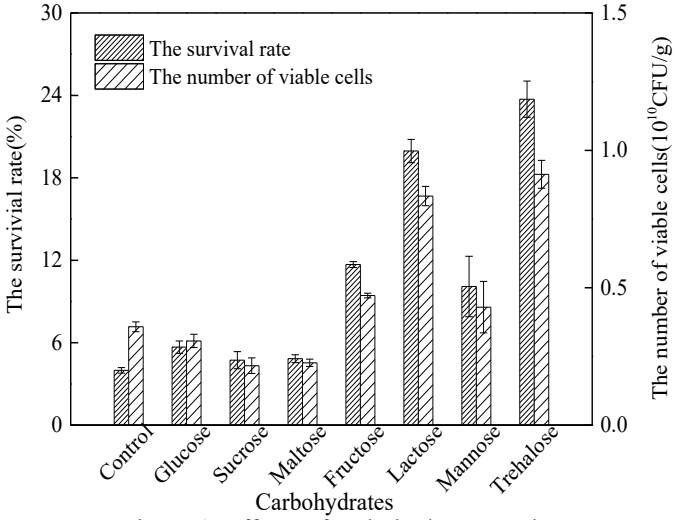

Figure 1. Effects of carbohydrates on the freeze-drying of *S. boulardii*

The physiological characteristics of the dry plasma membrane in the presence of carbohydrate protectant and the hydrated membrane in the presence of no protective agent are similar. For instance, trehalose can effectively improve the survival rate of microorganisms after cryophilization. It has been considered as a carbon source of microorganisms for a long time (Benaroudj N et al., 2001). However, more research results show that trehalose plays an important role in stabilizing cell membrane structure and protein structure, the change of cell membrane physical state and protein denaturation are the direct causes of microbial death (Leslie S B et al., 1995). Yang et al. studied *B. longum* and found that 10g / 100mL trehalose had the best protective effect when the protective agent was used alone, and its survival rate reached 53.22% ± 2.21% (Yang C Y et al., 2012). Choi et al. observed that the highest survival rate of *Saccharomyces cerevisiae* was obtained by using the saccharose and trehalose as protective agents (Choi et al., 2008).

Effects of prebiotics on the survival of *S. boulardii* during freeze-drying

The prebiotics, including isomalto-oligosaccharide (IMO), xylo-oligosaccharide (XOS), raffinose, stachyose, inulin, galacto-oligosaccharide (GOS) and fructo-oligosaccharide (FOS) were added to the *S. boulardii* precipitation collected by centrifugation, and the additive amount of each one was 0.4% (w/w), then the phosphate buffer was added, so that the total amount of the phosphate buffer and the protective agent was 1:1 with the precipitation of *S. boulardii*. The mixtures were uniformly mixed, and then were preceded to freeze-drying. The results are shown in Figure 2. When the protective agent was not added, the survival rate of the freezed-dried *S.boulardii* powder was only 3.54%, and the number of viable cells was 0.38×10^{10} CFU/g, which was lower than that of the test group with the added protective agent. When the above prebiotics were added as protective agents, the result showed that different prebiotics had different effects on the survival rate and the number of viable cells. Among them, XOS and inulin had better protective effect on *S. boulardii*, the freeze-dried survival rate was 20.70% and 19.41%, respectively, the number of viable cells reached 0.85×10^{10} CFU/g and 0.73×10^{10} CFU/g, respectively. The effect of prebiotic protective agents for *S.boulardii* was from large to small: XOS> inulin> stachyose> GOS> FOS> IMO> raffinose.

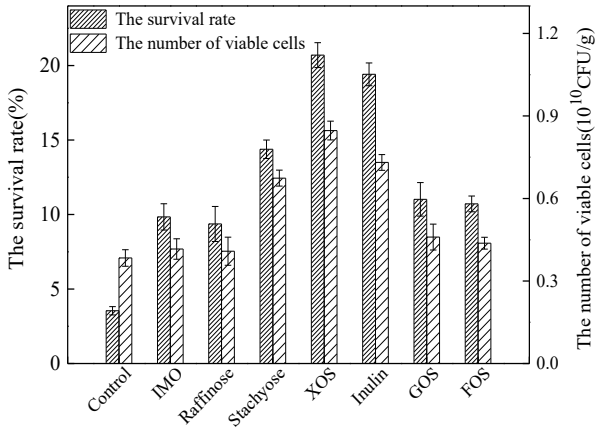

Figure 2. Effects of prebiotics on the freeze-drying of *S. boulardii*

Chen et al. used the response surface center combination experiment to obtain the optimized formula of bifidobacteria prebiotics lyophilized protective agent. The optimized formula was: inulin content of 13%, the content of stachyose was 11% and the xylooligosaccharide content was 7%. Under these conditions, the lyophilization survival rate of *Bifidobacterium bifidum* was (88.7 ± 1.3)% (Chen et al., 2017). Products that use prebiotics as a cryoprotectant have a positive effect on the health of the body.

Effects of salts on the survival of *S. boulardii* during freeze-drying

The selected salts, including $NaHCO_3$, $MgSO_4$, sodium glutamate, sodium ascorbate, and phosphate buffer were added to the *S. boulardii* yeast precipitation as a protective agent,

wherein the concentration of $NaHCO_3$ was 0%, 0.3%, 0.6%, 0.9%, 1.2%, 1.5%; the concentration of $MgSO_4$ was 0%, 0.1%, 0.2%, 0.3%, 0.4%, 0.5%; the concentration of sodium glutamate was 0%, 0.4%, 0.8%, 1.2% , 1.6%, 2.0%; the concentration of sodium ascorbate was 0%, 2%, 4%, 6%, 8%, 10%, the ratio of phosphate buffer addition to bacterial sludge weight was 0:1, 0.2:1 , 0.4:1, 0.6:1, 0.8:1, 1:1. In addition to the phosphate buffer test group, sterile saline should be added to make the phosphate buffer and the total amount of sterile physiological saline and the precipitation of *S. boulardii* 1:1. The other four salts need to be added with phosphate buffer, so that the total amount of the phosphate buffer and the protective agent was 1:1 with the bacterial sludge, and then were proceeded to vacuum freeze-drying. The results are shown in Figure 3.

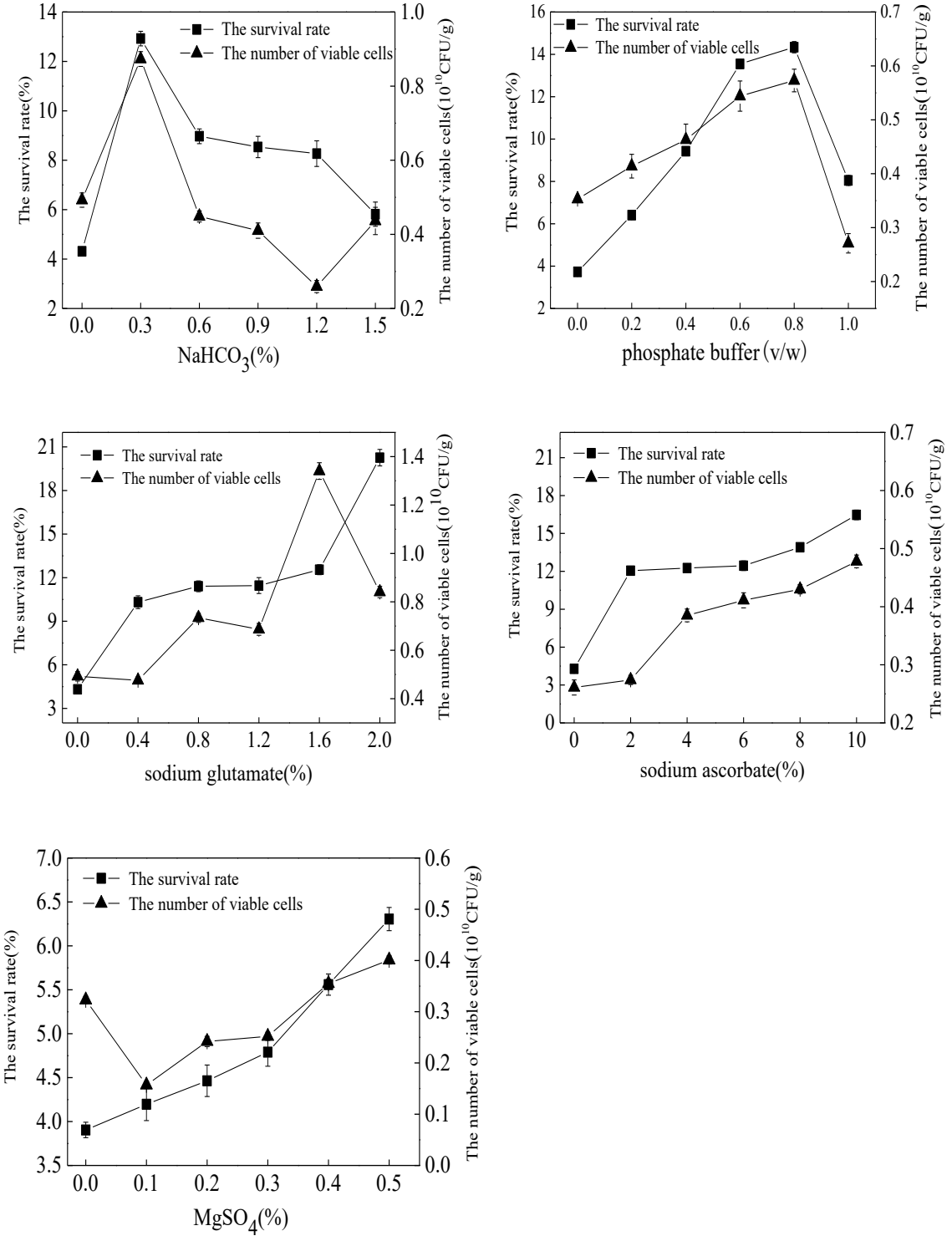

Figure 3. Effects of salts on the freeze-drying of *S. boulardii*

According to the results showed in Figure 3, when the protective agent was not added, the survival rate of the freeze-dried powder was low, roughly distributed around 4%. When different concentrations of salts were added as protective agents, with the increase of salt concentration, the freeze-dried survival rate and the number of viable cells showed different trends. The freeze-dried survival rate of $NaHCO_3$ and phosphate buffer group increased and then decreased. When the amount of $NaHCO_3$ was 0.3% (w/w), the freeze-dried survival rate reached the maximum value of 12.92%, which was about 3 times that of the control sample. The number of viable cells was 0.87×10^{10} CFU/g; when the ratio of phosphate buffer to precipitation of *S. boulardii* was 0.8:1, the freeze-dried survival rate reached a maximum of 14.14%, which was about 3.6 times that of the control sample, it was 0.573×10^{10} CFU/g. Qi et al. reported that $NaHCO_3$ had a protective effect on the viability of *Bifidobacterium bifidum* BB01 (Qi et al., 2017).

However, the survival rate increased with the concentration of sodium glutamate increasing. When the additive amount of sodium glutamate was 2%, the survival rate was 20.26%, the number of viable cells of *S. boulardii* reached 0.84×10^{10} CFU/g. When the concentration of sodium glutamate increased, the number of viable counts decreased and the freeze-dried survival rate increased. The reason was that the addition of sodium glutamate increased the weight of bacteria powder, which led to the decrease of the number of viable cells, but increased the protective effect on bacteria, so the total number of viable cells increased and the survival rate increased.

In addition, the survival rate and the number of viable cells of freeze-dried powder increased with the concentration of sodium ascorbate and $MgSO_4$ increasing. 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. In case of $MgSO_4$, the number of viable cells decreased at the addition proportion of 0.1 % and then increased at 0.2%. The possible reason is that, when adding magnesium sulfate and then proceeding to vacuum freeze-drying, the weight of the freeze-dried powder was heavier than that of the control sample. And the optimal concentration of sodium glutamate, sodium ascorbate and $MgSO_4$ groups were 2%, 10%, 0.5% respectively; the survival rate reached 20.26%, 16.47% and 6.29%, respectively. The results showed that these five salts acted as protective agents to significantly improve the survival rate of the freeze-dried *S. boulardii* powder.

According to the study of Kandylis et al. that sodium glutamate gave the best viability of immobilized on potato pieces S. cerevisiae AXAZ-1 cells after freeze-drying (Kandylis et al., 2014). Shu et al. reported that phosphate buffer had a protective effect on the viability of *Lactobacillus bulgaricus* (Shu et al., 2015).

CONCLUSIONS

In this study, experiments were studied about effect of carbohydrates, prebiotics and salts on survival of *Saccharomyces boulardii* during freeze-drying and screened the cryoprotectant contents for *S. boulardii* by using single factor analysis. The result showed that lactose and trehalose had a better protective effect on the *S. boulardii* yeast, the survival rate was 19.95% and 23.72%, respectively, and the number of viable cells reached 0.83×10^{10} CFU/g and 0.91×10^{10} CFU/g, respectively; XOS and inulin had better protective effect on *S. boulardii* yeast, the freeze-dried survival rate was 20.70% and 19.41%, respectively, the number of viable cells reached 0.85×10^{10} CFU/g and 0.73×10^{10} CFU/g, respectively; the addition amount of $NaHCO_3$ was 0.3%, the freeze-dried survival rate reached the maximum value of 12.92%, the phosphate buffer additive amount and the bacterial sludge weight were 0.8:1, the freeze-dried survival rate reached a maximum of 14.14%, the freeze-dried survival rate of sodium glutamate, sodium ascorbate and $MgSO_4$ groups was increasing, reaching a maximum of 20.26% 16.47% and 6.29% when the addition amount was 2%, 10%, 0.5%. These results provide a reference for protective agent selection and also provide a basis for the subsequent research of *S. boulardii.*

ACKNOWLEDGMENTS

The work was partially supported by Science and Technology Project of Weiyang District, Xi'an City (No.201831) and the science and technology project of Weinan city (No.2017CXFC-27).

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