Investigating Cryoprotectant Effects on *Saccharomyces boulardii* as Probiotic Strain during Freeze-Drying for Tablet Development

Azhar M.A.
Faculty of Chemical and Process Engineering Technology, Universiti Malaysia Pahang Al-Sultan Abdullah (UMPSA), MALAYSIA
akmalazhar@umpsa.edu.my

Abstract
Freeze-drying is an effective method to maintain the therapeutic properties of probiotics in a tablet form by solidifying and then sublimating frozen water in the culture to increase their shelf life and stability. However, this method can also damage probiotic cells and can impact their viability. Cryoprotectants can improve the strain preservation of probiotics during drying, yielding a more superior and potent final product, but their efficacy depends on the microorganisms, cryoprotectant types and freeze-drying conditions. The present study looked at how different cryoprotectants affected a chosen probiotic strain Saccharomyces boulardii on its ability to survive after being freeze-dried for tablet manufacture. The yeast strain was harvested, mixed with cryoprotectants of different concentrations (sorbitol, lactose, sucrose and skim milk) and freeze-dried using a vacuum freeze dryer. After the powder was rehydrated, a plate dilution method was conducted to determine the number of viable cells. The results showed that using a cryoprotectant agent during freeze-drying significantly improved the survival rate by up to 40% compared to the control (3.65%).

The study found that 10% skim milk exhibited the highest cell viability after freeze-drying followed by 5% skim milk, 20% lactose, 10% sucrose, 10% sorbitol, 2.5% sorbitol and 2% skim milk, indicating the beneficial role of cryoprotectants in preventing ice formation and membrane damage during the process. The findings of this study provided valuable insights into the preservation of probiotic viability during processing and storage. These findings also have significant implications for the probiotic supplement industry and can help to develop high-quality and effective probiotic tablet formulations by identifying the most effective cryoprotectants for use in tablet formulations.

Keywords*:* Probiotics, Freeze-drying, Cryoprotectant, *Saccharomyces boulardii*, Viability.

Introduction

Probiotics have become a popular topic in nutrition and health in recent years due to their beneficial properties. According to the World Health Organization (WHO), probiotics comprise live microorganisms that can contribute greatly to human health when consumed sufficiently [9]. They are widely used in various forms including dietary supplements, functional foods and as ingredients in animal feed. Studies have shown that probiotics boost intestinal microbiome diversity and function, reduce inflammation and enhance metabolic health[8]. Probiotics may also help to control a variety of chronic conditions including respiratory disease, metabolic syndrome and specific types of tumours [19]. Often, these beneficial microorganisms are formulated as dietary supplements in a tablet form to make them more accessible and convenient for consumers.

Nevertheless, the survival rate of these microorganisms is a major concern in the preparation of these tablet formulations. One of the important factors for success in probiotic formulation development is maintaining the high viability and sustainability of cells during the production process. Therefore, effective strategies for ensuring the stability of probiotic components must be implemented to make these tablets a viable option.

Freeze-drying is an effective way to ensure probiotics retain their therapeutic properties, even when consumed in a tablet form. This process involves solidifying a probiotic culture through freezing and then subjecting it to a low-pressure atmosphere which causes the frozen water within the culture to be directly sublimated, transforming the culture from a solid state into a vapour state [14]. This process helps to preserve probiotic cells and their activity, allowing for longer shelf life and improved final product stability. Freeze-drying method is one of the most widely used processes to produce dried powders for sensitive materials such as microbial cells. The method involves dehydration at very low temperatures that can cause damage to the cell wall of probiotic cells and can result in cell death.

This is due to the osmotic shock, oxidative stress and mechanical contraction on the cells that lead to dehydration toward the microbial cells. In short, the freeze-drying method causes cell membrane damage because of the high pressure, drying and freezing factors[16]. Therefore, the idea to apply the protective agent is essential to maintain the cell's vitality during the process. Several strategies can be used to minimise the loss of probiotics during freeze-drying including cryoprotectants. Cryoprotectants are compounds that protect biological materials such as probiotics from damage during the freezing and drying processes. These cryoprotectants function by reducing the ice's melting point,

preventing ice crystals' formation and providing osmotic protection to the cells[5].

Cryoprotectants can enhance the integrity and activity of the probiotic strains while they are being dried, resulting in a high-quality and effective end product. They can also help to maintain the stability of the cells while drying [6]. Various types of cryoprotectants can be added to improve the cell's survivability by reducing the cell death rate and cell damage. Examples of cryoprotectants include dimethyl sulphoxide, non-reducing disaccharide, skim milk, sugar alcohols, lactose, polysaccharides, glycerol, amino acids, betaine, proteins and adonitol However, the effectiveness of cryoprotectants can vary depending on the specific microorganisms, the types of cryoprotectant used and the conditions of freeze-drying.

In this study, we examined the effectiveness of cryoprotectants on the viability of selected probiotic strains during the lyophilisation process for tablet production. Several cryoprotectants were evaluated to determine the most effective one at sustaining the survivability of probiotic strains during this process. This study was crucial for the quality control and formulation of probiotic supplements to provide valuable insights into the preservation of probiotic viability during processing and storage. The findings from this study can have significant implications for the probiotic supplement industry and will contribute to the development of high-quality and effective probiotic tablet formulations.

Material and Methods

Preparation of Culture: In this study, a yeast strain known as *Saccharomyces boulardii* was utilised. The strain was obtained from a kefir drink and was previously identified as a probiotic strain through isolation and screening [3]. The strain was grown in a yeast extract peptone dextrose (YEPD) medium at 37 °C for 24 h at 150 rpm in a shake flask culture. The medium composition was 2% peptone, 2% dextrose and 1% yeast extract.

Effect of cryoprotectant on probiotic survival after freeze-drying: The cultivated yeast strain was harvested using a high-speed centrifuge for 15 min with a speed of 4,000 rpm. The strain was then added with an equal volume of cryoprotectant agents namely 2.5% sorbitol, 10% sorbitol, 20% lactose, 10% sucrose, 2% skim milk, 5% skim milk and 10% skim milk. All the components were first sterilised either using an autoclave or filter sterilisation. The suspension was subjected to freezing for 24 h at −20 °C. After that, it was freeze-dried by a vacuum freeze dryer until it totally powderise in around 20–40 h. The probiotics were lyophilised and ground into powder before being stored at 4 °C.

Cell viability measurement: Next, YEPD broth was used to rehydrate the cells following freeze-drying at room temperature for 30 min, followed by a viable colony analysis. Plate dilutions with plate count agar were used to quantify the colony-forming units per gram. The survival rate of the cell was evaluated by measuring the count of viable cells post-rehydration (Na) to the count of viable cells prior to freeze-drying (Nb). The rate was expressed as a percentage (Na/Nb × 100).

Results and Discussion

Freeze-drying or lyophilisation is an excellent method to conserve many thermally-sensitive materials such as tissues, microbes, plasma and proteins. In this study, the active *S. boulardii* cells were harvested using a high-speed centrifuge and the drying process used the freeze-dried method to transform the harvest into a powder form. In this process, the frozen solvent and water molecules were removed from the material via sublimation and desorption processes. The probiotic powder was produced using a sublimation process where the vapor was converted from the solid phase (ice) without passing across a liquid phase. The product, which was the probiotic cells, was totally frozen in a test tube or vial. Then, deep vacuum took place before heat energy was added to sublimate and vaporise the ice to produce powder.

As part of this study, different types of cryoprotectant agents were used to analyse the cells' viability after the freeze-drying process. These agents were in different concentrations which were 10% skim milk, 5% skim milk, 2% skim milk, 10% sucrose, 20% lactose, 10% sorbitol and 2.5% sorbitol. Double distilled water was used as a control. These materials are safe and cheap and are generally used in the food industry. The selected concentration range and the assessment for this individual component were chosen based on the positive impact toward the probiotic cells in the freeze-drying process[7,11,15]. As shown in figure 1, the usage of a cryoprotectant agents during the freeze-drying process was found to improve the survival rate up to 40% compared to the control which was only about 3.65%. These findings indicated that the cryoprotectant agents were highly beneficial in improving the success of the freeze-drying process.

The results of this study supported the results reported by Jalali et al[12] and Li et al[13] who found that the freeze-dried probiotic strain without the addition of excipient as a cryoprotectant showed percentages of survivability of 3% and 3.59% respectively. The usage of 10% skim milk showed significantly higher cell viability after freeze-drying process, followed by 5% skim milk, 20% lactose, 10% sucrose, 10% sorbitol, 2.5% sorbitol and 2% skim milk. Generally, the added component was attracted to the membrane of the microbial cell to develop a viscous layer surround it. Therefore, it prevented the intracellular formation of ice and prevented eutectic freezing injuries and membrane damage[4].

In this study, it was found that both types of cryoprotectant and their concentration played a significant role in the preservation of the viable state of the microorganism during freeze-drying.

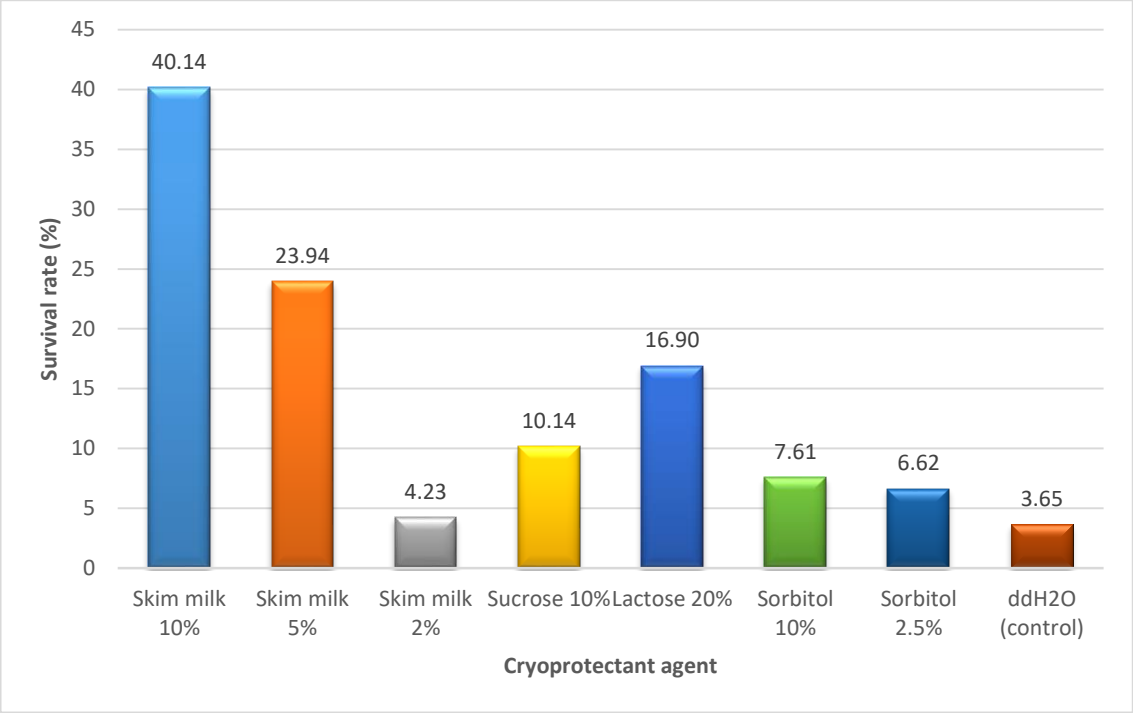

Figure 1: Survival rate after freeze-drying with different cryoprotectant agents.

Between lactose, sucrose and sorbitol, it was found that lactose was the most effective in protecting the cells while sorbitol was the least effective. The results were consistent with those reported in previous studies by Siaterlis et al[18] and Zhao et al[21]. These studies found that disaccharides such as lactose and sucrose are the best options for cryoprotection for various types of bacterial cells. The freeze-drying process causes a change in the structure of proteins. Physical changes in a membrane within a microbial cell damage the cell's biological system as a result of these changes.

Siaterlis et al also suggested that the presence of disaccharides could increase the flexibility of the lipid bilayer. This could explain the reason that the presence of disaccharides can improve the stability and functionality of proteins and enzymes[18]. With the addition of 2.5% and 10% sorbitol, the survival rate was found to be at around 6–7%, which was better than the control. The incorporation of sorbitol can elevate the balance between unsaturated and saturated fatty acids to preserve the structural integrity of the microbial cell membrane when it was freeze-dried.

Sorbitol has beneficial effects on the viability of the cell. Sorbitol can stabilise protein function and can increase membrane permeability for water during the drying process, which can help to preserve the structural integrity of the microbial cell membrane. This can lead to an increase in the survival rate of the cells after drying. However, the findings obtained in this present study seemed to indicate that the polyol, which is sorbitol, was not good enough for the preservation of *S. boulardii* for lyophilisation because the survival rate was still not significant (<30%) to maintain higher viability for the powdered cells after freeze-drying process.

Among all components, skim milk showed more positive results compared to other cryoprotectant agents added to the *S. boulardii* cells. These results were consistent with a study by of Gisela et al[10], who indicated that 24% skim milk added as a cryoprotectant significantly increased the probiotic strains' viability. Another study by FAO-WHO[9] found an optimum viability (90%) obtained for *Bifidobacterium bifidum* after the freeze-drying process by adding 25% skim milk as a cryoprotectant.

By using skim milk as a cryoprotectant, the yeast cells survived at low temperatures by up to 40%. There are two key functions of skim milk in maintaining the survival of freeze-dried cells. First, it forms a dry residue within the freeze-dried powder, thus facilitating the rehydration process by acting as a support material[18].

Secondly, it enhances the survivability of the cells by forming a protective coating around them, thereby stabilising the yeast cell membrane and preventing damage during the freezing and subsequent rehydration process[17].

Figure 1 shows that the yeast's survival rate increased with the increase of skim milk percentage. However, in this study, the maximum concentration can only be limited to 10% skim milk. This was because the skim milk would get curdled, coagulated and browned (Figure 2) after the autoclaving process if the skim milk concentration was more than 10%. Moreover, the sterile filter method cannot be used because the thickness of skim milk did not allow it to pass through the filter. Therefore, because of these limitations, the highest concentration for skim milk was only up to 10%. Normally, when heat treatment (sterilisation) is applied on milk, a Millard reaction usually occurs.

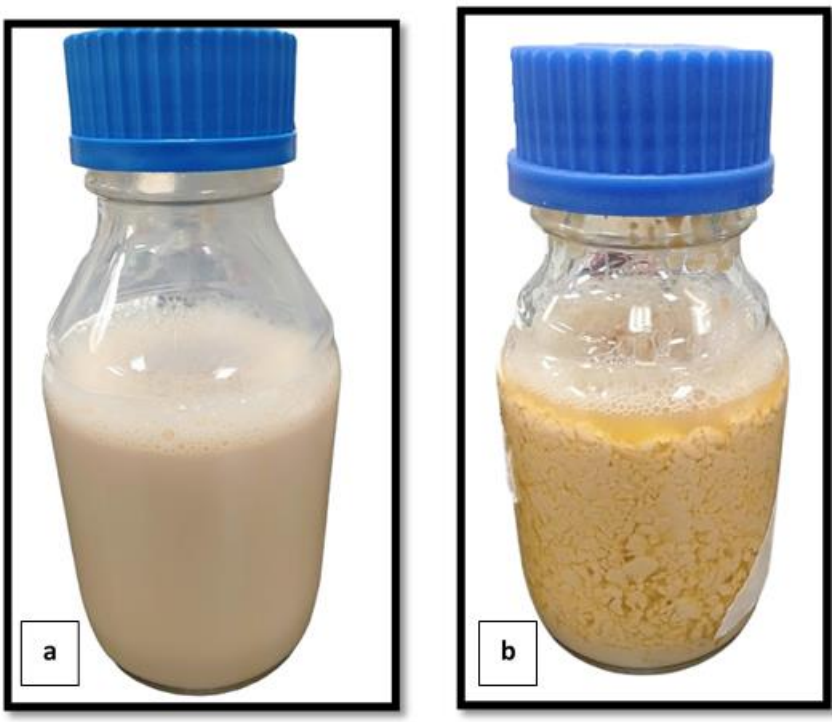

Figure 2: Skim milk solution after autoclave. (a) 10% skim milk. (b) >10% skim milk with curdled structure and browning

It is an extremely complex reaction between the carbonyl group and the amino group that falls under the nonenzymatic reaction. The present study found that when the milk was heated, coagulation occured to casein, where the reactants were mainly lysine and lactose residues[20]. Moreover, as reported by Alvarez et al[2], when the carbohydrate and protein mixture is heated under controlled water activity and below the protein denaturation temperature, the protein-polysaccharides conjugates will form by covalent bonding.

At the end of the Millard reaction, the product is generally affected physically, chemically and biologically and the reaction normally causes a change in color and flavour. Therefore, in this study, in order to avoid this kind of reaction, the skim milk concentration was limited to only 10% for autoclave sterilisation before proceeding with the cryoprotectant agent application. With the addition of 10% skim milk as a cryoprotectant agent, the *S. boulardii* can maintain a survival rate of around 40% following the freeze-drying stage with the CFU/g of around 2.85×10^{10}.

Conclusion

In conclusion, this study demonstrated the importance of using cryoprotectants in the preservation of *S. boulardii* during the freeze-drying process. Our results showed that the addition of cryoprotectant agents can significantly increase the survival rate of *S. boulardii* up to 40% compared to the control. Among the tested cryoprotectants, 10% skim milk showed the highest survival rate followed by 5% skim milk, 20% lactose and 10% sucrose. Other than that, 10% sorbitol and 2.5% sorbitol showed moderate protection to the cells, while the control with double distilled water showed the lowest survival rate.

The results also provided useful information for freeze-dried probiotics. The choice of appropriate cryoprotectants and their concentrations can thus play a crucial aspect in maintaining the survivability of probiotics during cryopreservation. In future studies, it would be interesting to investigate the effect of cryoprotectants on other probiotic strains and to evaluate the impact of different drying conditions such as pressure and temperature on the survival rate of probiotic cells. Other than that, the formulation and encapsulation of probiotics into different delivery systems can also be explored to improve their stability and shelf life.

Acknowledgement

The funding for this work was obtained from the internal grant of Universiti Malaysia Pahang Al-Sultan Abdullah (RDU220345).

References

1. Abadias M., Benabarre A., Teixidó N., Usall J. and Vias I., Effect of freeze drying and protectants on viability of the biocontrol yeast Candida sake, *Int J Food Microbiol*, **65**, 173–182 **(2001)**

2. Álvarez C., García V., Rendueles M. and Díaz M., Functional properties of isolated porcine blood proteins modified by Maillard's reaction, *Food Hydrocoll*, **28**, 267–274 **(2012)**

3. Azhar M.A. and Abdul Munaim M.S., Identification and Evaluation of Probiotic Potential in Yeast Strains Found in Kefir Drink Samples from Malaysia, *International Journal of Food Engineering*, **15**, 20180347 **(2019)**

4. Bagad M., Pande R. and Ghosh A.R., Determination of viability of Pediococcus spp. GS4 after storage Into hard gelatin capsule and

its survival under in vitro simulated gastrointestinal condition, *Int J Res Ayurveda Pharm*, **3**, 233–237 **(2012)**

5. Bhattacharya S., Cryopretectants and Their Usage in Cryopreservation Process, In Cryopreservation Biotechnology in Biomedical and Biological Sciences **(2018)**

6. Casas-Godoy L., Arellano-Plaza M., Kirchmayr M., Barrera-Martínez I. and Gschaedler-Mathis A., Preservation of non-Saccharomyces yeasts: Current technologies and challenges, *Compr Rev Food Sci Food Saf*, **20**, 3464–3503 **(2021)**

7. Chen H. et al, Optimization of composite cryoprotectant for freeze-drying Bifidobacterium bifidum BB01 by response surface methodology, *Artif Cells Nanomed Biotechnol*, **47**, 1559–1569 **(2019)**

8. Das T.K., Pradhan S., Chakrabarti S., Mondal K.C. and Ghosh K., Current status of probiotic and related health benefits, *Applied Food Research*, **2**, 100185 **(2022)**

9. FAO-WHO, Report of a joint FAO/WHO expert consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, *FAO Food Nutr Pap*, **85**, 1–4 **(2001)**

10. Gisela G. et al, Enhacement of the Viability of Lactobacillus plantarum during the Preservation and Storage Process Based on the Response Surface Methodology, *Food Nutr Sci*, **5**, 1746–1755 **(2014)**

11. Gul L.B., Con A.H. and Gul O., Storage stability and sourdough acidification kinetic of freeze-dried Lactobacillus curvatus N19 under optimized cryoprotectant formulation, *Cryobiology*, **96**, 122–129 **(2020)**

12. Jalali M. et al, Stability evaluation of freeze-dried Lactobacillus paracasei subsp. tolerance and Lactobacillus delbrueckii subsp. bulgaricus in oral capsules, *Res Pharm Sci*, **7**, 31–36 **(2012)**

13. Li B. et al, Effects of cryoprotectants on viability of Lactobacillus reuteri CICC6226, *Appl Microbiol Biotechnol*, **92**, 609–616 **(2011)**

14. Nowak D. and Jakubczyk E., The freeze-drying of foods - the characteristic of the process course and the effect of its parameters on the physical properties of food materials, *Foods*, **9**, 1488 **(2020)**

15. Romyasamit C., Saengsuwan P., Boonserm P., Thamjarongwong B. and Singkhamanan K., Optimization of cryoprotectants for freeze-dried potential probiotic Enterococcus faecalis and evaluation of its storage stability, *Drying Technology*, **40**, 2283–2292 **(2022)**

16. Sang Y. et al, Influence of Temperature during Freeze-Drying Process on the Viability of Bifidobacterium longum BB68S, *Microorganisms*, **11**, 181 **(2023)**

17. Selmer-Olsen E., Birkeland S.E. and Sørhaug T., Effect of protective solutes on leakage from and survival of immobilized Lactobacillus subjected to drying, storage and rehydration, *J Appl Microbiol*, **87**, 429–437 **(1999)**

18. Siaterlis A., Deepika G. and Charalampopoulos D., Effect of culture medium and cryoprotectants on the growth and survival of probiotic lactobacilli during freeze drying, *Lett Appl Microbiol*, **48**, 295–301 **(2009)**

19. Śliżewska K., Markowiak-Kopeć P. and Śliżewska W., The role of probiotics in cancer prevention, *Cancers* (Basel), **13**, 1–22 **(2021)**

20. Van Boekel M.A.J.S., Effect of heating on Maillard reactions in milk, *Food Chem*, **62**, 403-414 **(1998)**

21. Zhao G. and Zhang G., Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying, *J Appl Microbiol*, **99**, 333–338 **(2005)**.

(Received 28th December 2023, accepted 07th March 2024)