INFLUENCE OF PROTECTANTS ON *Lactobacillus plantarum* SUBJECTED TO FREEZE-DRYING

Vu Quynh Huong[1*], Bee May[2]

[1]Faculty of Food Science and Technology, Vietnam National University of Agriculture
[2]School of Applied Sciences, RMIT University, 124 La Trobe St, Melbourne, Victoria 3001, Australia

Email[*]: vqhuong@vnua.edu.vn

Received date: 12.04.2016 Accepted date: 10.08.2016

ABSTRACT

Lactobacillus plantarum is commonly found in many fermented food products and is an ideal candidate for the development of probiotics, which have healthy benefits for the body. The starter cultures have to be prepared to maintain their activity and stability to make use of the advantages of this species. Freeze-drying is a widely used technique for the preservation and storage of heat sensitive biological materials. However, bacterial cells can suffer from dehydration stress as water is removed. Therefore, to reduce adverse effects, protective substances can be added to samples before being freeze-dried to minimize stress associated with freeze-drying and to increase survival rate. Solutions of trehalose, lactose, trehalose + lactose, skim milk, and 2X lyophilization reagent were used as protective media for *Lactobacillus plantarum* A17 during freeze-drying. The survival rate, moisture content, and fermentation efficiency after freeze-drying were examined. The results showed that trehalose provided the highest survival rate followed by the combination of trehalose:lactose (64% and 61%, respectively). The moisture contents at the end of the freeze-drying cycle were less than 5% for all protectants tested. The efficiency of fermentation was significantly different ($P < 0.01$) between freeze-dried cells with and without protectants.

Keywords: Freeze-drying, fermentation, *Lactobacillus plantarum*, protectants, viability.

Ảnh hưởng của các chất bảo vệ đến *Lactobacillus plantarum* trong sấy thăng hoa

TÓM TẮT

Lactobacillus plantarum, được tìm thấy trong rất nhiều các sản phẩm lên men, bao gồm rất nhiều loài có hoạt tính probiotic, mang lại nhiều lợi ích về sức khỏe cho cơ thể con người. Sấy thăng hoa là một kỹ thuật được sử dụng rộng rãi để bảo quản và lưu trữ các vật liệu sinh học nhạy cảm với nhiệt. Tuy nhiên, các tế bào vi khuẩn có thể bị tổn thất khi tiến hành quá trình loại nước khi sấy. Vì vậy, để giảm bớt tác hại không mong muốn, các chất bảo vệ được thêm vào mẫu trước khi sấy thăng hoa để giảm thiểu tổn thất và làm tăng tỷ lệ sống của vi khuẩn sau khi sấy. Các dung dịch trehalose, lactose, trehalose + lactose, sữa gầy và 2X lyophilization được sử dụng để bảo vệ cho *Lactobacillus plantarum* A17 trong quá trình sấy thăng hoa. Tỷ lệ sống, độ ẩm và hiệu quả lên men sau khi sấy thăng hoa đã được nghiên cứu. Kết quả cho thấy sử dụng trehalose làm chất bảo vệ thì tỷ lệ sống của *Lactobacillus plantarum* là cao nhất, tiếp theo là hỗn hợp của trehalose và lactose (lần lượt là 64% và 61%). Độ ẩm vào cuối quá trình sấy thăng hoa là dưới 5% cho tất cả các mẫu có chứa chất bảo vệ. Hiệu quả của quá trình lên men đã có sự khác biệt đáng kể ($P < 0,01$) giữa các tế bào sấy thăng hoa có và không có chất bảo vệ.

Từ khoá: Chất bảo vệ, *Lactobacillus plantarum*, sấy thăng hoa, sự lên men, tỷ lệ sống.

1. INTRODUCTION

Lactobacillus plantarum, in general, is known as a type of probiotic that can be beneficial in the human body by, for instance, protecting the body against pathogenic microorganisms and helping the body fight diseases. *Lactobacillus plantarum* is used in many fermented foods such as yogurt, cheese, and instant fruit powder (Nualkaekul *et al.*, 2012). In order to make use of the advantages of this type of lactic acid bacteria, the starter

cultures have to be prepared to maintain their activity and stability.

Freeze-drying is the most convenient and successful method for the preservation and storage of heat sensitive biological materials such as bacteria, yeast, and fungi (Berny and Hennebert, 1991). Miao *et al.* (2008) stated that freeze-drying is especially attractive because it results in the production of a powder with a desired appearance, high specific surface area, and therefore, a fast re-hydration rate. Moreover, many advantages of freeze-drying, including protection of bacteria from contamination or infestation during storage, long viability, and ease of strain distribution, are reported by Passot *et al.* (2011).

However, bacteria cells can suffer from dehydration stress as water is removed during freeze-drying. Therefore, to reduce the adverse effects and to increase survival rate, protective substances can be added before freeze-drying. In the study of Hubalek (2003), protectants helped retain cellular viability during freeze-drying and increased the efficiency of bacteria to carry out fermentation. He also showed that trehalose, lactose, and skim milk are commonly used as effective protectants for bacteria, yeast, and mold.

The present research aimed to determine the effect of trehalose, lactose, skim milk, the combination of trehalose:lactose (Tre:Lac), and lyophilisation reagent (2X) on the survival rate and fermentation efficiency of freeze-dried *L. plantarum* A17.

2. MATERIALS AND METHODS

2.1. Materials

Trehalose and lactose monohydrate (L3625) were obtained from Sigma-Aldrich, Australia. Lyophilisation reagent (2X) was obtained from OPS Diagnostics, LLC. Other chemicals utilized in this study were of analytical grade. deMan Rogosa Sharpe (MRS) agar and broth were obtained from Oxoid, Australia. Unless otherwise stated, deionized water (Milli-Q system QGARD00R1, Millipore, Australia) was used in all experiments.

2.2. Microorganism

The test strain of *Lactobacillus plantarum* A17 was received from the culture collection of the Microbiology Laboratory, RMIT. It was maintained frozen at -80^{0}C in MRS Broth (Oxoid, Australia) with 40% (v/v) glycerol. The bacterial cells were grown in MRS broth at 30^{0}C (De Man *et al.*, 1960).

2.3. Methods

2.3.1. Preparation of bacterial cells

One colony of each of the working cultures was grown in different MRS broths (5 ml) for 24 h at 30°C. Cell suspensions (2% v/v) were re-grown in freshly prepared MRS broths at 30°C for another 17 h to reach the end of the growth phase. The actively growing cells were harvested under aseptic conditions by centrifugation at 4.000 g for 10 min followed by washing with 0.85% saline water. The washed cell pellets served as the seed culture for microencapsulation.

2.3.2. Preparation of protectant solutions

Lactose and trehalose solutions were prepared by adding 10 g of each type into 90 g of water, which had been sterilized. In addition, a mixture of Tre:Lac in a ratio of 9:1 was prepared by dissolving 9 g of trehalose in 90 g of water, adding 1 g of lactose powder and mixing well, and then the solution was autoclaved at 121^{0}C for 15 min. The skim milk solution was prepared at the concentration of 8.8% (w/w). A commercial protectant, lyophilisation reagent (2X) (OPS Diagnostics, LLC), and distilled water were used as control media for comparison.

2.3.3. Freeze-drying procedure

Each of the strains of seed culture harvested in 2.3.1 was mixed with 1 mL protectant solution at room temperature (approximately 23°C) for half an hour prior to freeze-drying. Each 1 mL resuspension was transferred into a sterilized McConkey bottle, and freeze-dried for 24 hours in a freeze-dryer (FreeZone Triad Freeze dry system, Labconco). The program was modified based on the methods of Tymczyszyn *et al.* (2012), which

involved the steps (i) pre-freezing for 3 hours, (ii) primary drying with ramping temperature at 2°C/min down to -15°C and holding for 16 hours, and (iii) secondary drying with ramping at the same rate of 2°C/min up to 20°C and holding for 3 hours.

2.3.4. Bacterial plate counts

Viable counts of cells were determined before freeze-drying and immediately after freeze-drying (zero time) by the spread plate method in duplicate using MRS agar medium. Bacterial cell count was enumerated by taking 1 ml of cell suspension in the feed solution prior to freeze-drying. After serial dilutions, 0.1 ml was transferred and plated on MRS agar and incubated at 30°C for 48 h. Cell counts before freeze-drying were calculated as CFU g^{-1} of dried matter based on the initial total solids content of the feed solution before freeze-drying.

Similarly, 0.1 g of freeze-dried powder was dissolved in peptone water, allowing 20-30 min for it to dissolve, followed by serial dilutions and plating. The bacterial count was expressed as CFU g^{-1} of dried powder and cell survival.

Calculations were done according to Australian Standards: AS 5013.1 (2004) using the below equation:

Number of colony forming units per mL = (N1 + N2) / v (n1 + n2 x v), wherein:

N1 (factor of first dilution), N2 (factor of second dilution), n1 (number of spreading plates for first dilution), n2 (number of spreading plates for second dilution), v (volume taken from sample for spreading).

The survival rate after freeze-drying was calculated as:

Percentage of survival rate (viability) = (N_f/N_0) x 100, where in:

N_0 and N_f are the survival rates before and after freeze-drying, respectively.

2.3.5. Moisture content determination

Moisture content of the freeze-dried samples was analyzed using a moisture analyzer MB45 (Ohuas Corporation, USA) with the standard method of moisture content analysis (Ohaus Corporation, 2011) by spreading the sample (approximately 1 g) on an aluminum pan and heating it up to a temperature of 105°C and holding the temperature until mass changes of less than 1 mg for 90 s were achieved.

2.3.6. Fermentation efficiency determination

After freeze-drying, 2% of samples were added to 5 ml MRS broth and incubated at 30°C. pH was measured every 2 hours for 30 hours using a pH meter.

2.3.7. Statistical analysis

All experiments were carried out in duplicate and the standard deviations calculated. Analysis of variance (ANOVA) was used to test data between treatments using Minitab 16 Software, State College, PA Inc. Comparison of means by Tukey methods was tested, and a p value of less than 0.05 was considered as statistically significant.

3. RESULTS AND DISCUSSION

3.1. Effect of protectants on the viability of bacteria after freeze-drying

The effective utilization of probiotic bacteria for functional food products depends on the ability to produce concentrated preparations of the probiotic culture that can resist the harsh conditions experienced during processing, and remain viable during storage of the product. Recently, live cultures in powder form have become an appealing option, however, maintaining viability after processing can be challenging. Freeze-drying is considered a suitable method for producing powders of biological materials because drying is carried out at low temperatures, reducing chemical reaction proportions and heat degradation. This study investigated the role of protectants such as trehalose, lactose, trehalose + lactose, skim milk, and Lyophilization 2X as potential cryoprotective additives during the freeze-drying process.

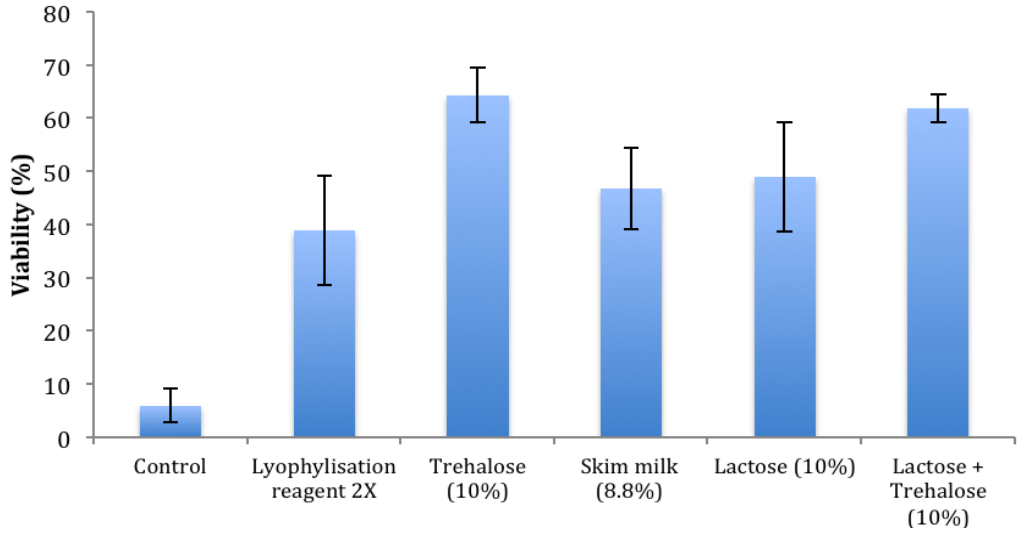

Figure 1. Effect of protectants on viability of bacteria after freeze-drying

Note: Control: no protectant

Figure 1 shows the percentage of viability of *Lactobacillus plantarum* A17 bacteria with and without protectants after freeze-drying.

The survival rate of *L. plantarum* A17 bacteria after freeze-drying when using protectants was extremely higher than without protectants (6%). The viability obtained was similar to the results of Zayed and Roos (2004) who found that the survival rate of *Lactobacillus salivarius* subsp. *salivarius* was very low (4%) when it was suspended in only water. Champagne and Gardner (2001) reported that without protectant, streptococci cells in alginate could not survive well after being freeze-dried.

From the results of the statistical analyses, there was a significant difference between samples with and without protectant. Many studies have shown the different impacts of using protective agents on bacteria. Castro *et al.* (1995) stated that protective agents could be used to stabilize the cell membrane components to avoid cell damage.

The presence of skim milk increased the viability rate to approximately 46.8%. According to Zayed and Roos (2004), two components in skim milk, proteins and calcium, can cover the cell wall proteins to protect as well as increase the viability of bacteria.

The addition of trehalose and the combination of Tre:Lac did not significantly affect ($P>0.05$) the viability of *L. plantarum* A17 when compared with lactose and skim milk on the survival of the bacteria. However, as can be seen from the results, trehalose and the mixture of Tre:Lac solution gave the highest viability (64% and 61%, respectively). The effectiveness of trehalose as a protectant has been observed in many studies. Trehalose was identified as a carbohydrate reserve (Benaroud *et al.*, 2001) and it was shown that it could prevent cell damage during freezing or freeze-drying of *Lactobacillus salivarius* subsp. *salivarius* by Zayed and Roos (2004). Reder-Christ *et al.* (2013) stated that trehalose can be used for the freeze-drying of proteins because it can prevent fusion and phase transitions. In addition, the protective ability of trehalose is better than lactose because of the difference between these two sugars. Trehalose, a non-reducing sugar, cannot undergo the browning reaction that causes denaturation of proteins, hence it is preferred for use as a protectant in freeze-drying (Elbein *et al.*, 2003; Jain and Roy, 2009). The difference in the survival rate between trehalose and Tre:Lac was not significant because the ratio of lactose to trehalose was too little (1:9). However, the partial replacement of trehalose by lactose could reduce the cost of the protectant.

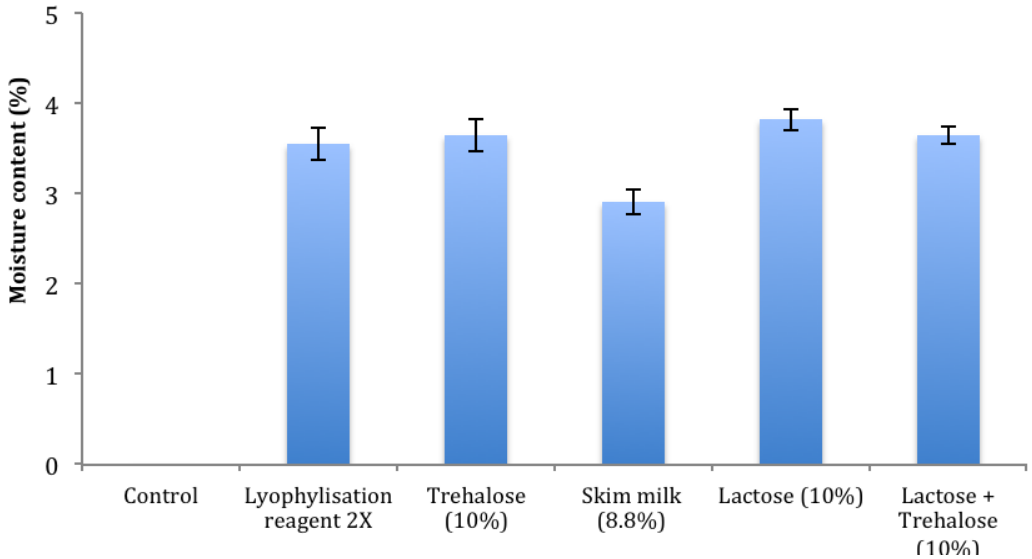

Figure 2. Effect of protectants on moisture content after freeze-drying

Note: Control: no protectant

3.2. Effect of protectants on moisture content after freeze-drying

The moisture content of products after freeze-drying affects the viability of bacteria as well as the rate of loss of viability during subsequent storage. Hence, a moisture content measurement was carried out and the results are shown in Figure 2.

Trelea *et al.* (2007) stated that a quality requirement of the final freeze-dried product is to reach a pre-specified residual moisture content, both under- and over drying results in damage. Therefore, if the moisture content of a sample, which only had added water before freeze-drying, was too low, it indicated a high injury level in the cell membranes of bacteria. In the literature, a variety of different critical moisture contents have been described, such as Jouppila and Roos (1994) who referred to a critical moisture content of dried milk powder of 7% for storage stability at 25°C based on the calculated glass transition temperature value. Zayed and Roos (2004) examined the effect of water content on the survival of bacteria in a mixture of skimmed milk, trehalose, and sucrose, and reported enhanced survival during storage for moisture contents within the range of 2.8-5.6%. As can be seen in Figure 2, the moisture contents at the end of the freeze-drying cycle were less than 5% for all protectants tested. There was a significant difference in the moisture content of freeze-dried cells with and without protectants ($P < 0.01$). Our results are similar to previous studies as well as the viability rate of freeze-dried cells with and without protectants presented in Section 3.2.1.

The comparison of different protectants in moisture content after freeze-drying showed that skim milk gave the lowest moisture content (2.9%) and it was significantly different with other protectants. The reduction in moisture content with skim milk may be due to the higher water content of the suspending medium. During freeze-drying, water was removed and hence, moisture content decreased.

3.3. Effect of protectants on fermentation efficiency

The effectiveness of protectants on protecting bacterial cells was also expressed through fermentation efficiency. The pH reduction by time is showed in Figure 3.

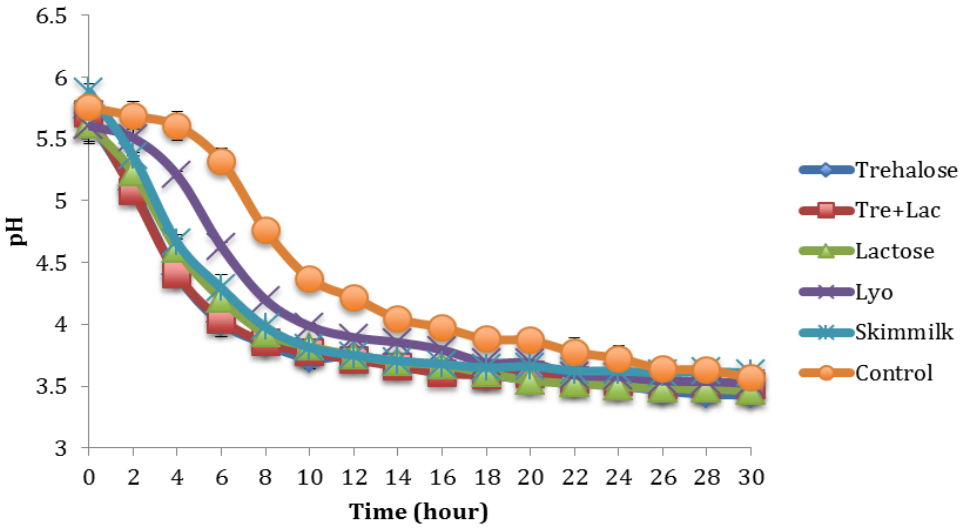

Figure 3. Effect of protectants on pH reduction during fermentation

Figure 3 indicates that during the first 12 hours, the pH of MRS broth solutions decreased rapidly in samples that had added protectants before freeze-drying. Meanwhile, the pH reduction of the sample that contained only bacteria and water prior freeze-drying was slow. The results also show that the efficiency of fermentation was significantly different ($P < 0.01$) between freeze-dried cells with and without protectants. This result is in accordance with the outcome found in the viability experiment. When the bacteria are damaged or have died, they cannot be active and as a consequence, they cannot ferment as well as strong bacteria. The study of Hedberg *et al.* (2008) also found that the fermentation ability of *L. plantarum* with the presence of trehalose and lactose was very good. In addition, it can be seen from the results that the fermentation efficiency of the sample containing Lyophilization 2X was lower than other protectants. This is also shown by the lower viability rate after freeze-drying of *L. plantarum* with this type of commercial protectant.

4. CONCLUSIONS

In the context of the current investigation, a good understanding of bacterial interaction with the encapsulation matrix is crucial. These preliminary results show that the survival of the bacterial strain tested could be affected by the addition of protectants before freeze-drying. Trehalose (10%w/w) and the mixture of Tre:Lac are suitable protectants to create appropriate moisture content as well as to enhance the efficiency of fermentation of *L. plantarum* A17 after freeze-drying.

ACKNOWLEDGEMENTS

Gratitude is expressed to the School of Applied Sciences, RMIT University, Australia for supporting this study and to the Australia Award Scholarship for supporting Vu Quynh Huong.

REFERENCES

Australian Standard: AS 5013.1 (2004). Food microbiology. Method 1: Examination for specific organisms-Standard plate count. Retrieved from http://www.saiglobal.com

Benaroudj, N., D. H. Lee, and A. L. Goldberg (2001). Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. Journal of Biological Chemistry, 276(26): 24261-24267.

Berny, J. F., and G. L. Hennebert (1991). Viability and stability of yeast cells and filamentous fungus spores during freeze-drying: effects of protectants and cooling rates. Mycologia, pp. 805-815.

Castro, H. P., P. M. Teixeira, and R. Kirby (1995). Storage of lyophilized cultures of *Lactobacillus bulgaricus* under different relative humidities and atmospheres. Applied Microbiology and Biotechnology, 44(1-2): 172-176.

Champagne, C. P., and N. J. Gardner (2001). The effect of protective ingredients on the survival of immobilized cells of *Streptococcus thermophilus* to air and freeze-drying. Electronic Journal of Biotechnology, 4(3): 7-8.

De Man, J. C., D. Rogosa, and M. E. Sharpe (1960). A medium for the cultivation of lactobacilli. Journal of applied Bacteriology, 23(1): 130-135.

Elbein, A. D., Pan, Y. T., Pastuszak, I., and Carroll, D. (2003). New insights on trehalose: a multifunctional molecule. Glycobiology, 13(4): 17R-27R.

Hubalek, Z. (2003). Protectants used in the cryopreservation of microorganisms. Cryobiology, 46(3): 205-229.

Hedberg, M., P. Hasslöf, I. Sjöström, S. Twetman, and C. Stecksén-Blicks (2008). Sugar fermentation in probiotic bacteria-an in vitro study. Oral Microbiology and Immunology, 23(6): 482-485.

Jain, N. K. and I. Roy (2009). Effect of trehalose on protein structure. Protein Science, 18(1): 24-36.

Jouppila, K. and Y. H. Roos (1994). Glass transitions and crystallisation in milk powder. Journal of Dairy Science, 77: 2907-2915.

King, V. E. and J. T. Su (1994). Dehydration of Lactobacillus acidophilus. Process Biochemistry, 28(1): 47-52.

Miao, S., S. Mills, C. Stanton, G. F. Fitzgerald, Y. Roos, and R. P. Ross (2008). Effect of disaccharides on survival during storage of freeze-dried probiotics. Dairy Science and Technology, 88(1): 19-30.

Nualkaekul, S., G. Deepika, and D. Charalampopoulos (2012). Survival of freeze dried *Lactobacillus plantarum* in instant fruit powders and reconstituted fruit juices. Food Research International, 48: 627-633.

Ohaus Corporation. (2011). Instruction manual MB45 moisture analyser. Accessed on 15 July 2013 from <http://www.scalenet.com/pdf/Ohaus_MB45_Moisture_Balance_Manual. pdf./>

Passot, S., F. Fonseca, S. Cenard, I. Douania, and I. C. Trelea (2011). Quality degradation of lactic acid bacteria during the freeze drying process: Experimental study and mathematical modeling. *In*: 11th International Congress on Engineering and Food 11th ICEF 2011 May 22-26, 2011 Athens - Greece "Food Process Engineering in a Changing World". Presented at ICEF 11, International Congress of Engineering and Food, Athens. Accessed on 20 June 2014 from <http://prodinra.inra.fr/record/355611>

Reder-Christ, K., P. Schmitz, M. Bota, U. Gerber, H. Falkenstein-Paul, C. Fuss,... and G. Bendas (2013). A dry membrane protection technique to allow surface acoustic wave biosensor measurements of biological model membrane approaches. Sensors, 13(9): 12392-12405.

Trelea, I. C., S. Passot, F. Fonseca, and M. Marin (2007). An interactive tool for the optimization of freeze-drying cycles based on quality criteria. Drying Technology, 25(5): 741-751.

Tymczyszyn, E. E., N. Sosa, E. Gerbino, A. Hugo, A. Gómez-Zavaglia, and C. Schebor (2012). Effect of physical properties on the stability of *Lactobacillus bulgaricus* in a freeze-dried galacto-oligosaccharides matrix. International Journal of Food Microbiology, 155(3): 217-221.

Zayed, G., and Y. H. Roos (2004). Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. Process Biochemistry, 39(9): 1081-1086.