



Investigation of cryoprotection on the viability of freeze dried probiotics

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ABSTRACT

Aims: The aim of the present work was to evaluate the effect of various cryoprotective agents on the survival of freeze-dried probiotic *Lactobacillus rhamnosus*.

Methodology and results: Investigation was done on the viability and stability of probiotics *L. rhamnosus*. The effect of different cryoprotective agents (namely, sodium chloride, sucrose, dextran, sorbitol, monosodium glutamate, glycerol, skim milk and skim milk with malt extract) with modified De-Man Rogosa Sharpe (MMRS) medium were examined. Commercial De-Man Rogosa Sharpe (MRS) medium was proved to be more expensive than the modified MRS medium with relatively low yield of probiotics *L. rhamnosus*. Significantly high viable counts were achieved with monosodium glutamate, skim milk and skim milk with malt extract, with optimum concentration at 0.3% w/v. There was a reduction in cell viability at concentration above 0.5% w/v, which could be attributed to cell shrinkage associated with osmotic pressure changes. The cells were found to be stable at room temperature (28 °C) for eight weeks. A significant growth of probiotics was produced from skim milk.

Conclusions: Modified MRS medium with skim milk is suggested for the remarkable growth and yield of probiotic lactobacilli.

Keywords: Cryoprotective agents, De-Man Rogosa Sharpe (MRS), *Lactobacilli*, probiotics, viability

INTRODUCTION

The uses of probiotics in biotechnological applications have attracted much attention due to their natural occurrence in the human gastrointestinal system. Probiotics are important in the dairy and nutraceutical industries due to its application in the maintenance of human and animal health (Cencic and Chingwaru, 2010). However, these beneficial microbes were losing viability or activity during the preservation process (Dianawati *et al.*, 2016). To avoid cellular damage during cryopreservation and subsequent thawing, a wide array of cryoprotective agents has been applied (Kerckhof *et al.*, 2014). The major factors of injury from drying of bacterial cells are probably due to osmotic shock with membrane damage and the removal of bound water which affects the properties of many hydrophilic macromolecules in cells (Selmer-Olsen *et al.*, 1999). Shu *et al.* (2015) reported that, a good cryoprotective agent should provide protection and efficient drying to the cells during the freeze drying process. It also provides a good matrix to allow stability and ease of rehydration.

Cryoprotectants such as skim milk, whey protein, trehalose, glycerol, betaine, adonitol, sucrose, glucose, lactose and polymers may be added during the process of freeze-drying lactobacilli species, these prevent

inactivation of the cells during drying and stabilize the microorganisms during storage (Jalali *et al.*, 2012).

Probiotic lactic acid bacteria (PLAB) are heterotrophic and fastidious with complex nutritional requirements. De-Man, Rogosa and Sharpe (MRS) (De Man *et al.*, 1960), medium is commonly used for cultivation of lactobacilli species (Sathyanarayanan *et al.*, 2011). The factors to consider in the choice of growth medium ability to produce large number of cells and are costs. These growth factors are usually supplied by a complex nitrogen source like yeast extract. Thus, there is a requirement of an inexpensive and easy to prepare media to grow lactic acid bacteria with maximum yield (Monteagudo *et al.*, 1993).

The aim of the present work was to evaluate the effect of various cryoprotective agents on the survival of freeze-dried probiotic *Lactobacillus rhamnosus*. In addition, the stability of freeze-dried probiotic *L. rhamnosus* powder at different temperatures was studied by determining the total viable count for a period of eight weeks.

MATERIALS AND METHODS

Bacterial culture and growth conditions

Probiotics culture was stabbed in De-Man Rogosa Sharpe Agar (MRSA) medium (Difco, USA) and stored at 4 °C.

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The strain was activated by two subcultures in 100 mL MRS broth prior to experimental use.

Preparation of modified MRS medium

The modified MRS broth medium was prepared using 20.0 g dextrose (R and M Chemicals, UK), 8.0 g Bacteriological peptone (Oxoid, UK), 8.0 g meat extract (Fluka, Germany), 4.0 g yeast extract (Fluka, Germany), 5.0 g sodium acetate (AJAX Chemicals, Sydney, Australia), 2.0 g disodium phosphate (Sigma, UK), 2.0 g ammonium citrate (Oxoid, UK), 1.0 g Tween 80 (Sigma, UK), 0.1 g magnesium sulfate (Sigma, UK) and 0.05 g manganese sulfate (Fluka, Germany) in 1 liter of distilled water. The mixture was boiled until the ingredients were completely dissolved. The pH of the mixture was adjusted with 1 M HCl to 6.0±0.1. The mixture was autoclaved at 121 °C for 15 min and cooled to 45 °C prior to use.

In addition, the modified MRS agar medium was prepared similar to that described for the modified MRS broth medium but with the addition of 15.0 g agar (Becton, Dickinson and Company, USA) per liter of distilled water.

Preparation of cryoprotective solutions

The cryoprotective solutions of 0.1% w/v were prepared in sterile distilled water and sterilized (Zayed and Roos, 2004). In addition, monosodium glutamate, skim milk and skim milk with malt extract at concentrations of 0.1 - 0.7% w/v were also prepared.

Cultivation of probiotic lactobacilli and freeze dried with cryoprotective solutions

Probiotic lactobacilli were cultivated in modified MRS medium for 36 h at 37 °C. After incubation, 20 mL of the medium was centrifuged at 3500 rpm for 10 min at 4 °C (Beckman J-6M/E, USA). Free-cell concentration was estimated by the pour plate method on modified MRS agar, after aerobic incubation at 37 °C for 36 h. After centrifugation, the pellet (cell biomass) was collected and washed with Ringer solution (Merck, Germany) twice. Each time, Ringer solution was discarded after centrifugation. The cell pellet was mixed with 0.02 mL distilled water or various cryoprotective solutions before freeze drying (Labconco Lyph Lock 6 Freezer Dryer, Shell Freeze System, USA).

Moisture content measurement

The moisture content was measured using moisture analyzer (Mettler Toledo Delta range, PM 480, Switzerland). Five grams of the freeze dried powder was measured for 10 min at 105 °C.

Determination of total viable count

The freeze-dried powder of 1.0 g was rehydrated separately by mixing with the original volume (20 mL) of modified MRS broth medium and then incubated at 25 °C

for 10 min (Sinha *et al.*, 1982). After serial dilution, probiotic lactobacilli was incubated aerobically at 37±1 °C for 72 h in modified MRS agar medium and the viable count was determined by pour plate method (Hekmat and McMahon, 1992).

Determination of percentage of loss in viable count

The percentage of loss in the viable counts of probiotic lactobacilli after freeze-drying with various cryoprotective agents was calculated using the following equation:

$$\text{Loss of the cells} = \frac{N_0 - N}{N_0} \times 100 \%$$

N_0 = Number of viable cells before freeze-drying.

N = Number of viable cells after freeze-drying

Stability evaluation of freeze-dried strains of probiotic lactobacilli

The freeze-dried probiotic lactobacilli were stored at 4 °C, 28 °C and 40 °C. Every 7th day, the powder was analyzed separately for 8 weeks. The stability of the powder was assessed by obtaining the total viable count.

Statistical analysis

All data are presented as mean ± standard deviation. Comparison of means of modified MRS and commercial MRS were analyzed by Student's t-test. One-way analysis of variance was used for statistical analysis (version 15.0, SPSS Inc. Software, USA), with a significance level at $P < 0.05$. When there was a statistically significant difference, post-hoc Tukey-HSD test was performed. The experiments were repeated three times.

RESULTS AND DISCUSSION

One of the methods which can be used as a dehydration process for bacteria in producing dry solid formulation is freeze-drying. The impact of modified MRS medium and cryoprotective agents to the viability of probiotics *L. rhamnosus* after freeze-drying, was studied in this work. The determination of total viable count in eight weeks times were performed by estimating the stability of freeze-dried probiotics *L. rhamnosus* powder at different temperature, and also estimating the influence of initial cell concentration at both strains.

Modified MRS medium

A better MRS medium than the commercial one has been resulted in this study. The modified MRS medium was found to be more economical because of less amount of nitrogen sources needed for the fermentation. In addition, the modified medium also resulted in higher yield of probiotics than the commercial MRS medium after incubation, as shown in Figure 1 ($P < 0.05$). Therefore, it is compulsory to select a raw material for industrial

production of probiotics with a number of characteristics such as low cost, rapid rate of fermentation, lowest amount of contaminants and high yields production (Ghaffar *et al.*, 2014). Schnierda *et al.* (2014) found that the selection and optimization of carbon and nitrogen sources are the critical steps in the development of low-cost fermentation process. Yeast extract and peptone have significant influence on the bacterial cell wall, as witnessed by changes in surface charge, hydrophobicity, and the nitrogen-to-carbon ratio (Schär-Zammaretti *et al.*, 2005). However, yeast extract is expensive if it used in large quantities. Many of studies being cited above concluded that peptone and yeast extract were important nitrogen sources in the probiotics production, i.e. improving growth production and decreasing incubation period. In the current study, although the concentration of peptone, yeast extract, and meat extract were decreased to 20%, the growth of probiotics was better than commercial MRS medium.

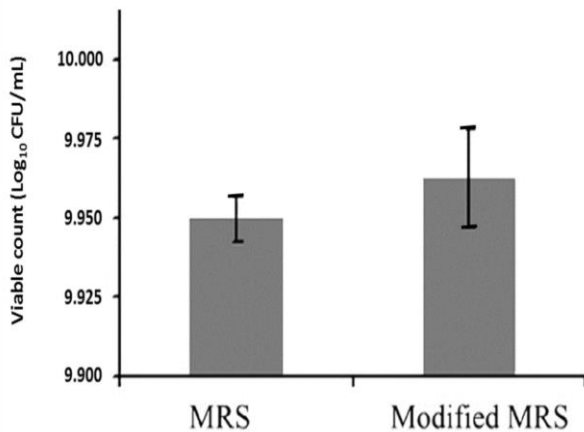


Figure 1: Viability comparison between MRS medium and modified MRS medium.

Moisture content study

One of the important parameters for the stability of dried cultures is water content (Peiren *et al.*, 2015). The microorganisms survive better at low-water activity, while the increasing of water activity decreased its viability (Kurtmann *et al.*, 2009). Figure 2 shows the results of moisture content values. The moisture content values of freeze-dried probiotics in the different presence of cryoprotective agents were between 1.88% and 1.99%. Ekdawi-Sever *et al.* (2003) reported that the moisture content in the range of 1.0 - 4.0% was acceptable for freeze-dried probiotics. Previously, Gardiner *et al.* (2000) found that the moisture content up to 4.0% was acceptable for freeze-dried probiotic *L. rhamnosus*.

Recovery and viability of freeze-dried probiotic lactobacilli

Another important process in the recovery of free freeze-dried microorganisms is rehydration. During rehydration,

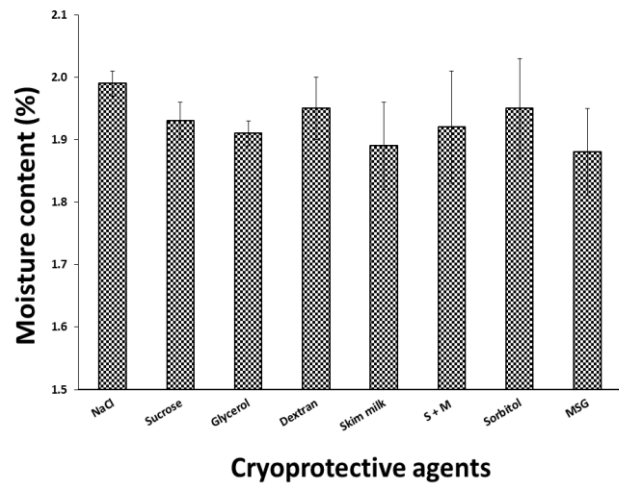


Figure 2: Moisture content of freeze-dried probiotic *Lactobacillus* species with different cryoprotective agents. Mean \pm SD, N=3. S+M, Skim milk with malt extract; MSG, Monosodium glutamate.

the viability of organism might decrease. Inadequate rehydration procedure might be resulted from poor recovery of cells. Therefore, rehydration is a critical step in the recovery of freeze-dried microorganisms, because cells that were subjected to sub lethal injury may not be able to repair said damage if they are rehydrated under inappropriate conditions (Sharma *et al.*, 2014). Wang and colleagues (2004) showed that the recovery of probiotics *Lactobacillus acidophilus* was improved by a higher rehydration temperature of 30-40 °C. De Valdez *et al.* (1985) found that the optimum rehydration temperature for freeze-dried lactic acid bacteria was 20 °C. In the present study, the optimum rehydration temperature was 25 °C.

Figure 3 shows the viable count of freeze-dried probiotic lactobacilli at different cryoprotective agents. Therefore, the presence of cryoprotective agents is suggested to improve the viability of the cells ($p < 0.05$). Monosodium glutamate, skim milk and skim milk with malt extract provided significantly higher viable counts than other cryoprotective agents ($p < 0.05$). Due to the low cost, safety and beneficial properties, the skim milk was preferred over monosodium glutamate and skim milk with malt extract. Khoramnia *et al.* (2011) investigated the usage of skim milk as cryoprotective agent on the survival rate of a probiotic *Lactobacillus* species during freeze-drying and storage. Adding cryoprotective agents such as monosodium glutamate before freeze-drying process attenuated the damaging effects of freezing, thus improving the bacterial resistance to drying (Coulibaly *et al.*, 2010).

Figure 4 shows the percentage of loss results in the viable counts of after freeze-drying with various cryoprotective agents. Freeze-dried probiotic lactobacilli without cryoprotective agents were lost 90% of their viability after freeze-drying. This is in agreement with the previous studies (Mitropoulou *et al.*, 2013), that found the

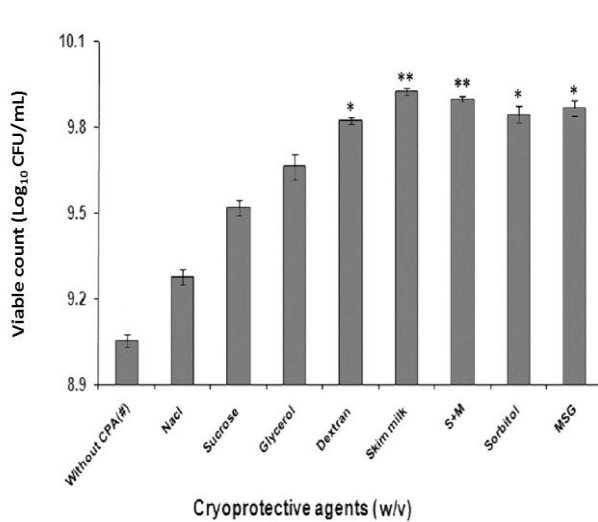


Figure 3: Effect of cryoprotective agents on the viability of probiotic *Lactobacillus* species. Mean \pm SD, N=3. Without CPA#, no cryoprotective agents; S+M, Skim milk with malt extract; MSG, Monosodium glutamate; **, significant when compared to the control ($P < 0.01$); *, significant when compared to the control ($P < 0.05$).

entrapment of probiotic cells without protective solution decreased the viability in freeze-drying. The decreasing of cell's viability without any cryoprotective agent is due to cell injury at several target sites, i.e. cell wall, cell membrane and DNA (Teixeira *et al.*, 1995), and also due to membrane lipid oxidation (Castro *et al.*, 1997). The primary cell damage in freeze-drying was suggested due to ice crystal formation, high osmolarity due to high concentrations of internal solutes with membrane damage, macromolecule denaturation and the removal of water that affected the properties of many hydrophilic macromolecules in the cells (Thammavongs *et al.*, 1996). Whilst in other report, cryoprotective agents could promote cell survival during freezing or drying process (Pehkonen *et al.*, 2008; Coulibaly *et al.*, 2010). The cryoprotective agents have two main functions in preserving the viability of freeze-dried cells: to provide a dry residue with definite physical structure acting as a support material and as a receptor in rehydration; and to protect the living cells against damage during freezing or drying (Larena *et al.*, 2003).

Storage stability of freeze-dried probiotic lactobacilli

One of the most important aspects of the freeze-dried probiotic productions is the stability (Chávarri *et al.*, 2012). The improvement of probiotic strain stability during storage is required in the functional food industry, especially at room temperature storage (Savini *et al.*, 2010). It was reported that the storage condition critically affected the viability and stability of freeze-dried cells (Yonekura *et al.*, 2014). The results showed that probiotic

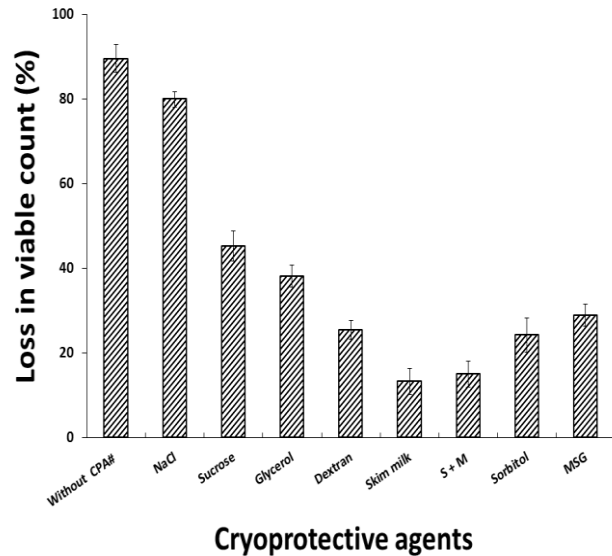


Figure 4: Loss in viable counts (%) of probiotic *Lactobacillus* species after freeze-drying. Mean \pm SD, N=3. Without CPA#, no cryoprotective agents; S+M, Skim milk with malt extract; MSG, Monosodium glutamate. Means with different uppercase letters are significantly different ($P < 0.05$).

lactobacilli were stable at 4 °C and 28 °C until eight weeks' time. After that period of time, a negligible decrease was observed in the viable counts. The viable counts were reduced to approximately 75% at the end of eight weeks when the probiotic lactobacilli stored at 40 °C.

CONCLUSION

The present study showed that the modified MRS medium produced higher yield of probiotics than the commercial one, thus resulted in better economic value without compromising the medium's quality in terms of viability and stability of the bacteria. The usage of cryoprotective agents showed improvement in the cell viability of probiotics *L. rhamnosus* significantly. In the current study, monosodium glutamate, skim milk and skim milk with malt extract combination with optimum concentration of 0.3% were the best cryoprotective agents that showed high viable counts. The probiotics *L. rhamnosus* was stable for eight weeks at 28 °C. Nevertheless, skim milk powder was selected as the most preferable cryoprotective agents due to its low cost, good availability, well-functioned, and also good nutritional value.

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REFERENCES

- Castro, H., Teixeira, P. and Kirby, R. (1997). Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze drying. *Journal of Applied Microbiology* **82**, 87-94.
- Cencic, A. and Chingwaru, W. (2010). The role of functional foods, nutraceuticals, and food supplements in intestinal health. *Nutrients* **2**, 611-625.
- Chávarri, M., Marañón, I. and Villarán, M. (2012). Encapsulation technology to protect probiotic bacteria. In: Probiotics. Rigobelo, E. (ed.). InTech Open Access Publisher, Croatia. pp. 501-540.
- Coulibaly, I., Dubois-Dauphin, R., Destain, J., Fauconnier, M., Lognay, G. and Thonart, P. (2010). The resistance to freeze-drying and to storage was determined as the cellular ability to recover its survival rate and acidification activity. *International Journal of Microbiology* **2010**, ID 625239.
- De Man, J., Rogosa, M. and Sharpe, M. (1960). A medium for the cultivation of *Lactobacilli*. *Journal of Applied Microbiology* **23**, 130-135.
- De Valdez, G., de Giori, G., de Ruiz Holgado, A. and Oliver, G. (1985). Rehydration conditions and viability of freeze-dried lactic acid bacteria. *Cryobiology* **22**, 574-577.
- Dianawati, D., Mishra, V. and Shah, N. (2016). Survival of microencapsulated probiotic bacteria after processing and during storage: A Review. *Critical Reviews in Food Science and Nutrition* **56**, 1685-1716.
- Ekdawi-Sever, N., Goentoro, L. and Pablo, J. (2003). Effects of annealing on freeze-dried *Lactobacillus acidophilus*. *Journal of Food Science* **68**, 2504-2511.
- Gardiner, G., O'Sullivan, E., Kelly, J., Auty, M., Fitzgerald, G., Collins, J., Ross, R. and Stanton, C. (2000). Comparative survival rates of human-derived probiotic *Lactobacillus paracasei* and *L. Salivarius* strains during heat treatment and spray drying. *Applied and Environmental Microbiology* **66**, 2605-2612.
- Ghaffar, T., Irshad, M., Anwar, Z., Aqil, T., Zulifqar, Z., Tariq, A., Kamran, M., Ehsan, N. and Mehmood, S. (2014). Recent trends in lactic acid biotechnology: A brief review on production to purification. *Journal of Radiation Research and Applied Sciences* **7**, 222-229.
- Hekmat, S. and McMahon, D. (1992). Survival of *Lactobacillus acidophilus* and *Bifidobacterium bifidum* in ice cream for use as a probiotic food. *Journal of Dairy Science* **75**, 1415-1422.
- Jalali, M., Abedi, D., Varshosaz, J., Najjarzadeh, M., Mirlohi, M. and Tavakoli, N. (2012). Stability evaluation of freeze-dried *Lactobacillus paracasei* subsp. *tolerance* and *Lactobacillus delbrueckii* subsp. *bulgaricus* in oral capsules. *Research in Pharmaceutical Sciences* **7**, 31-36.
- Kerckhof, F., Courtens, E., Geirnaert, A., Hoefman, S., Ho, A., Vilchez-Vargas, R., Pieper, D., Jauregui, R., Vlaeminck, S., de Wiele, T., Vandamme, P., Heylen, K. and Boon, N. (2014). Optimized cryopreservation of mixed microbial communities for conserved functionality and diversity. *PLoS One* **9**(6), e99517.
- Khoramnia, A., Abdullah, N., Liew, S., Sieo, C., Ramasamy, K. and Ho, W. (2011). Enhancement of viability of a probiotic *Lactobacillus* strain for poultry during freeze-drying and storage using the response surface methodology. *Animal Science Journal* **82**, 127-135.
- Kurtmann, L., Carlsen, J. and Skibsted, L. (2009). Storage stability of freeze-dried *Lactobacillus acidophilus* (La-5) in relation to water activity and presence of oxygen and ascorbate. *Cryobiology* **58**, 175-180.
- Larena, I., De Cal, A., Linan, M. and Melgarejo, P. (2003). Drying of *Epicoccum nigrum* conidia for obtaining a shelf-stable biological product against brown rot disease. *Journal of Applied Microbiology* **94**, 508-514.
- Mitropoulou, G., Nedovic, V., Goyal, A. and Kourkoutas, Y. (2013). Immobilization technologies in probiotic food production. *Journal of Nutrition and Metabolism* **2013**, ID 716861.
- Monteagudo, J., Rincon, J., Rodriguez, L., Fuertes, J. and Moya, A. (1993). Determination of the best nutrient medium for the production of L-lactic acid from beet molasses a statistical approach. *Acta Biotechnologica* **13**, 103-110.
- Pehkonen, K., Roos, Y., Miao, S., Ross, R. and Stanton, C. (2008). State transitions and physicochemical aspects of cryoprotection and stabilization in freeze-drying of *Lactobacillus rhamnosus* GG (LGG). *Journal of Applied Microbiology* **104**, 1732-1743.
- Peiren, J., Buyse, J., De Vos, P., Lang, E., Clermont, D., Hamon, S., Bégaud, E., Bizet, C., Pascual, J., Ruvira, M., Macián, M. and Arahál, D. (2015). Improving survival and storage stability of bacteria recalcitrant to freeze-drying: A coordinated study by European culture collections. *Applied Microbiology and Biotechnology* **99**, 3559-3571.
- Sathyanarayanan, J., Kunthala, J. and Gurumurthy, K. (2011). Optimization of MRS media components using response surface methodology for the riboflavin production by *Lactobacillus fermentum* isolated from yoghurt sample. *International Food Research Journal* **18**, 149-158.
- Savini, M., Cecchini, C., Verdenelli, M., Silvi, S., Orpianesi, C. and Cresci, A. (2010). Pilot-scale production and viability analysis of freeze-dried probiotic bacteria using different protective agents. *Nutrients* **2**, 330-339.
- Schär-Zammaretti, P., Dillmann, M., D'amico, N., Affolter, M. and Ubbink, J. (2005). Influence of fermentation medium composition on physicochemical surface properties of *Lactobacillus acidophilus*. *Applied and Environmental Microbiology* **71**, 8165-8173.
- Schnierda, T., Bauer, F., Divol, B., Rensburg, E. and Görgens, J. (2014). Optimization of carbon and nitrogen medium components for biomass production

- using non-*Saccharomyces* wine yeasts. *Letters in Applied Microbiology* **58**, 478-485.
- Selmer-Olsen, E., Birkeland, S. and Sørhaug, T. (1999).** Effect of protective solutes on leakage from and survival of immobilized *Lactobacillus* subjected to drying, storage and rehydration. *Journal of Applied Microbiology* **87**, 429-437.
- Sharma, R., Sanodiya, B., Thakur, G., Jaiswal, P., Sharma, A. and Bisen, P. (2014).** Standardization of lyophilization medium for *Streptococcus thermophilus* subjected to viability escalation on freeze drying. *Microbiology Research* **5**, 502-504.
- Shu, G., Hui, Y., Chen, S., Wan, H. and Chen, H. (2015).** Application of Plackett-Burman design in screening freeze drying cryoprotectants for *Lactobacillus bulgaricus*. *Annals of the University Dunarea de Jos of Galati. Fascicle VI : Food Technology* **39**, 70-80.
- Sinha, R., Shukla, M. and Ranganathan, B. (1982).** Rehydration of freeze-dried cultures of lactic streptococci. *Journal of Food Science* **47**, 668-669.
- Teixeira, P., Castro, M., Malcata, F. and Kirby, R. (1995).** Survival of *Lactobacillus kdelbrueckii* ssp. *bulgaricus* following spray-drying. *Dairy Science* **78**, 1025-1031.
- Thammavongs, B., Corroler, D., Panoff, J., Auffray, Y. and Boutibonnes, P. (1996).** Physiological response of *Enterococcus faecalis* JH2-2 to cold shock: Growth at low temperatures and freezing/ thawing challenge. *Letters in Applied Microbiology* **23**, 398-402.
- Wang, Y., Yu, R. and Chou, C. (2004).** Viability of lactic acid bacteria and bifidobacteria in fermented soymilk after drying, subsequent rehydration and storage. *International Journal of Food Microbiology* **93**, 209-217.
- Yonekura, L., Sun, H., Soukoulis, C. and Fisk, I. (2014).** Microencapsulation of *Lactobacillus acidophilus* NCIMB 701748 in matrices containing soluble fibre by spray drying: Technological characterization, storage stability and survival after in vitro digestion. *Journal of Functional Foods* **6**, 205-214.
- Zayed, G. and Roos, Y. (2004).** Influence of trehalose and moisture content on survival of *Lactobacillus salivarius* subjected to freeze-drying and storage. *Process Biochemistry* **39**, 1081-1086.