Effect of pretreatment, lyophilization parameters and different cryoprotectants on the efficiency of probiotic freeze-drying immobilization

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Abstract

Lyophilization is an excellent process to increase the shelf life of food products or preserve probiotics. Living cells prefer mild conditions, and any deviation (vacuum, high or low temperatures) leads to cell damage. This paper examined the influence of different freeze-drying process parameters on the survival of probiotic *Lactobacillus plantarum* immobilized on the activated charcoal pad. In specific, the process included several phases in which different pretreatments, freezing temperatures, cryoprotectants and phase durations were investigated. Activation of *L. plantarum* in De Man, Rogosa and Sharpe (MRS) broth before freezing, resulted in the increased initial number of living cells, but also positively affected the cell survival after the lyophilization process. Freezing the culture in liquid nitrogen did not significantly affect cell viability after lyophilization compared to deep freezing at -80 °C, while incubation of the culture in a refrigerator for 2 h before lyophilization increased the probiotic viability. Also, the prolonged duration of lyophilization from 5 to 48 h had a slight impact on probiotic viability. The use of milk showed a significant increase in culture survival, while sucrose, maltose, and trehalose showed cryoprotectant ability, but significantly lower than that of milk. The best lyophilization protocol resulting in the highest viability of *L. plantarum* included the culture activation by MRS incubation, followed by either deep freezing in liquid nitrogen or by precooling for 2 h at 7 °C and then deep freezing at -80 °C in milk, and ending with lyophilization for 5 h.

Keywords: Lactobacillus plantarum; viability; milk; liquid nitrogen; preservation

Available on-line at the Journal web address: http://www.ache.org.rs/HI/

ORIGINAL SCIENTIFIC PAPER

UDC: 664.8.047+579.864

Hem. Ind. **00(0)** 000-000 (2025)

1. INTRODUCTION

Cell preservation is one of the main challenges in the development of efficient probiotic systems aimed at different applications, such as pharmaceuticals, functional foods, *etc*. The primary goal of preserving cells is to maintain their viability while ensuring that their biochemical, morphological, physiological, and genetic characteristics are not altered [1]. Even though there are several efficient cell preservation strategies, lyophilization or freeze-drying is considered the gold standard in the biopharmaceutical sector, especially when the goal is to preserve sensitive microbial cells. During the process of freeze-drying, bacterial cells are exposed to low temperatures and consequent removal of water within the cells. However, the freezing step may lead to cellular damage due to the formation of ice crystals and osmotic stresses. To protect cells against such damage, different protectants may be added to the drying media. The role of protectants is to stabilize the cells during water removal and to provide a good matrix that allows shape maintenance of the entire sample during and after processing. In this way, protectants conserve viability of the cells and allow their stability and easy rehydration. Many different substances have been used for testing protective ability, including skimmed milk, polyols, polysaccharides, disaccharides, amino acids, proteins, minerals, salts of organic acids, vitamins, *etc*. [2-4]. However, sensitive microorganisms like lactic acid bacteria (LAB), even in the presence of effective protectants, can lose viability and stability [5] since the microbial cell survival during this process is dependent on other

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Paper received: 2. April 2025; Paper accepted: 7 June 2025; Paper published: xx June 2025.
https://doi.org/10.2298/HEMIND250402010K

factors as well, such as the initial microorganism concentration, growth conditions, freezing rate, rehydration conditions, *etc.* [6,7].

To overcome these limitations, novel methodologies have been sought, and one of the simple approaches is immobilization of microorganisms on different carriers *via* physical or chemical methods [8]. Unlike free cells, immobilized microorganisms exhibit higher cell density and biological activity as well as higher resistance to environmental influences [9]. However, viability and function of the immobilized microorganisms are affected by the type of carrier used [10]; therefore, selecting an appropriate carrier for immobilization of microorganisms is crucial. Carbon materials such as granular activated charcoal, biochar, carbon nanotubes, graphene, and their derivatives are widely used for adsorbing pathogenic microorganisms in wastewater treatment and purification. Their high mechanical strength, well-developed porosity, large specific surface area, and tunable surface chemistry make them particularly suitable for microbial attachment [9]. Moreover, these materials are shown to support the stability and viability of immobilized microorganisms [11-14], which have been extensively investigated for biodegradation of different contaminants in water and soil. To a much lesser extent, carbonaceous materials have been used in other fields, including biomedical applications. For example, activated carbon fabric (ACF) has been examined for cell therapy and stem cell culture support [15] which shows that these materials are not just excellent carriers for prokaryotic but for eukaryotic cells as well. In addition, immobilized bacteria have been observed to continue to multiply on activated charcoal and, over time, release into the surrounding solution [16] which suggests that carbon materials hold significant potential for broader biomedical applications, including the development of efficient delivery systems for live biological agents.

The aim of this study was to investigate functionalization of ACF with probiotic microorganisms and to evaluate the influence of various factors on the survival rate of immobilized cells during freeze-drying. Precisely, the aim was to immobilize probiotics by simple adsorption onto ACF, freeze-dry produced biocomposites, and afterwards investigate the cell viability in physiologically relevant solutions. In addition, the aim was also to examine different factors that influence the survival rate of freeze-dried immobilized microbes, such as initial growth conditions, different freezing temperatures, duration of the freeze-drying process, efficacy of different protective agents, as well as modification of the environmental factors e.g. temperature. In this study, we used the bacteria strain *Lactobacillus plantarum* 299v (*Lp*299v) as a model microorganism, a typical representative of probiotic bacteria which has been extensively characterized in the literature and widely used in medicine.

2. EXPERIMENTAL

2. 1. Bacterial strain

Lactobacillus plantarum (LAB *Lp299v* DSM 9843) stabilized with starch (Flobian®, Abela Pharm, Croatia) in the form of lyophilized powder was directly dissolved in 10 mL of normal saline solution (NS) (0.9 % w/v NaCl), diluted 1:100 and further used either for immobilization or for preparation of frozen stock as described previously [17]. Bacteria from the frozen stock were later incubated under anaerobic conditions in De Man, Rogosa and Sharpe (MRS) broth (Torlak, Serbia) at 37 °C for 18 h two times consecutively and then washed in 10 mL of NS 3 times. The obtained cultures, non-activated (directly from lyophilized powder) and activated ones (from frozen stock), respectively, were then centrifuged at 4000 rpm for 5 min, and bacterial pellets were further re-suspended in 1 ml of different freeze-drying media with/without protectant ability.

2. 2. Freeze-drying medium

Four different variants of freeze-drying medium with protectant ability were used: 10 % w/v skimmed milk (Imlek, Serbia), 10 % w/v sucrose (Centrohem, Serbia), 10 % w/v trehalose dehydrate (Carl Roth, Germany), and 30 % w/v maltose monohydrate (Carl Roth, Germany). Suspensions of freeze-drying media with disaccharides (sucrose, trehalose, and maltose) were prepared in NS. Two freeze-drying media were used as controls: NS, which does not exhibit protectant ability, and formulation Reagent 18 (R18) recommended by the American Type Culture Collection (ATCC), which generates samples that freeze dry well and are very effectively preserved. Precisely, R18 is prepared by mixing 0.75 g trypticase

soy (TS) broth (Torlak, Serbia), 10 g sucrose, and 5 g bovine serum albumin fraction V (Sigma, USA) to 100 mL deionized water [17]. All freeze-drying media were filter-sterilized through a 0.2 µm filter before mixing with cells.

2. 3. Immobilization of bacterial cells on activated carbon pads

Activated carbon (AC) pads were prepared in a circular shape (12 mm in diameter, 0.5 mm thick) by punching from ACF (ConvaTec, USA). The obtained pads were placed in a glass Petri dish, sterilized by hot air at 160 °C for 2 h, and then used for immobilization of bacterial cells. Concretely, 100 µl of a freeze-drying medium mixed with cells (concentration 10^7 and 10^{10} for non-activated and activated cultures, respectively) was aseptically added on top of each pad and left to settle for 15 min at room temperature.

2. 4. Freeze-drying

The samples were put in a plastic Petri dish and freeze-dried using a freeze dryer, Beta 2-8 LD plus (Christ, Germany). The freeze-drying parameters and the influence of pretreatments were investigated in three experimental series.

In the first experimental series, AC pads with immobilized non-activated probiotics with different protectants (skimmed milk, R18, or 10 % sucrose) or without (NS only) were frozen at -80 °C and then freeze-dried for either 5 h or 48 h (-40 °C, 12 Pa).

In the second experimental series, AC pads with immobilized activated probiotics with different protectants (skimmed milk, R18, 10 % trehalose, or 30 % maltose) or without (NS only) were frozen in liquid nitrogen and then freeze-dried for 5 h (-40 °C, 12 Pa)

In the third experimental series, AC pads with immobilized activated probiotics with a protectant (skimmed milk) or without (NS only) were precooled at 7°C for either 2 h or 10 h, followed by freezing at -80 °C and then freeze-dried for 5 h (- 40 °C, 12 Pa).

The design of all three experimental series is presented in Figure 1. AC pads with immobilized cells after freeze-drying were gold-coated and visualized by a MIRA 3 XMU Field Emission Scanning Electron Microscope (Tescan USA Inc., Cranberry Twp, PA).

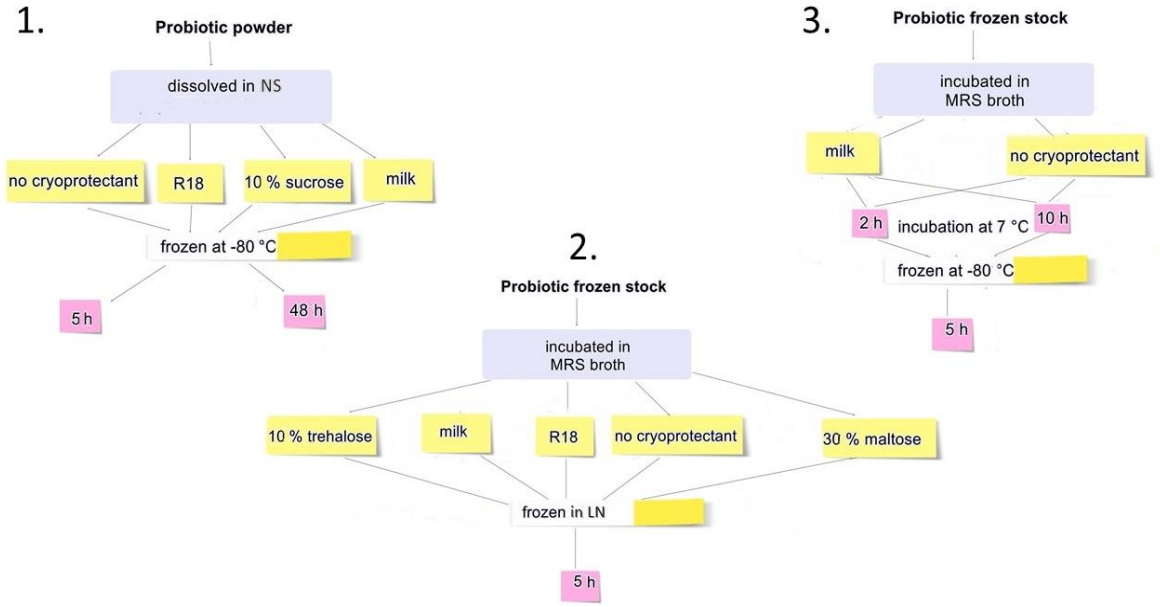

Figure 1. Scheme of the study, divided into three phases (NS is normal saline solution and LN is liquid nitrogen)

2. 5. Determination of cell viability after the freeze-drying process

The pads with the immobilized probiotic after freeze-drying were incubated in 2 ml of NS each at 37 °C for 10 min and then examined for live cells. The cell number was determined by the pour plate counting method and was expressed in $\log_{10}$ CFU/ml.

2. 6. Statistical analysis

Experiments were performed in triplicate. All values are expressed as a mean ± standard deviation. Mean values were analyzed using one-way ANOVA. The Tukey post hoc test was performed for means comparison (Origin Pro 8 software package) [18]. Data was considered significantly different when p <0.05.

3. RESULTS AND DISCUSSION

AC pads made by punching were approximately 12 mm in diameter (Fig. 2) and weighed 0.017 ± 0.001 g. Immobilization was achieved by simple seeding of bacterial cell suspensions with/without protectants on each AC pad (100 µl). Immobilized cells without protectants were visualized after freeze-drying by field emission scanning electron microscopy (FE-SEM, Fig. 2).

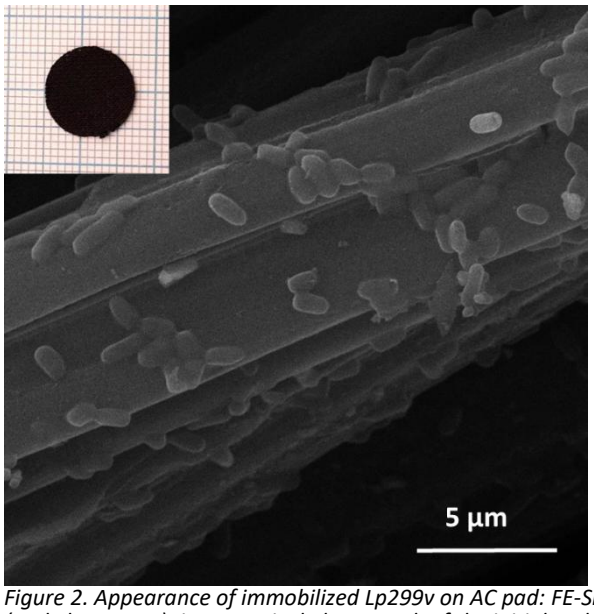

Figure 2. Appearance of immobilized Lp299v on AC pad: FE-SEM micrograph of the pad after lyophilization without any protectant (scale bar: 5 µm); inset: optical photograph of the initial pad

In our study, ACF has been chosen as a suitable carrier for freeze-drying of immobilized probiotics because carbon materials are shown to be excellent thermal conductors that secure proper thermal distribution into the samples, thus reducing the chance of damaging the cells [19].

In the first experimental series, non-activated probiotic cells were successfully immobilized on AC pads in a concentration of approximately 10^6 CFU/pad, and samples were freeze-dried for either 5 or 48 h. Skimmed milk and sucrose were used as protective agents, while the controls were R18 and NS. The resulting cell viabilities are shown in Figure 3.

Skimmed milk and R18 showed the highest protective capacity with approximately 87 % probiotic viability after the first time point in comparison to approximately 62 and 77 % for samples without protectant and the ones with sucrose, respectively. However, prolongation of freeze-drying up to 48 h adversely affected viability of the immobilized bacteria in all tested samples except in the ones containing R18. *Oluwatosin et al*. also demonstrated that skimmed milk exhibited the highest protective effect on *L. plantarum* during freeze-drying compared to various saccharides. Specifically, 10 % (m/v) skimmed milk, inulin, maltodextrin, and sucrose were evaluated as protective agents, and among the saccharides tested, inulin provided the highest protective capacity [20].

The standard protocol for lyophilization of cells in a vial implies a duration of the process of 18 h [17]. However, lyophilization is a very energy-consuming process and one of the ways to freeze-dry faster is to increase the surface area of the frozen sample by spreading out the sample and pre-freezing it in a thin layer thus allowing a larger contact for heat input as well as shorter distances in which the drying front has to move through the sample.

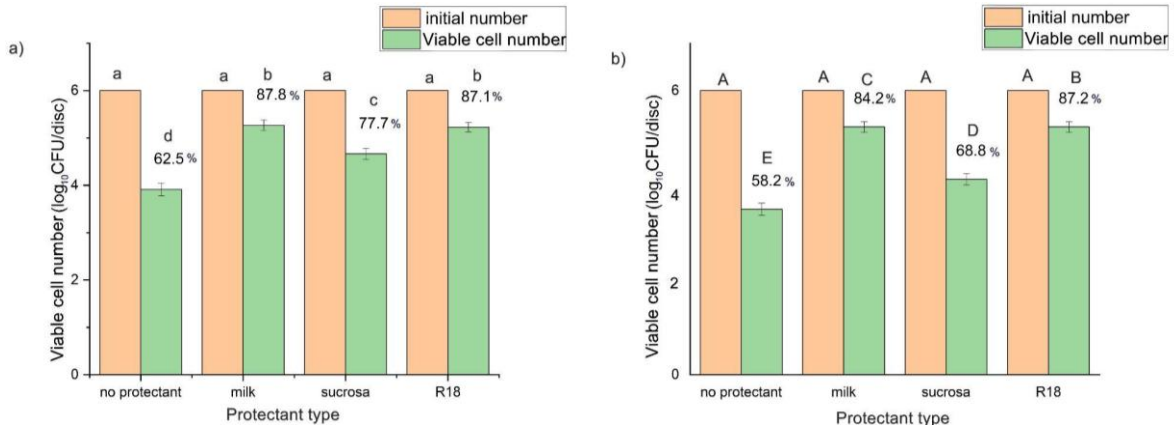

Figure 3. Viable cell number before and after lyophilization of samples with or without a protective agent (milk, sucrose, and R18), frozen at -80 °C and lyophilized for: a) 5 h, b) 48 h (experimental values are mean ± standard deviation (SD < 2 %) of n=3). Statistically different groups are marked with different letters

Since our samples were in the form of thin discs with the external surface area of approximately 1 cm^2 (Fig. 2), reduction of freeze-drying time was suitable. This was confirmed by the obtained results, which showed that a lyophilization time of 5 h is more favorable than a longer period of 48 h. Furthermore, in this experimental series it was noticed that samples containing sucrose were very difficult to manipulate because of the stickiness and stiffness of the material. So, even though sucrose is the most commercially used protectant for freeze-drying probiotic cultures in pharmaceutical products, we had to substitute it with other disaccharides instead.

To enhance bacterial viability during the freeze-drying process, approximately 18 h-old bacterial cultures are usually used [17]. So, in the next step, we examined freeze-drying of the immobilized activated culture. Activated probiotic cells were successfully immobilized in a concentration of approximately 10^9 CFU/pad. Freezing sensitive and high-value materials such as biological formulations, vaccines, bacteria, viruses, *etc*., in liquid nitrogen is the best way to preserve materials that cannot withstand oxygen or heat. Since *Lactobacillus* spp. is microaerophilic, in this experimental series the samples were immediately frozen at an ultra-low temperature. Skimmed milk, trehalose, and maltose were used as protective agents while the controls were R18 and NS. Trehalose is an exceptional and extensively used protectant in the food, medical, pharmaceutical, and cosmetic industries. Even though modern advancements in enzyme production technology have significantly decreased the production costs of trehalose, it is still more costly than common sugars like glucose or sucrose. However, lower-priced maltose in a concentration of 30 % w/v was shown to be a good alternative to trehalose in lyophilizing sensitive biological materials [21]. The obtained results are shown in Figure 4.

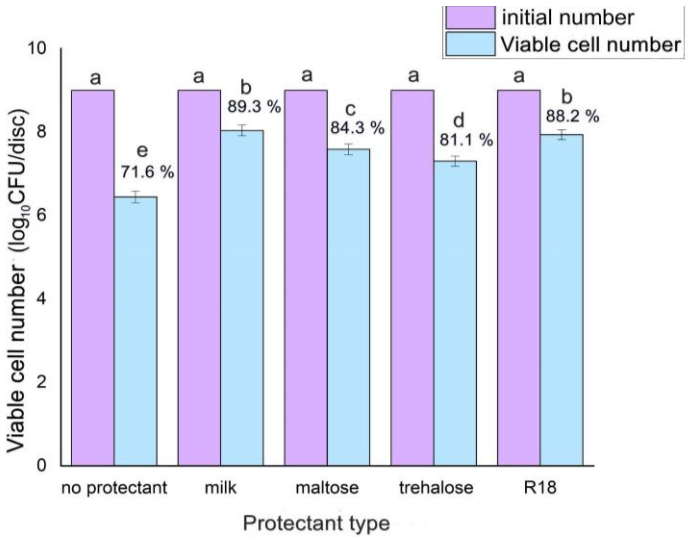

Figure 4. Viable cell numbers before and after 5 h of lyophilization for samples with or without a protective agent (milk, maltose, trehalose, and R18), frozen in liquid nitrogen (experimental values are mean ± standard deviation (SD < 2 %) of n = 3). Statistically different groups are marked with different letters

As expected, activation of probiotics (cultivation in MRS broth 2 times, consequently for 18 h) increased the total initial number of living bacteria and consequently the number of immobilized cells per pad (approximately 10^9 CFU/pad for activated culture *vs*. approximately 10^6 CFU/pad for non-activated ones). Effects of activation on the cell survival after the lyophilization process could be seen by comparing Figures 3a and 4. In specific, the largest increase in cell viability as a consequence of activation was achieved in samples without a protectant (from 62.5 to 71.6 %, *i.e.* ~10 % increase). In the case of skimmed milk and R18, the increase was negligible by 1 to 2 % (from 87.8 to 89.3 % for milk and from 87.1 to 88.2 % for R18). In the case of samples containing disaccharides, the increase in cell viability compared to non-activated cell samples in sucrose was modest, ranging from approximately 4% to 8%. The obtained results show that skimmed milk and R18 still had the highest level of protection, and that activation of probiotics as well as freezing in liquid nitrogen had only a minor beneficial influence with respect to cell viability (up to 2 %). However, the total initial number of immobilized cells is significantly higher as a result of activation, which enables the cells to have greater potential to exhibit their beneficial effect in later use. The use of trehalose and maltose with prior activation of the bacteria, as well as immediate freezing at ultra-low temperatures, resulted in much better survival compared to the use of sucrose as a protectant without cell activation. This increase can be attributed to the activation of bacteria and the use of liquid nitrogen rather than the protectant itself, since there was a significant viability increase of almost 10 % in the samples with cells alone without any protectant (Figure 3a and 4). In addition, maltose, similarly to sucrose, also showed undesirable properties, namely the stickiness and stiffness of the samples. Since the freezing step is critical in the lyophilization process due to potential cell damage, different freezing strategies are explored in the literature. For example, in a recent study the authors investigated the effects of pre-freezing at -80 *vs*. -196 °C in liquid nitrogen on the quality and storage stability of several probiotic cultures, including *L. plantarum* CN2018, and found that the latter pre-freezing did not cause cell death during the freezing phase [22]. However, in case of some probiotic strains (*e.g. B. breve* CCFM1025) liquid nitrogen pre-freezing increased the melting enthalpy of the cell suspension and consequently decreased the survival rate, which suggests that the impact of pre-freezing methods on probiotic viability following lyophilization is strain-dependent [22].

In the third experimental series, activated probiotic cells were successfully immobilized in a concentration of approximately 10^9 CFU/pad. The samples were first precooled at 7°C for either 2 h or 10 h, followed by freezing of the samples at -80 °C. This freezing temperature was chosen since freezing in liquid nitrogen did not show significant beneficial effects on cell survival in samples with milk and R18, and at the same time, it is economically very demanding. Skimmed milk was used as a protectant while NS was used as a control. The results of this experimental series are shown in Figure 5.

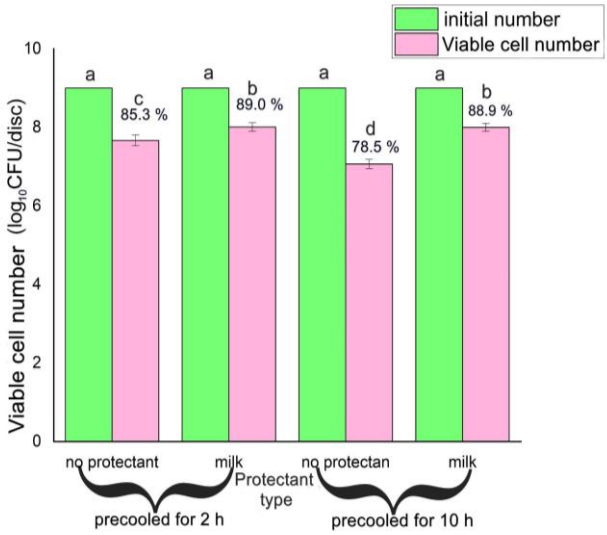

Figure 5. Viable cell numbers before and after 5h of lyophilization for samples with and without a protective agent (milk), precooled for 2 h or 10 h, at 7 °C before freezing at -80 °C (experimental values are mean ± standard deviation (SD < 2 %) of n = 3). Statistically different groups are marked with different letters

Precooling of the samples at 7 °C for both durations contributed significantly to the survival of the probiotics without a protectant added and resulted in even better survival than in the case of freezing in liquid nitrogen (85.3 and 78.5% *vs*. 71.6 % for precooling for 2 and 10 h *vs*. freezing in liquid nitrogen, respectively). These results are in accordance with literature data, which show that cryotolerance can be acquired after an adaptation period. Concretely, the viability of *Lactococcus lactis* subsp. *lactis* cultured at 37 °C improved significantly after it was cold shocked at 10 °C for 2 h prior to freezing at -20 °C [23,24]. It is well known that microorganisms can adapt to environmental stresses by the induction of proteins and other products. So, by modification of the cell environment, such as temperature, the composition of cells can be phenotypically altered in a number of ways, which can play a role in the resistance to freezing [25]. Although cooling prior to pre-freezing and lyophilization positively affects cell viability and the overall efficiency of the lyophilization process, the duration of exposure to low temperatures before freezing at -80 °C plays a critical role. In the experiment conducted, as shown in Figure 6, keeping the bacteria at 7 °C for 10 h proved to be too long, yielding poorer results compared to a 2 h cooling period.

Interestingly, survival of probiotics protected with milk was the same in the case of precooling and freezing at -80 °C as in the case of freezing in liquid nitrogen without the precooling step (Figs. 4 and 5), which confirms that precooling can be a very efficient and at the same time inexpensive strategy inducing cryoresistance in LAB.

4. CONCLUSION

This study investigated the influence of different pretreatment procedures, freeze-drying durations, freezing modes, and protective agents on the survival of immobilized probiotics. Pretreatments such as activation of *Lp*299v in MRS broth and precooling, as well as freezing at ultra-low temperatures, significantly affected the survival of immobilized cells without any protective agent. The activation of probiotics was shown to be an important pretreatment step because it increased the initial number of live bacteria and consequently the number of cells immobilized on the ACF. This study also showed that precooling is very beneficial in inducing cryo-resistance and that, in comparison to costly freezing in liquid nitrogen, it results in higher survival in the case of samples without any protectant. However, all these beneficial effects are not so pronounced in the samples protected with skimmed milk and formulation R18, which both exhibited very high protective ability. Furthermore, skimmed milk was shown to be equally efficient providing the same degree of protection for a short 5-hour freeze-drying time as the costly formulation R18. Since it is inexpensive and widely available, it is a better option for the protection of immobilized cells on ACF. To sum up, activation, short precooling treatment with skimmed milk as a protective agent, resulted in a very high total number of immobilized live cells after deep freezing and consequent 5 h freeze-drying. Thus, this process enables a greater capacity of microbial cells to exhibit their positive effects in potential pharmaceutical and medical applications.

Acknowledgement: *This research was supported by the Science Fund of the Republic of Serbia, Grant No 9802, Activated Charcoal as a Carrier of Probiotics: A New Approach for Pathogen Elimination in Wounds-ProHealingAC.*

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Uticaj predtretmana, parametara liofilizacije, korišćenja različitih krioprotektanata na preživljavanje imobilisanih probiotika tokom postupka liofilizacije

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(Naučni rad)

Izvod

Liofilizacija je odlična metoda koja omogućavo produženje roka trajanja prehrambenih proizvoda kao i očuvanje probiotskih kultura. Žive ćelije preferiraju blage uslove i svako odstupanje (npr. vakuum, visoka ili niska temperatura) dovodi do oštećenja ćelija. U ovom radu je ispitan uticaj različitih parametara kao što su predtretmani, trajanje liofilizacije, tip smrzavanja, primena krioprotektanata na preživljavanje probiotika imobilisanih na tkaninu od aktivnog uglja tokom procesa liofilizacije. Aktivacija *L. plantarum* u De Man, Rogosa and Sharpe (MRS) bujonu pre imobilizacije značajno povećava broj živih ćelija, ali i njihovo preživljavanje tokom liofilizacije. Zamrzavanje imobilisanih ćelija tečnim azotom nije dalo željeno povećanje procenta preživelih probiotskih ćelija nakon liofilizacije u poređenju sa dubokim smrzavanjem na -80 °C, dok je inkubacija ćelija 2 h u frizideru pre dubokog smrzavanja doprinela povećanju broja ćelija koje prežive liofilizaciju. Dužina trajanja (5 i 48 h) samog procesa sušenja imala je blagi uticaj na stepen preživljavanja probiotika. Upotreba mleka kao krioprotektanta značajno je povećala stepen preživljavanja, dok su saharoza, maltoza i trehaloza pokazale dobru zaštitnu moć, ali znatno manju u poređenju sa mlekom. Najviši stepen preživljavanja *L. plantarum* obezbeđuje procedura koja podrazumeva inkubaciju ćelija u MRS bujonu, korišćenje mleka kao krioprotektanta, inkubaciju 2h u frizideru, duboko smrzavanje i liofilizaciju u trajanju od 5 h.

Ključne reči: Lactobacillus plantarum; preživljavanje; mleko; tečni azot; prezervacija