Lyoprotectants for *Lactobacillus brevis* B144 and Their Capability to Maintain the Expression of Surface Layer Protein

**Abstract.** *Lactobacillus brevis* as a probiotic has been reported to facilitate micronutrient such as minerals absorption in the intestine via the surface layer protein. Negatively charged-surface layer protein from the bacteria that adheres on the intestinal mucosa binds to the positively charged-minerals, e.g., calcium, zinc, iron, magnesium and potassium via an ionic interaction. In this study, several sugar-based lyoprotectants (glucose, galactose, and lactose) and skim milk were implemented in the *L. brevis* B144 InaCC freeze drying. The dried *L. brevis* B144 InaCC was kept in the refrigerator up to three months and monthly tested for the cell viability. The dried *L. brevis* B144 InaCC was re-culture and then its surface layer protein was isolated using 8 M guanidine hydrochloride. In general, combinations of skim milk and the sugar-based lyoprotectant provided higher protection than the skim milk alone. Meanwhile, without any lyoprotectant, the *L. brevis* B144 InaCC lose its ability to grow. In regards to maintain the capability of the surface layer protein expression, the *L. brevis* B144 InaCC which was protected with the combination of 20% skim milk and 10% galactose showed the highest protein expression level among the other lyoprotectants.

**Keywords:** cryoprotectant, *Lactobacillus brevis*, surface layer protein

1. **Introduction**

Probiotics in functional foods are currently not only limited in fermented and dairy-based foods, such as cheese, yogurt, tempe, and buttermilk, but they are now included in all types of foods and beverages, for instance snacks, fruit and vegetables, juices, and the other vegan beverages [1]. Therefore, dried probiotic has been developed for more applicable formulations to those varieties of healthy foods and beverages [2]. A number of drying processes have been used to dehydrate microorganism used as probiotics, e.g., lactic acid bacteria, such as freeze drying, spray drying, vacuum oven drying, fluid bed drying or combination of those methods [2]. Freeze drying and spray drying are preferable for industrial scale than the other drying methods [2]. However, dried microorganisms might lose its viability and survival rate. Therefore, lyoprotectants are needed in the drying process to decrease the stress exposure to the microorganism to maintain its viability [3].

In this study, sugar-based-lyoprotectants (glucose, galactose, and lactose); skim milk; and combinations of them were used to decrease the harmful effect of the freeze drying on the *L. brevis* B144 InaCC. After freeze drying, the bacteria were stored in cold temperature approximately 4-10°C in the refrigerator up to three months. The cell viability test was carried out on month 1; 2; and 3 using total plate count (TPC) with duplication. The living bacteria was then tested for their capability to express the surface layer protein, the main feature of the lactic acid bacteria for self-protection and host-adherence. Overall, the cell viability was higher by the combination of skim milk and sugar based-lyoprotectants than that of by
the skim milk. The highest surface layer protein expression level was found on the bacteria protected by combination of 20% skim milk and 10% galactose.

2. Materials and Methods
2.1 Materials
*L. brevis* B144 (InaCC); de Man, Rogosa, and Sharpe or MRS medium (Merck); glucose, galactose, and lactose (Sigma); skim milk (Tropicana Slim); sterile distilled water (Ikapharmindo); guanidine hydrochloride (Biobasic); dialysis membrane cut off 100 – 500 (Spectra/Por®).

2.2 Methods
2.2.1 Preparation of cell culture
*L. brevis* B144, a strain with the highest Slp expression among the other *L. brevis* strains was used [4]. A fresh single colony of the *L. brevis* B144 was cultured at 30°C, 24 h, 160 rpm in two test tubes containing 1 mL MRS medium, respectively. All of those cultures were added to 18 ml MRS medium in a 250 mL Erlenmeyer flask and then incubated at 30°C, 48 h, 160 rpm. The second cell culture (10 ml) was added to 90 mL MRS medium in 1 L Erlenmeyer flask and re-cultured at the same condition with the previous step. The OD of final culture was measured at 600 nm using spectrophotometer (GeneQuant Pro, Amershams Biosciences).

2.2.2 Preparation of lyoprotectant solutions
The lyoprotectant solutions were prepared by weighing 8 g of each of sugar based lyoprotectants (glucose, galactose, and lactose) and 20 g skim milk, then added to 16 mL and 40 mL sterile distilled water, respectively in order to obtain 50% lyoprotectant solution. The sugar based lyoprotectants were sterilized in autoclave at 121°C, 15 min, meanwhile the skim milk was autoclaved at 110°C, 30 min.

2.2.3 Freeze-drying of *L. brevis* with various lyoprotectant
The cell pellet was obtained by centrifugation of 10 mL cell culture at 6,000 rpm, 6 min, room temperature and washed twice with 10 mL sterile distilled water. It was then resuspended in 10 mL of various sugar based-lyoprotectants and skim milk were used in different concentrations (Table 1). The cell suspensions were kept in -80°C freezer for at least 2 days or until completely frozen before freeze drying. The lyophilization was performed by using the standard method of the instrument: freezing -80°C, 20 min, 1 bar; primary drying -60°C (Martin Christ Alpha 1-4 LSCbasic), at least 24 h or until the cells were completely dried. The dried cells were stored in refrigerator (4-10°C) (Sharp SCH250FS Showcase). Before the freeze drying, 100 μL of each cell suspension was collected and tested for the cell viability as control.

| Table 1. Formulations of lyoprotectants for *L. brevis* B144 freeze drying |
|-----------------|-----------------|-----------------|
| Formulation    | 50% Skim milk | 50% Sugar based-Lyoprotectant | Sterile distilled water |
| F1             | 2              | 2               | 6               |
| F2             | 2              | 2               | 6               |
| F3             | 2              | 2               | 6               |
| F4             | 4              | 2               | 4               |
| F5             | 4              | 2               | 4               |
| F6             | 4              | 2               | 4               |
| F7             | 2              | 0               | 8               |
| F8             | 4              | 0               | 6               |
| F9             | 0              | 0               | 10              |
2.2.4 Cell viability test
The dried *L. brevis* B144 was completely resuspended in sterile distilled water (mg/100 μL) and added to 900 μL sterile distilled water, then thoroughly mixed by using vortex at least 1 min (10^4 dilution). The cell suspension (100 μL) from the 10^-1 dilution was added to another 900 μL sterile distilled water and mixed until homogenous, similar with the previous procedure. The dilution was continued until the 10^-6 dilution. The final dilution (100 μL) was spread on MRS agar medium. The number of colonies was counted after incubation 48 h at room temperature. Statistical analysis was done by using two-way ANOVA (α = 0.05) with GraphPad Prism ver 8.3.0.

2.2.5 Isolation and characterization of surface layer protein
Isolation of the surface layer protein referred to Viljanen & Palva (2002) with some modifications [4,5]. A regrowth single colony of the freeze-dried *L. brevis* B144 was cultured in 1 ml MRS and then incubated at 30°C, 160 rpm, overnight. In the following day, the culture was added to 10 ml MRS and reincubated at 30°C, 160 rpm, 48 h. The medium was separated with the cell pellet by centrifugation at 6,000 rpm, room temperature, 6 min. Afterwards, solubilization of the cell pellet was carried out using 2 M guanidine HCl 1.8 mL, followed by incubation at 37°C for 2 h. The high concentrated salt was eliminated by dialysis in sterile distilled water with gently stirring at 4-10°C, 48 h. The insoluble materials were removed by filtration using a sterile membrane filter with cut-off 0.20 μm. The protein in the soluble fraction was collected by centrifugation at 12,000 rpm, room temperature, 15 min. The pellet of surface layer protein was then denatured in 30 μL 2×Laemmli sample buffer. The SDS PAGE was performed using separating gel (13%) and stacking gel (4%), at 90 V, 2.5 – 3 h.

3. Results and Discussion
Freeze drying is a dehydration process by sublimation the ice water crystal of a frozen product, without passing through the liquid phase under high vacuum or below the triple point of water [6]. The triple point of water is a condition of temperature and pressure at which all phases of the water (liquid water, ice water, water vapor) are at equilibrium state [7].

Freeze drying is commonly applied in food and pharmaceutical industry for biological, chemical, or biotechnological products [6]. The process consists of three crucial steps, i.e., freezing, primary drying, and secondary drying [6,8–10]. In the freezing step, liquid water is supercooled and solidified by ice nucleation followed by forming ice crystals. The ice crystals are removed by sublimation during the primary drying step at high temperature and pressure. The remain unfrozen water is desorbed in the secondary drying stage which is performed at higher temperature and lower pressure [8]. Extremely changes of temperatures and pressure during the freeze drying might affects chemical (solute concentration, pH, chemical gradient, hydrogen bond with water and other excipients); physical (osmotic pressure, thermal gradient, and pressure from ice); and mechanic environment (stiffness, elasticity, and toughness) of any biomaterials including bacterial cells [11]. Several types of excipients such as monosaccharides, disaccharides, polysaccharides, proteins, polyglycol, sulfoxide, polyphenol, and alcohol are reported for their protections on biomaterials during the freeze drying [11]. In this study, we compared the lyo-protection effect of monosaccharides (glucose and galactose); disaccharide (lactose); and protein (skim milk) towards damages caused by the freeze drying.

3.1 Effects of lyoprotectants on the *L. brevis* B144 cell viability
The protection mechanisms of sugar based-lyoprotectants including monosaccharide and disaccharide are vitrification and water replacement [12]. Vitrification is solidification of liquid into glass-like state or noncrystalline of amorphous phase that avoids formation of ice crystals [13]. The sugar lyoprotectants provides the vitrification because of its capability to dehydrate the biomaterial. Simultaneously, they maintain the structural and functional of biological membrane integrity [14]. The sugar based lyoprotectants substitute the hydrogen bond of water during the drying process, thus they could minimize the unacceptable effect of chemical alteration of the biomaterials [15].
The culture of *L. brevis* B144 was harvested at optical density (OD$_{600}$) = 5.57 × 10$^6$. The final products of dried *L. brevis* B144 InaCC were yellowish and coarse powder as depicted in Figure 1. Figure 2 shows the average of cell viability from the first to the third month after freeze drying. Lyoprotectants were crucial for the bacteria viability after freeze drying. Without any lyoprotectants, the *L. brevis* B144 InaCC could not grow after freeze drying (F9) (Figure 2). In general, no significant interaction between lyoprotectant formula and storage time (p value = 0.9414; α = 0.05). Significant differences were found between each of formulas and between storage time, with p value = 0.0006 and 0.0444; α = 0.05, respectively. Combinations of sugar based-lyoprotectant and skim milk were better than the skim milk only, as it was reported than skim milk showed the lowest protection among the other reported types of lyoprotectant [3]. However, all of the formula gave the protection more than the minimum requirement for the probiotic viability (>10$^6$ cfu per mL or g) until three months of storage at cold temperature [1].

![Figure 1. *L. brevis* B144 after freeze drying with various lyoprotectants](image)

**Table 1.**

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![Figure 2. The number of the *L. brevis* B144 colonies after freeze drying and stored in the refrigerator (4-10°C) up to 3 months. (F1) 10% skim milk and 10% galactose; (F2) 10% skim milk and 10% lactose; (F3) 10% skim milk and 10% glucose; (F4) 20% skim milk and 10% galactose; (F5) 20% skim milk and 10% lactose; (F6) 20% skim milk and 10% glucose; (F7) 10% skim milk; (F8) 20% skim milk; (F9) sterile distilled water. n = 2](image)

3.2 Surface layer protein the *L. brevis* B144 after freeze drying with different lyoprotectants

Surface layer protein expressed by lactic acid such as *L. brevis* B144 is important for its adherence and transit in the gastrointestinal tract. The protein shields the bacteria against gastro-acidic and enzymatic degradation [16]. So, beside the cell viability, the expression level of the surface layer protein is also requested for probiotic characterization to ensure its effectivity [16].
In a previous study, the surface layer protein of the *L. brevis* B144 was observed at 45 – 50 kD and had the closest similarity with the surface layer protein from *L. brevis* KB290 [4]. In this study, we found the thickest band of the surface layer protein was obtain from a single colony that had been previously dried with 20% skim milk and 10% galactose (Figure 3). Galactose has been reported in biofilm formation of *Bacillus subtilis* via UDP-galactose conversion [17]. In addition, surface layer protein is known as the dominant biomaterials in the biofilm formation [18].

![Figure 3. Surface layer protein of the re-cultured dried *L. brevis* B144. (M) protein marker; (1-8) F1 to F8. Dried *L. brevis* B144 with the F4 lyprotectant shows the thickest protein band approximately at 45 kD](image)

4. Conclusion

Lyoprotectants are mandatory in the *L. brevis* B144 freeze drying because the bacteria could not maintain its viability after freeze dried without any lyoprotectants. Combination of sugar based lyoprotectant and skim milk showed relatively higher cell viability than the skim milk alone after cold storage for three months. *L. brevis* protected with 20% skim milk and 10% galactose expressed the highest amount of surface layer protein among those of lyoprotectants.

5. Acknowledgement

References


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[16] Meng J, Zhang Q X and Lu R R 2018 Identification and analysis of the function of surface layer proteins from three Lactobacillus strains Ann Microbiol 68 207–16
