



Optimal combination of multiple cryoprotectants and freezing-thawing conditions for high lactobacilli survival rate during freezing and frozen storage

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ABSTRACT

The optimal cryoprotectants combination, freezing rate and thawing temperature were investigated for preserving *Lactobacillus plantarum* and *Lactobacillus casei* during freezing and frozen storage. It is indicated that, among all cryoprotectants applied singly, 150 g/L and 100 g/L sucrose provided the best protective effect for *L. plantarum* and *L. casei*, respectively (survival rate = 74.4% and 84.8%, respectively). By using orthogonal experimental design for cryoprotectant mixtures, the mixture of trehalose, sucrose, glycerol and skimmed milk at final concentration of 12.5, 12.5, 37.5 and 25.0 g/L, respectively, appeared to enhance significantly the survival rate of *L. plantarum* (92.8%) and *L. casei* (91.2%). Among all freezing and thawing conditions examined, the survival rate of *L. plantarum* was the highest at a freezing rate of $-10^{\circ}\text{C}/\text{min}$ and thawing temperature of 0°C ; and that of *L. casei*, at a freezing rate of $-1^{\circ}\text{C}/\text{min}$ and thawing temperature of 0°C . The cryoprotectant effect exerted by keeping the integrity of cell membrane and therefore maintaining the lactobacilli survival.

1. Introduction

Currently, developing novel food products containing probiotics especially lactic acid bacteria (LAB) has drawn many interests (Dimitrellou, Kandyli, & Kourkoutas, 2016) based on the consideration of promoting human health. The most widely used LAB are the genera *Lactobacillus* commonly from human intestine (Tripathi & Giri, 2014). Technically, freezing is importantly and commonly applied to preserve the viability of LAB in the dairy industry and laboratory for a long time, for maintaining most of the bacterial survival and functionality (Fonseca, Béal, & Corrieu, 2001; Kandil & Soda, 2015). It also has been described as the initial and most critical step within the freeze-drying process (Stephan, Silva, & Bisutti, 2016). Nevertheless, it is still challenging to keep bacteria in high survival.

The bacterial survival during freezing is generally dependent on several major factors, such as cell nature (Fonseca et al., 2001; O'Brien, Aryana, Prinyawiwatkul, Ordóñez, & Boenke, 2016), cryoprotectant formulation (Chavarri, De Paz, & Nunez, 1988; Fonseca et al., 2001), and freezing-thawing conditions (Carvalho et al., 2004; Fonseca et al., 2001). Different species, even strains of LAB, exhibit various survival

after freezing, relating to cell size, shape and structure (Bozoglu, Ozilgen, & Bakir, 1987; Klaenhammer & Kleeman, 1981). Bigger and more complicated bacteria are less resistant to freezing damage than the smaller and simpler ones. Various compounds have been generally added as cryoprotectants to protect some LAB against freezing damage, such as trehalose for *L. casei* (Dimitrellou et al., 2016), sucrose for *Lactobacillus delbrueckii* subsp. *bulgarius* (Carvalho et al., 2010), skimmed milk for *Lactobacillus salivarius* (Zayed & Roos, 2004), and glycerol for *Streptococcus thermophilus* and *L. delbrueckii* subsp. *bulgarius* (Fonseca et al., 2001). A mixture of 280 g/L skim milk, 240 g/L lactose, and 48 g/L sodium ascorbate has been shown to improve significantly the survival rate of *L. delbrueckii* subsp. *bulgarius* (Chen, Chen, Li, & Shu, 2015). And, a mixture of 98.5 g/L skimmed milk and 106.5 g/L sucrose has too for *L. salivarius* (Ming, Rahim, Wan, & Ariff, 2009). For freezing conditions, the optimal freezing rate for high bacteria survival should be properly slow to maintain equilibrium between inside and outside of cells, minimizing the formation of ice crystals (Berny & Hennebert, 1991; Mazur, 1977). Thawing temperature after freezing process may also affect bacterial survival importantly via changes in electrolyte concentrations or water recrystallization during thawing

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(Mazur, 1970; Brashears & Gilliland, 1995; Fernandez Murga, De Ruiz Holgado, & De Valdez, 1998). Besides, storage temperature is also crucial to the viability of LAB. These bacteria are quite stable under -80°C (Fonseca, Béal, & Corrieu, 2000; Karlsson & Toner, 1996; Mazur, 1970).

Generally, LAB survival after freezing is strongly dependent on the presence of cryoprotectants and freezing-thawing condition. However, the studies on strain-dependent optimal combination of multiple cryoprotectants are still limited in literature. There has been little work on LAB survival relating to strain-dependent freezing-thawing conditions, especially thawing temperatures (Carvalho et al., 2003; Fonseca et al., 2001). *Lactobacillus* is one of most important LAB groups. Some *Lactobacillus* species, such as *Lactobacillus plantarum* and *Lactobacillus casei* are used as starter cultures in industry for controlled fermentation in the production of yogurt, cheese, beer and other fermented foods. *Lactobacillus plantarum* ST-III and *Lactobacillus casei* LC2W are widely used in the dairy industry in China. But it is still challenging to keep them with high survival during freezing and thawing processes.

Therefore, the purpose of this study was to investigate optimal cryoprotectants combination and freezing-thawing conditions for maximizing the survival rates of *L. plantarum* and *L. casei*. The factors investigated included cryoprotectants, freezing rate, thawing temperature, and storage time. The physiological damage of the lactobacilli examined during freezing was also preliminarily studied.

2. Materials and methods

2.1. Bacteria culture and samples preparation

L. plantarum ST-III and *L. casei* LC2W were provided by the Shanghai Engineering Research Center of Food Microbiology, University of Shanghai for Science and Technology (Shanghai, China). *L. plantarum* and *L. casei* were previously subcultured in de Man, Rogosa and Sharpe (MRS) culture medium, then inoculated in MRS broth, and incubated at 37°C for 12 h. Cells during the logarithmic phase of growth were harvested by centrifugation at $1180 \times g$ for 20 min, following by successively washing twice in phosphate buffer solution (PBS; pH 6.5), centrifuging, and resuspending in sterile PBS (pH 6.5) for subsequent centrifugation to collect bacteria mass. Aliquots (0.8 mL) of bacterial suspension (10^8 CFU/mL) were prepared and loaded to cryo tubes (2.0 mL).

2.2. Influence of single cryoprotectants on lactobacilli survival

The cryoprotectants, including trehalose (100, 200, 300 g/L), sucrose (100, 200, 300 g/L), glycerol (100, 200, 300 g/L), and skimmed milk (100, 200, 250 g/L), were singly added into bacterial suspension in the cryo tubes at a ratio of 1:1 (v/v). The final concentrations of trehalose, sucrose and glycerol were 50, 100 and 150 g/L. And, those for skimmed milk were 50, 100 and 125 g/L. The mentioned concentration of signal cryoprotectant is the final concentration in the following. The resultant bacterial suspension (10^8 CFU/mL) were immediately frozen at -80°C for 72 h in an ultra-deep-freeze equipment (Revco™, Thermo Scientific, Waltham, USA) before measurement of survival rate.

2.3. Influence of multiple cryoprotectants on lactobacilli survival by orthogonal experimental design

Orthogonal experimental design is one of the most effective and time-saving methods for studying an optimized combination and significant factors of the target product (Dargahi, Kazemian, Soltanieh, Hosseinpour, & Rohani, 2012). An orthogonal matrix L9 (3^4) design was adopted to determine an optimal combination of cryoprotectants. Four factors including trehalose (T), sucrose (S), glycerol (G) and skimmed milk (M) at three levels were shown in Table 1. For every trial, four cryoprotectants were mixed well (each 0.2 mL, totally 0.8 mL) and then

loaded to the cryo tubes containing bacterial suspension (10^8 CFU/mL) at a ratio of suspension: cryoprotectant = 1:1 (v/v). The concentrations of cryoprotectants in the mixture refer to the final concentration. The resultant mixtures were immediately frozen at -80°C for 72 h before measurement of survival rate.

2.4. Co-influence of freezing rate and thawing temperature on lactobacilli survival

A CryoMed controlled rate freezer (Kryo360–1.7, Planer plc, UK) was used in this study. The mixture of trehalose, sucrose, glycerol, and skimmed milk was added into the cryo tubes containing bacterial suspension (10^8 CFU/mL) at a ratio of suspension: cryoprotectant = 1:1 (v/v). The final concentration of trehalose, sucrose, glycerol and skimmed milk in the mixture is 12.5, 12.5, 37.5 and 25.0 g/L, respectively. The resultant mixture was soon subjected to freezing from -4 to -80°C at a rate of -1 , -10 , -20 , or $-40^{\circ}\text{C}/\text{min}$. The given -80°C -samples were immediately taken out and frozen at -80°C . After -80°C storage for 72 h, the samples were thawed in a water bath at 0°C (in an ice-water bath), 20°C , or 40°C before measurement of survival rate. For the control, the freshly prepared mixtures were soon subjected to liquid nitrogen for immediately freezing before transforming to a -80°C freezer.

2.5. Co-influence of cryoprotectant and freezing time on lactobacilli survival

The single cryoprotectant (50 g/L trehalose, 100 g/L trehalose, 150 g/L sucrose, 200 g/L glycerol, or 100 g/L skimmed milk) or PBS buffer (negative control) was used. Then the samples were frozen at -80°C . The lactobacilli survival rate was measured after freezing of 3, 6, and 9 days.

2.6. Survival rate examination

Total viable cell counts were determined prior to freezing and after freezing-thawing process. For the later, frozen cells were thawed in an ice-water bath (0°C) before total cell counts analysis. All samples were serially diluted in sterile saline (9 g/L NaCl), plated on MRS agar plates, and then anaerobically incubated at 37°C . Cell counts were determined after 48 h of 37°C anaerobic incubation. The survival rate was expressed as $N_1/N_0 \times 100\%$, where N_0 and N_1 were, respectively, the numbers of viable cells before and after freezing-thawing process.

2.7. Cell membrane integrity of lactobacilli after freezing-thawing

The LIVE/DEAD BacLight Bacterial Viability Kit L7012 (Molecular Probes, Invitrogen, USA) was used to determine the integrity of cell membrane (Li et al., 2011; Zahedi, Icaran, Yuan, & Pijuan, 2016). The kit contains green-fluorescent nucleic acid stain SYTO[®] 9 and red-fluorescent nucleic acid stain propidium iodide (PI). When used alone, SYTO[®] 9 stain generally labels all bacteria with intact or damaged membranes. But, PI stain stains only dead cells by penetrating damaged membranes of bacteria. When using an appropriate mixture of SYTO[®] 9 and PI, PI will cause a reduction in SYTO[®] 9 stain fluorescence, resulting in viable bacteria (with intact cell membranes) in green and dead ones (with damaged cell membranes) in red.

Equal volumes of stains A (SYTO[®] 9) and B (PI) were added to a microfuge tube and mixed thoroughly. Three microliters of the dye mixture were added to 1 mL of bacterial suspension, mixed thoroughly, and incubated at room temperature in the dark for 15 min. Ten microliters of the stained bacterial suspension were then trapped between a slide and 18-mm square coverslip for observation in a fluorescence microscope (Leica DM2000, Wetzlar, Germany).

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