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Effect of acids produced from carbohydrate metabolism in cryoprotectants on the viability of freeze-dried *Lactobacillus* and prediction of optimal initial cell concentration

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For the industrial production of probiotics powder, various sugars have been used as cryoprotectants to preserve probiotics during freeze-drying. Some of these sugars can be metabolized by *Lactobacillus* with the production of acids during the mix. In this study, we investigated the effect of acids on ATPase, β-galactosidase, lactate dehydrogenase (LDH), integrity and fluidity of cell membrane and the survival rate of *Lactobacillus* during freeze-drying. In the presence of *Lactobacillus*, acids were produced from cryoprotectants containing fermentable sugars before freezing, resulting in a decrease in the pH of the bacterial suspension to below 5.0. During freeze-drying, the acids caused a loss of viability of *Lactobacillus* due to aggravated damage to ATPase, β-galactosidase and cell membrane fluidity, but not LDH and cell membrane integrity. This finding implied that cryoprotectants that do not lead to the production of acids are effective in improving the survival rate of freeze-dried *Lactobacillus*. Here, a new formula was proposed for a protectant containing whey protein isolate (WPI) and rhamnose, which were not metabolized. In addition, linear-regression analyses were performed on the proportion of cryoprotectants (M) against cell paste (m), total cell count (N), total surface area (S_t) and total volume (V_t) of bacteria for 100% survival rate. The total surface areas of bacteria were found to be highly correlated with the amount of proposed cryoprotectant. The following prediction equation was established for the optimal initial cell concentration for a 100% survival rate of freeze-dried *Lactobacillus*: $N\,(4\pi r^2 + 2\pi l) = (0.66 \pm 0.03)M$.

[**Key words:** *Lactobacillus*; Cryoprotectant; Acids; Initial cell concentration; Prediction equation]

Lactobacillus is important organisms that are widely used as probiotics that offer health-promoting properties to humans and in fermentation starter cultures. They have been widely studied due to their potential effects on various digestive diseases (1), chronic kidney disease (2), immunomodulation (3) and lowering cholesterol levels (4). They are also frequently used in fermentative foods including dairy products, sausages, vegetables and fruit (5). The concentration and biological stability of probiotics during long-term storage are important for the wide application of *Lactobacillus*. Freeze-drying (lyophilisation) is the most effective way to maintain the stability of *Lactobacillus* (6), and widely used for the industrial production of lactic acid bacteria powder.

However, bacteria are susceptible to desiccation at low-temperatures. The damage to bacteria caused by lyophilisation has two major causes: changes in the structure of sensitive proteins, leading to a loss of key metabolic enzyme activity, and changes in the physical condition of cell membrane lipids, which impair cell membrane integrity and fluidity (7). Cryoprotectants are therefore used to reduce the unavoidable loss of viability of probiotic cells during freeze-drying. Generally, cryoprotectants contain polysaccharides, disaccharides (e.g., the sugars trehalose, lactose, maltose), organic polymers, proteins (e.g., casein, whey protein), amino acids, sugar alcohols and complex mixtures such as skimmed milk (8,9). The most commonly used cryoprotectants are skimmed milk, sucrose and trehalose (6). However, sucrose, trehalose, lactose in skimmed milk and many saccharides can be metabolized by *Lactobacillus* with formation of acids. It is well known that organic acids can diffuse across the cell membrane, damaging the membrane and leading to acidification of the cytosol (10). Before freezing, high concentrations of bacteria may metabolize the fermentative saccharides contained in cryoprotectants to produce a large amount of organic acids, which decrease the pH of the cell suspension. The organic acids and low pH may aggravate the damage to the cells caused by freeze-drying. In addition, different bacterial strains exhibit different tolerance levels to lyophilisation treatment, which can be attributed to various factors such as species, morphology, initial cell concentration and cell size (11,12). To achieve a 100% survival rate, different *Lactobacillus* strains require different optimal initial concentrations during

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freeze-drying under the same protective agent. It is essential that the optimal initial bacterial concentration per unit of cryoprotectant can be predicted for industrial production.

This study investigated the effect of acids on the viability of *Lactobacillus* during freeze-drying. A cryoprotectant that did not lead to the production of acids was developed, and correlations between the morphological properties of different strains and the cryoprotectant were analysed. An equation to predict the optimal initial cell concentration per unit of cryoprotectant was established.

MATERIALS AND METHODS

Microorganisms and growth conditions *Lactobacillus plantarum* CCFM 8610, *L. plantarum* CCFM 8661, *L. rhamnosus* CCFM 1107, *L. rhamnosus* CCFM 319, *L. casei* CCFM 30, *L. helveticus* CCFM 310 and *L. bulgaricus* CCFM 4 were obtained from the Culture Collections of Food Microbiology at Jiangnan University (Wuxi, China). *L. plantarum* ATCC 14917 was generously provided by Professor Heping Zhang (Inner Mongolia Agricultural University, China). The strains were maintained at −80°C. Each strain was activated by sub-culturing twice in MRS medium. *L. rhamnosus* and *L. casei* were cultured at 37°C, whilst *L. plantarum*, *L. bulgaricus* and *L. helveticus* were respectively cultured at 35°C, 44°C and 40°C. The activated strains were then injected into Erlenmeyer flasks containing 2 L of MRS broth at 5% and statically incubated at the respective optimum growth temperatures.

Sample preparation for freeze-drying The growth of the bacterial cultures was stopped at the end of the log phase. Strains were collected by centrifugation (RC BIOS 10, Thermo Fisher Scientific, Waltham, MA, USA) at 8000 ×*g* for 10 min at room temperature. The pellets were washed twice in a 1% (w/v) neutral aqueous solution of peptone (OXOID LP0042, Basingstoke, Hampshire, England), which had been previously sterilised at 121°C followed by centrifugation. Different masses of the cell pastes were added to cryoprotectant A (1.2 g skimmed milk, 0.2 g sucrose, 0.2 g trehalose, pH 7.0); cryoprotectant B (0.6 g whey protein isolate (WPI 90, Hilmar, USA), 1.0 g rhamnose, pH 7.0); and cryoprotectant C (0.6 g WPI, 1.0 g rhamnose, different pH values (7.0, 6.0, 5.0 and 4.0) adjusted by addition of 1 M lactic acid solution). The final volume of each bacterial suspension was maintained at 10 mL.

Freeze-drying procedure For lyophilisation of cell, a previously developed freeze-drying procedure was used in a Lyostar II freeze-dry system (Virtis, Warminster, PA, USA). Specifically, 1 mL of the formulated cell suspensions was added to 7-mL penicillin bottles, in which the liquid level did not exceed 1 cm. After all the samples were laid out, the shelf temperature in the freeze-dryer was reduced rapidly to −40°C in the shortest possible time. The temperature of −40°C was held for 4 h. Primary drying was carried out at a shelf-temperature of −20°C at the lowest degree of vacuum reached by this system for 20 h. Then, the shelf-temperature was increased to 20°C in 1 h and was held at the above vacuum for 20 h. After the freeze-drying procedure was over, the bottles were sealed with rubber plugs before removal. The samples were then kept at 4°C for further use.

Cell counts and pH detection Viable cell counts were measured before freezing and after freeze-drying. The freeze-dried samples were rehydrated by adding sterile deionised water to match the sample weight before freeze-drying, and then the pH of the samples was measured immediately. The viable cell counts were performed by tenfold serial dilutions in 10-mM phosphate buffer solution (PBS; pH 7.2) and plate assays on MRS plates. After incubation at optimum growth temperatures for 48 h, plates with 30–300 colonies were enumerated. The survival rate was reported as the ratio between colony forming units (CFU) per millilitre before and after freeze-drying and given as % values. The survival data were determined based on the means of duplicate samples of each of three biologically separate experiments.

Cell-free extract Dried cells were rehydrated to match their original weight in autoclaved deionised water (dH_2O) and harvested by centrifugation. The supernatant was used to measure extracellular enzymes. The pellets were then washed twice with dH_2O and then resuspended to the original volume in dH_2O. The resuspended cells were mechanically disrupted by mixing with 100-μm sterile glass beads (1:1.5 w/v) in a high-throughput tissue grinder (SCIENTZ-48, Scientz biotechnology Polytron Technologies Inc., Zhejiang, China) for 10 cycles of 1 min with a 5 min break on ice (13). Subsequently, the suspensions were centrifuged (10,000 ×*g*, 30 min, 4°C) to remove the glass beads and cell debris. The supernatant was immediately used for intracellular enzyme assays. The total protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit (BCA P0011, Beyotime, Shanghai, China). An equal amount of bacterial suspension at pH 7.0 before freeze-drying was treated as above as the control.

Assay of ATPase ATPase activity was assayed using an ATP assay kit (A016-1, Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's instructions. One unit of ATPase activity was defined as 1 μmol inorganic phosphate (Pi) released after incubation of 1 g protein per minute. That is, 1 U = 1 μmol Pi/g protein/min.

Assay of β-galactosidase The method described by Bhowmik et al. (14) was used to determine the activity of β-galactosidase based on the established standard curve (μmol o-nitro-phenol (ONP) vs. OD_{420}). The amount of ONP liberated was measured by OD_{420}. One unit of activity was defined as 1 μmol ONP/g protein/min.

Assay of lactate dehydrogenase Lactate dehydrogenase (LDH) activity was measured by monitoring the rate of decrease in the absorbance of NADH following reduction of pyruvate to lactate at 340 nm at 25°C during the first 3 min (15). The reaction mixture (3 mL) contained 0.63 mM pyruvate and 50 mM phosphate buffer (pH 7.5). The reaction was initiated by addition of 100 μL crude enzyme and 50 μL NADH solution (11.3 mM). The absorbance at 340 nm was recorded at exactly 1, 2 and 3 min after the reaction was initiated. One unit of activity was defined as 1 ΔE/g protein/min.

Determination of cell membrane fluidity Cell membrane fluidity was characterised by fluorescence anisotropy using a fluorescence spectrophotometer (Hitachi F-7000, Hitachi, Tokyo, Japan) as described by Louesdon et al. (16) and deduced by fluorescence polarisation (P), microviscosity (η) and anisotropy (r), which were calculated according to Li et al. (6). Higher P, η and r values signify less fluidity of the cell membrane. The bacteria before freeze-drying were used as the control.

Cell size measurement The morphological properties of the bacteria were determined using a microscope (Leica DM 2000, Leica Microsystems Wetzlar GmbH, Wetzlar, Germany) linked to a video camera (Leica DFC425 C). The cells at the end of the log phase of bacterial growth were treated with Gram staining. Leica Qwin V3 software was used to measure the width and length of individual cells. The results were reported as the arithmetic means of 100 measurements. The surface area (S) and volume (V) were calculated for each kind of cell according to Young (17). The calculations for *Lactobacilli*, assumed to be capped by two equal and symmetrical hemispherical ends, are as follows: surface area $S = 4\pi r^2 + 2\pi rl$; volume $V = \frac{4}{3}\pi r^3 + \pi r^2 l$, where l and r are the length and radius, respectively, of the cells (in μm). $S_t = NS$; $V_t = NV$, where S_t, V_t and N are total surface area, total volume and total bacterial count, respectively.

Statistical methods The experimental results were expressed as means ± standard errors of means. The linear-regression analysis was carried out using Origin 8.6 software (OriginLab, Northampton, MA, USA). Paired, two-tailed t-tests were conducted to determine the statistical significance of the differences using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). A p value below 0.05 was considered to indicate statistical significance.

RESULTS AND DISCUSSION

Effects of cryoprotectant A on survival rate of *Lactobacillus* and pH of samples Cells of *L. plantarum* CCFM 8610, *L. casei* CCFM 30 and *L. rhamnosus* CCFM 1107 were freeze-dried in cryoprotectant A. The survival rate and pH of the cell suspensions rehydrated after freeze-drying are shown in Table 1. Significant differences were discovered in the viabilities of different bacteria after freeze-drying. The survival rate decreased with the increase in the cell pastes. The pH of all samples was lower than 5.0.

As the pH of the cryoprotectant was adjusted to 7.0 and the cell pellets were washed in a neutral aqueous solution of peptone before the two were mixed, the lower pH indicates that the saccharides in cryoprotectant A were metabolized by *Lactobacillus* to produce acids in the short time before freezing. The more cell pastes were mixed, the more acids were produced. As noted in the introduction, organic acids can damage the cell membrane and acidulate the cytoplasm. A low pH may thus aggravate the cell damage caused by freeze-drying.

Effects of cryoprotectant B on survival rate of *Lactobacillus* and pH of samples It is clear from Table 2 that the survival rate of the same mass of cell pastes in cryoprotectant B was higher than that in cryoprotectant A. The pH of all samples was higher than 6.0, except for the sample containing 4.0 g/10 mL of *L. plantarum* CCFM 8610. The survival rate reached 100% with less than 1.0 g/10 mL of *L. plantarum* CCFM 8610, 2.0 g/10 mL of *L. casei* CCFM 30 and 4.0 g/10 mL of *L. rhamnosus* CCFM 1107. Compared with previous reports, cryoprotectant B also showed better protective effect. The protective medium containing 10% sorbitol and 10% skim was considered by Lee et al. (18) to be the optimal cryoprotectant of *L. plantarum* JH287. However, only a survival rate of 86.37% was reached with $2-3 \times 10^{10}$ cfu/mL (equivalent to 0.5–1.0 g/10 mL) of bacteria. According to the

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