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IMPROVING VIABILITY OF FREEZE-DRIED LACTIC ACID BACTERIA USING LYOPROTECTANTS IN COMBINATION WITH OSMOTIC AND COLD ADAPTATION

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ABSTRACT

Effect of lyoprotectants on survival of *Lactococcus lactis* and *Lactobacillus sakei* after freeze drying was investigated. Their survivals in 9.1% w/w of skim milk, soy milk, egg yolk, sucrose, lactose, glucose, trehalose and sorbitol were compared, and the protectants caused high viability were selected to develop formulations of mixed protectants. Compared to soy milk and sorbitol, higher viability of *L. lactis* was observed with lactose, skim milk and sucrose. For freeze-dried *L. sakei*, the survival in skim milk and soy milk was greater than that in trehalose, sucrose and lactose. Among all protectants tested, glucose and egg yolk provided the lowest protection to these bacteria. Of all lyoprotectant formulations, the highest number of *L. lactis* survivors was found in mixed lyoprotectants containing 3.97 g lactose, 3.97 g sucrose, 3.97 g trehalose, 8.73 g skim milk and 79.36 g distilled water. The protectant mixture provided the highest viability of *L. sakei* contained 3.97 g lactose, 3.97 g sucrose, 3.97 g trehalose, 8.73 g skim milk and 79.40 g soy milk with distilled water (1:1). Compared to single protectant, the number of *L. lactis* and *L. sakei* survivors in these formulations was increased by 11.69-20.15% and 9.51-18.15%, respectively. To improve viability, osmotic adaptation alone (with 0.3 M sucrose) or in combination with cold adaptation (10°C) induced cross-protection of *L. lactis* and *L. sakei* in selected protectant mixtures after freeze drying and storage for 28 days at -80°C were studied. These adaptation treatments did not provide protection of *L. lactis* and *L. sakei* cells after freeze drying and storage.

KEYWORDS: *Lactococcus lactis*, *Lactobacillus sakei*, lyoprotectant, freeze drying, adaptation

1. INTRODUCTION

Lactic acid bacteria (LAB) are commonly used as starter cultures for food fermentation. Freeze drying is usually used in the preservation of LAB starters. However, this technique brings about undesirable side effects such as changes in the physical state of membrane lipids and structure of sensitive proteins and decreasing of cell viability [1]. Consequently, some compounds such as polyols, polysaccharides, disaccharides, amino acids, proteins, vitamins, and various salts have been examined for their potential role to improve the survival of LAB throughout freeze drying process [2]. Glucose, lactose, sucrose, sorbitol, trehalose, skim milk and egg yolk have been used

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For cryopreservation to improve survival of certain bacterial cultures [3]. However, soy milk which contains several nutrients including protein, fat, carbohydrate, calcium, phosphorus, riboflavin, iron, thiamin and niacin has never been studiedfor its protective effect on microorganisms during freeze drying. Therefore, understanding the effect of these lyoprotectants and their formulations is important to develop viable freeze-dried *Lactococcus lactis* and *Lactobacillus sakei* as starter cultures.

Stress adaptation of microbial cells enables the cells to survive better when they are subsequently exposed to the same stress or other types of stresses [4]. Lactic acid bacteria have been shown to induce adaptive response after exposing to some stresses. Panoff *et al.* [5] demonstrated that following cold adaptation at 10°C, *L. lactis* ssp. *lactis* showed increased resistance to freezing stress. Bâati *et al.* [6] reported that preincubation of *Lactobacillus acidophilus* at low temperature (22°C) for 6 h led to development of cryotolerance during freezing treatment at -80°C for 24 h. This bacterial species was reported to be protected from osmotic stress by glycine betaine, and intracellular osmolyte [7].

This work aimed at quantifying the effect of lyoprotectants and lyoprotectant formulations on survival of *L. lactis* and *L. sakei* after freeze drying and storage at -80°C, and determining the effect of osmotic and cold adaptation on survival of these bacterial strains.

2. MATERIALS AND METHODS

2.1 Bacterial Strains

Two LAB strains isolated from fish fillet, *Lactococcus lactis* 13IS3 and *Lactobacillus sakei* 13IS4 were used in this study. They were antimicrobial substance- producing strains. Stock cultures were maintained on MRS slopes (de Man Rogosa Sharpe medium, pH 7.0±0.2, Difco Laboratories) at 37°C, subcultured every week and subsequently stored at 4°C.

2.2 Inoculum Preparation

A loopful of 24 h surface growth of each LAB strain on MRS slope was transferred to 10 ml MRS broth and incubated at 37°C for 24 h. Cells were then collected by centrifugation at 3,000 rpm for 20 min, washed twice with 0.1% peptone water and resuspended in 10 ml of the same solution. The turbidity of each suspension was adjusted to match the turbidity of 4 McFarland standard (10^8-10^9 CFU/ml).

2.3 Effect of Lyoprotectants and Lyoprotectant Formulations on Survival of *Lactococcus lactis* and *Lactobacillus sakei* after Freeze Drying

Each sterile lyoprotective agent (9.1% (w/w)) including skim milk, soy milk, egg yolk, sucrose, lactose, glucose, trehalose, and sorbitol in distilled water was prepared. Inoculum suspension (1 ml) of each strain was added into 9 ml of each lyoprotective solution. The original cell number (before freeze drying) was determined by pour plating with MRS agar and incubated at 37°C for 48 h in microaerophilic condition. Then, the mixture of each lyoprotective agent and bacterial cells was frozen at -80°C for 18 h and freeze dried for 3 h in a freeze dryer (Heto LyoLab, model 3000). Cell viability after freeze drying was determined using the same method. Percent survival was calculated, with 100% viability representing the colony counts before freeze drying.

Six lyoprotectants, including skim milk, soy milk, sucrose, lactose trehalose and sorbitol were selected to develop nine formulations of mixed lyoprotectants. Survival of *L. lactis* and *L. sakei* in nine formulations of lyoprotectants before and after freeze drying was evaluated using the same method as indicated above.

2.4 Effect of Osmotic and Cold Adaptation on Survival of *Lactococcus lactis* and *Lactobacillus sakei* after Freeze Drying and Storage

The cell suspension of *L. lactis* and *L. sakei* was prepared as previously described. Then, osmotic adaptation of these LAB was induced by addition of 1 ml cell suspension into 9 ml of MRS broth with 0.3 M sucrose and incubated at 37°C for 24 hrs. The osmotic adapted cells were collected by centrifugation at 3,000 rpm for 20 mins, washed twice, and resuspended in 10 ml of 0.1% peptone water, and adjusted the turbidity to match 4 McFarland standard. Nonadapted cells were prepared by growing the cells at 37°C for 24 hrs, then using the same procedure as previously indicated, but no osmotic challenge. The inoculum suspension (1 ml) of each cell type (osmotic adapted cells and nonadapted cells) was added into 9 ml of selected lyoprotectant formulation, frozen at -80°C for 18 hrs, and dried for 3 hrs. in the freeze dryer.

In case of osmotic adaptation in combination with cold adaptation, the adapted cells of each LAB strain were first prepared by using the same method as osmotic adapted cells. Then, cold adaptation was subsequently induced by adding 1 ml of osmotic adapted cell suspension into 9 ml of selected lyoprotectant formulation, and incubated at 10°C for 2 h before freeze drying. All cell types of freeze-dried LAB were stored at -80°C for 28 days. Viability of *L. lactis* and *L. sakei* was determined before and after freeze drying, and during storage at time intervals (0, 3, 7, 14, 21 and 28 days) using pour plate technique with MRS agar. The plates were incubated at 37°C for 24 h in microaerophilic condition. Percent survival of these freeze-dried LAB during storage was calculated, with 100% survival representing colony counts immediately after freeze drying (at the beginning time of storage).

2.5 Statistical Analysis

Data from three replications were analysed by using analysis of variance to determine if significant differences ($P \leq 0.05$) existed between mean values and using Duncan multiple range test to compare between treatment means**.**

3. RESULTS AND DISCUSSION

3.1 Effect of lyoprotectants and lyoprotectant formulations on viability of *Lactococcus lactis* and *Lactobacillus sakei*

After freeze drying, viability of *L. lactis* in lactose (64.17%), skim milk (61.56%) and sucrose (60.96%) was higher than that in other lyoprotectants tested (Table 1). For the survival of *L. sakei*, skim milk and soy milk are the best lyoprotectant which provided higher viability after freeze drying compared to trehalose, sucrose and other compounds. *L. lactis* and *L. sakei* in glucose and egg yolk had the lowest viability after freeze drying.

Disaccharide, lactose and sucrose caused high survival of *L. lactis* after freeze drying. This was probably because of high amount of unfrozen water in an amorphous state during freezing of lactose and sucrose at very low temperature. In the frozen state, the amount of unfrozen water varies depending on types of lyoprotectants. Riedel [8] reported that lactose had 40.80% unfrozen water of total water, which was higher than that of sucrose (35.90%), sorbitol (18.70%) and trehalose (16.70%) during freezing. Thus, the substances which have high amount of unfrozen water may reduce lethal effect of microbial cells, resulting from ice crystal forming during the first step of freeze drying. During deep freezing, proteins may be stabilized by their interaction with lactose. Carpenter and Crowe [9] reported that hydrogen bonding of disaccharide with the proteins induced protein unfolding, while cells lost water, thereby preventing cell dehydration. Chavarri *et al.* [10] found that lactose (1-10%) provided higher viability of *L. lactis* during storage at -20°C to -70°C, compared to glycerol.

L. lactis and *L. sakei* had high viability in skim milk and soy milk after freeze drying. This was probably because they contained high amount of nutrients, especially protein and carbohydrate which may provide protection to the cells. Skim milk contains 32.0-35.7% protein and 48.4-54.1%lactose [11], while soy milk has high protein (40-45% crude protein) and some vitamins and minerals [12]. However, *L. lactis* and *L. sakei* had lower viability in egg yolk and glucose, compared to other lyoprotective agents. This may be because of high amount of frozen water in frozen egg yolk. Riedel [8] reported that egg yolk contains high proportion of frozen water (87%) during freezing at -30°C. This may cause cell damage or injury due to ice crystals. Similarly, glucose may not be a good lyoprotectant. Miyajima [13] found that maltose was more effective to maintain stability of egg yolk phosphatidylcholine than glucose. Therefore, almost all lyoprotective agents tested, except for egg yolk and glucose were used as ingredients in formulations of lyoprotectants.

Table 1 Survival of *Lactococcus lactis* and *Lactobacillus sakei* in eight lyoprotective agents after freeze drying

Lyoprotective agents (9.1% w/w in distilled water)	Survival (%)[a] ± SD	
	Lactococcus lactis	*Lactobacillus sakei*
egg yolk	44.57 ± 3.38C[b]	49.23 ± 0.96D
glucose	46.25 ± 4.01C	46.35 ± 4.73D
lactose	64.17 ± 3.00A	56.42 ± 2.35BC
skim milk	61.56 ± 0.56A	65.06 ± 1.13A
sorbitol	59.72 ± 1.47AB	55.87 ± 1.87C
soy milk	60.39 ± 0.61AB	62.80 ± 1.67A
sucrose	60.96 ± 1.56A	58.54 ± 1.65BC
trehalose	55.71 ± 2.98B	59.43 ± 1.04B

[a]Data are means of three replications.
[b]Means within a column with different letters are significantly difference ($P<0.05$).

Survival of *L. lactis* and *L. sakei* in nine formulations of lyoprotectants after freeze drying were determined. Among all formulations, the highest survival of *L. lactis* (75.86%) was found in formulation 8 of mixed lyoprotectants. Similarly, the formulation 5 and 8 provided the greatest viability of *L. sakei* with 74.57% and 74.47% survival, respectively (Table 2). There were significant differences in the number of survival cells between these formulations and the others ($P<0.05$).

The reason that the lyoprotectant formulations 5 and 8 provided highest survival of these bacteria is probably because of the protective effect of skim milk, an ingredient in both formulations. In addition, mixing of saccharides such as lactose, sucrose, trehalose and sorbitol with skim milk and/or soy milk caused higher viability of *L. lactis* and *L. sakei* as compared to each single protectant (Table 3.1). Font de Valdez *et al.* [14] suggested that those lyoprotectants in the mixture may protect the cytoplasmic membrane by increasing the concentrated layers between the cytoplasmic membrane and cell wall to inhibit ice crystal forming and ice crystal growth inside the cells. In economical point of view, only skim milk (not soy milk) may be enough to add in the lyoprotectant formulations with some disaccharides to provide high viability.

The results of this study were in agreement with the previous finding. Palmfeldt *et al.* [15] reported that mixed lyoprotectants containing 150 g skim milk, 50 g trehalose and 1 g ascorbic acid provided higher viability of *Pseudomonas chlororaphis* (4.3% survival) as compared to a single 200g/l lyoprotectant, skim milk (0.6% survival).

Table 2 Survival of *Lactococcus lactis* and *Lactobacillus sakei* in nine formulations of lyoprotectants after freeze drying

Lyoprotectant formulations	Composition of lyoprotectant formulations (g/100 g total weight)	Survival (%)[a]± SD	
		Lactococcus lactis	*Lactobacillus sakei*
1	0.97 g lactose, 0.97 g sucrose, 0.97 g trehalose, and 97.10 g soy milk	64.69 ± 1.23CD[b]	65.25 ± 2.58EF
2	4.35 g lactose, 4.35 g sucrose, 4.35 g trehalose, and 86.96 g soy milk	69.10 ± 5.084BC	67.59 ± 1.44DE
3	0.96 g lactose, 0.96 g sucrose, 0.96 g trehalose, 0.96 g sorbitol, and 96.15 g soy milk	61.70 ± 3.49D	70.04 ± 1.34CD
4	9.65 g skim milk, 0.88 g lactose, 0.88 g sucrose, 0.88 g trehalose, and 87.72 g soy milk with distilled water (1:1)	65.95 ± 1.58BCD	64.19 ± 0.65F
5	8.73 g skim milk, 3.97 g lactose, 3.97 g sucrose, 3.97 g trehalose, and 79.40 g soy milk with distilled water (1:1)	70.36 ± 4.66ABC	74.57 ± 0.86A
6	9.57 g skim milk, 0.87 g lactose, 0.87 g sucrose, 0.87 g trehalose, and 86.96 g soy milk with distilled water (1:1)	64.82 ± 0.35CD	73.95 ± 1.08AB
7	9.65 g skim milk, 0.88 g lactose, 0.88 sucrose, 0.88 g trehalose, and 87.72 g distilled water	71.88 ± 2.90AB	71.28 ± 1.41BC
8	8.73 g skim milk, 3.97 g lactose, 3.97 g sucrose, 3.97 g trehalose, and 79.36 g distilled water	75.86 ± 3.41A	74.47 ± 2.88A
9	9.57 g skim milk, 0.88 g lactose, 0.88 g sucrose, 0.88g trehalose, 0.88 g sorbitol, and 86.96 g distilled water	71.18 ± 3.06AB	70.11 ± 1.40CD

[a]Data are means of three replications.
[b]Means within a column with different letters are significantly difference ($P<0.05$).

3.2 Osmotic and cold adaptation of *Lactococcus lactis* and *Lactobacillus sakei* after freeze drying and storage

In this study, the lyoprotectant formulation 8 and formulation 5 were selected to be the freeze-dried suspending medium of *L. lactis* and *L. sakei*, respectively. Of these two bacterial species, some differences in their survival after freeze drying was observed. In *L. sakei*, both adaptation treatments provided higher viability as compared to nonadapted treatment, but not for *L. lactis* (Table 3). However, no significant difference was observed between treatments for both bacterial strains ($P>0.05$).

Table 3 Effect of osmotic and cold adaptation on survival of *Lactococcus lactis* and *Lactobacillus sakei* after freeze drying in lyoprotectant formulation

Types of cells	Survival (%)[a]± SD	
	Lactococcus lactis[b]	*Lactobacillus sakei*[c]
nonadapted cells	75.01 ± 2.96A[d]	71.27 ± 4.96A
osmotic adapted cells	73.18 ± 0.79A	79.16 ± 4.24A
osmotic and cold adapted cells	75.46 ± 0.84A	77.22 ± 1.89A

[a]Data are means of three replications. Percent survival was calculated, with 100% viability representing the colony counts prior to freeze drying.
[b]Freeze dried suspending medium for *L. lactis* contained 8.73 g skim milk, 3.97 g lactose, 3.97g sucrose, 3.97 g trehalose, and 79.36 g distilled water.
[c]Freeze dried suspending medium for *L. sakei* contained 8.73 g skim milk, 3.97 g lactose, 3.97 g sucrose, 3.97 g trehalose, and 79.40 g soy milk with distilled water (1:1).
[d]Means within a column with different letters are significantly difference ($P<0.05$).

After storage at -80°C, viable counts of freeze-dried *L. lactis* osmotically adapted cells and cells treated with both osmotic and cold adaptation were higher than those of nonadapted cells throughout the 28-day storage, but no significant difference ($P>0.05$) was observed (Figure 1a). This indicates that osmotic and cold adaptation may not induce protective effect of *L. lactis* during deep-freezing storage. Similarly, both adaptation treatments did not enhance survival of freeze-dried *L. sakei* during deep-freezing storage (Figure 1b). Osmotic and cold adaptation did not provide cross-protection of *L. lactis* and *L. sakei* after freeze drying. This may be because these bacterial strains may not accumulate compatible solutes inside the cells during osmotic challenge. Glassker *et al.* [16] found that *Lactobacillus plantarum* and other lactic acid bacteria were unable to carry out de novo synthesis of compatible solutes including glycine betaine and proline during exposure to stress condition. These solutes were proved to affect stability of intracellular enzyme [17]. Compatible solutes are solutes accumulated in cells during osmotic stress conditions. They reduce the intracellular a_w without being toxic to enzyme activity and without accumulating an accompanying toxic compound even at high concentration. These solutes can either be synthesized in the cytoplasm or be transported from the culture medium [18]. Therefore, addition of glycine betaine or proline into medium is suggested to enhance survival of *L. lactis* and *L. sakei* after freeze drying and storage. Uguen *et al.* [19] reported that addition of 0.1 mM glycine betaine into medium with 0.4 M NaCl caused higher growth rate of *L. lactis* spp. *lactis* ADRIA 85L030, compared to the growth in the same medium without glycine betaine.

The combination of osmotic (0.3 M sucrose) and cold adaptation (10°C for 2 hrs) did not enhance survival of both bacterial species after freeze drying. Temperature and time for cold adaptation may not be appropriate enough to induce synthesis of cold shock protein which may cause cell protection against ice crystal damage during deep freezing [20].

In conclusion, lactose, sucrose, trehalose, skim milk and soy milk are good lyoprotectants for freeze-dried *L. lactis* and *L.sakei*. The mixture of these compounds could be used to improve viability of these freeze -dried LAB starter.

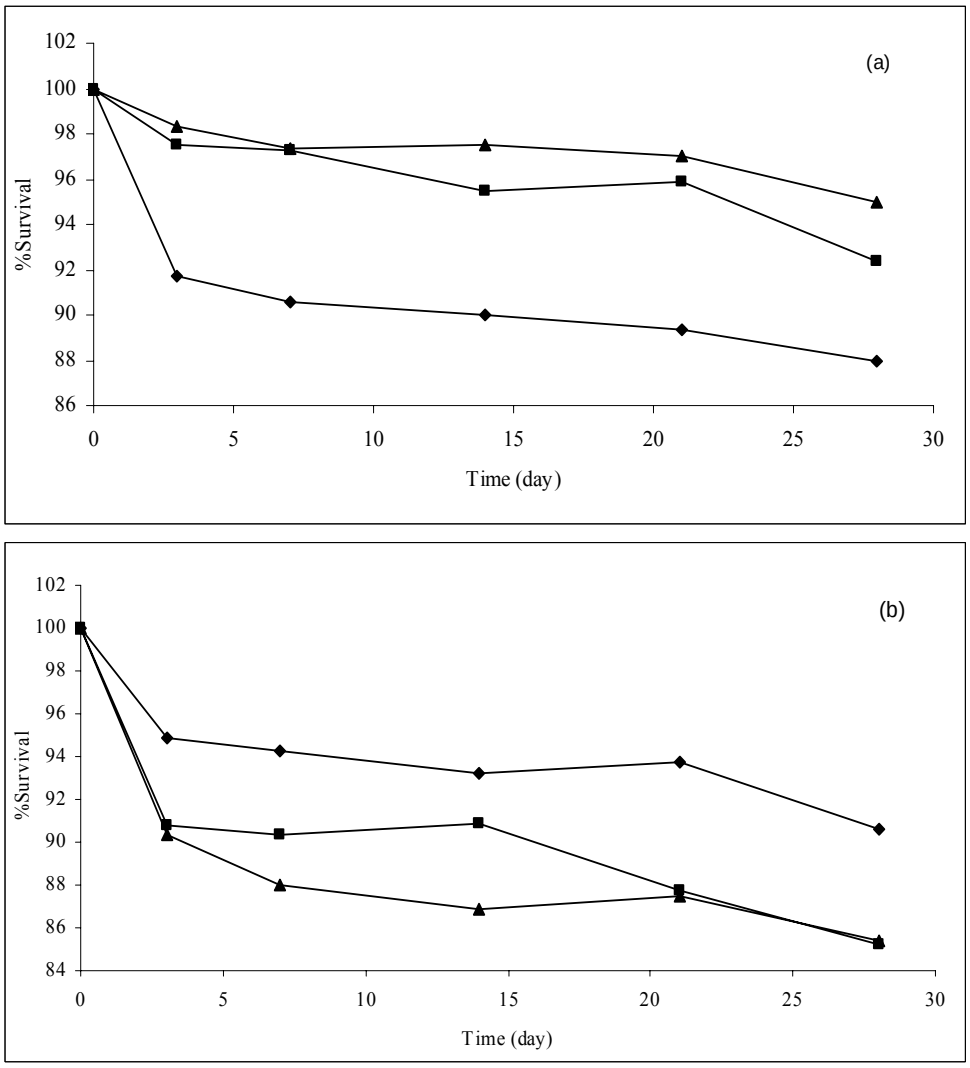

Figure 1 Effect of osmotic and cold adaptation on survival of freeze-dried *Lactococcus lactis* (a) and *Lactobacillus sakei* (b) in lyoprotectant formulations duringstorage at - 80 ° C (Symbol: ♦, nonadapted cells ; ■, osmotic adapted cells ; ▲, osmotic and cold adapted cells)

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