The Effects of Freeze Drying and Rehydration on Survival of Microorganisms in Kefir

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ABSTRACT : The purpose of this research was to study the effect of freeze drying on the microorganisms in kefir. Influences of lyoprotectants and rehydrated media (water at 4°C, 25°C; 10% reconstituted milk at 4°C, 25°C) on the viability of lactic acid bacteria and yeasts in freeze-dried kefir were investigated. Kefir was made from cow milk which was inoculated with 5% kefir grains, and incubated at 20°C for 20 h. Lyoprotectants (galactose, lactose, maltose, sucrose and trehalose) were added independently before dehydration of kefir by freeze drying. Results indicated significant loss in viability of microorganisms in kefir after freeze-drying. Addition of 10% galactose or 10% sucrose as lyoprotectants significantly increased the survival rates of both lactic acid bacteria and yeasts ($p<0.05$). The 4°C rehydration temperature showed the best viabilities for yeasts, however, viability was not significantly affected by rehydration media ($p>0.05$). ***(Asian-Aust. J. Anim. Sci. 2006. Vol 19, No. 1 : 126-130)***

Key Words : Kefir, Freeze-drying, Lyoprotectant

INTRODUCTION

Freeze drying has been a method of choice for the long-term preservation of bioactive materials. This dehydration method causes little shrinkage and results in a completely soluble product that is easily rehydrated. Moreover, lyophilization is frequently used to preserve lactic acid bacterial starter cultures involved in dairy and food fermentations (Lodato et al., 1999). But, not all strains survive the process (Abadias et al., 2001). The major causes of loss cell viability in freeze-drying are probably ice crystal formation and high osmolarity (Conrad et al., 2000). Microbial cell survival during the freeze drying process is dependent on many factors, including protective additives and conditions during rehydration (Font de Valdez et al., 1983). Many compounds have been tested to improve the survival of lactic acid bacteria during freeze drying, including polysaccharides, disaccharides, amino acids and proteins (Champagne et al., 1991). These compounds were in most cases found to be effective toward protection of different lactic acid bacteria (Leslie et al., 1995; Linders et al., 1997; Carvalho et al., 2002). Zayed and Roos (2004) studied the influence of lyoprotectants on survival of *Lactobacillus salivarius* and found that trehalose and sucrose in addition to skim milk were the most efficient materials.

Kefir originated in the Caucasus Mountains of Russia centuries ago and has been credited with various health-promoting properties (Liu et al., 2005). This cultured milk beverage is the result of microbial action of a wide community of microorganisms present in kefir grains on milk. Kefir has a uniform creamy consistency, a slightly acidic taste caused mostly by lactic acid, some effervescence due to carbon dioxide, a minute (<2%) concentration of alcohol due to the action of yeast cells present in the grains, and a variety of aromatic substances including acetaldehyde, acetoin and diacetyl which give it a characteristic flavour. However, lactic acid concentration, acetaldehyde, acetoin and gas production increased during storage (Guzel-Seydim et al., 2000) which limited the market values of kefir drink products. Irigooyen et al. (2005) studied the sensory characteristics of kefir during storage and found that kefir samples revealed maximum acceptability levels in the first 2 days.

Dehydration of kefir and production of instant powders may provide the solution to extend the market values of this drink. However, viable cell number of lactic acid bacteria is an important factor of fermented milk quality (FAO/WHO, 1977). It is also a good index of damage caused by freeze drying. Thus, the objectives of this study were to develop an instant kefir powder product and evaluate the effect of lyoprotectants, rehydration media and temperatures on viability of lactic acid bacteria and yeasts when subjected to a freeze drying and rehydration processes. The endpoint was to extend the shelf life of kefir and increase the market value for this fermented milk product.

MATERIALS AND METHODS

Kefir grains

Kefir grains were collected from Shinchu in northern Taiwan (Lin et al., 1999). Microflora from samples of Taiwanese kefir grain were isolated and identified in our laboratory. The lactic acid bacteria isolated from kefir grains were identified as *Lactobacillus helveticus* and

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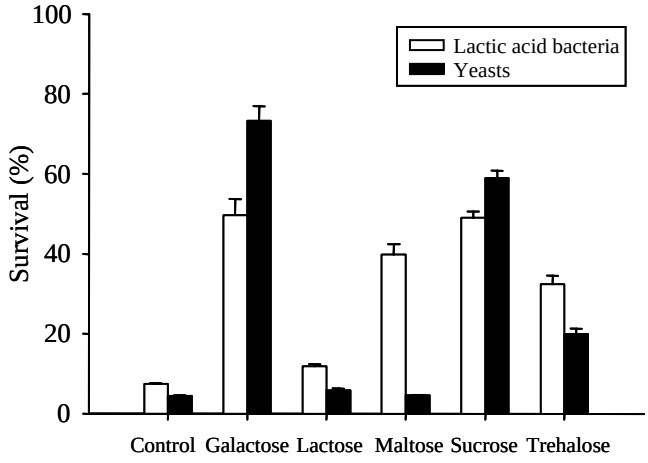

Figure 1. Survival of lactic acid bacteria and yeasts of freeze-dried kefir using 10% lyoprotectants.

Leuconostoc mesenteroides, and the yeasts were identified as *Kluyveromyces marxianus* and *Pichia fermentans*. In the laboratory, they were propagated at 20°C for 20 h with twice-or thrice-weekly transfers in sterilized milk, and kept at 4°C and -80°C for short and long-term storage, respectively.

Kefir preparation

Raw milk was obtained from the National Taiwan University Dairy Farm and heated to 90°C for 30 min in a water bath, before cooling to inoculation temperature. The heat-treated milk was inoculated with 5% (wt/vol) kefir grains and incubated at 20°C for 20 h.

Freeze drying process

For each treatment, 10-ml samples were distributed in 10 autoclaved vials and placed at -20°C for 12 h. After complete freezing, samples were freeze-dried (Labconco Freezone 4.5 liter Benchtop, USA) at a condenser temperature of -40°C for 24 h at a pressure of 1.5-3 mmHg. The freeze-dried samples were rehydrated with different rehydration conditions immediately.

Effect of lyoprotectants and rehydration conditions used in assays

Five sugars [D(+)-galactose, β-lactose, maltose, sucrose and D(+)-trehalose (Sigma, USA)] were selected as lyoprotectants in this study. Fresh kefir was mixed with 10% (wt/vol) of each sugar and freeze-dried. The freeze-dried samples were rehydrated to original volume with deionized water and evaluate the viable cell counts. Two lyoprotectectants (galactose and sucrose) were selected to study the effect of different concentrations (0-10%) of lyoprotectants and rehydration media (deionized water or 10% (wt/vol) skim milk) and temperatures (4 and 25°C) on the viabilities of kefir microorganisms. After all samples were dehydrated, the rehydration was preformed immediately by addition of deionized water or 10% (wt/vol) skim milk at 4 or 25°C. All experiments were repeated three times.

Enumeration of microorganisms

Lactic acid bacteria were enumerated on MRS agar (Oxoid, England) with 200 ppm cycloheximide (Sigma, U.S.A.) to inhibit the growth of yeasts. The plates were incubated at 37°C for 4 days. Yeasts were examined on potato dextrose agar (Difco, Detroit, USA) with 100 ppm chlortetracycline (Sigma, USA) to inhibit the growth of other bacteria. The plates were incubated at 25°C for 2 days (Lin et al., 1999). The resulting colonies from samples taken before and after freeze drying were counted and the percentages of surviving lactic acid bacteria and yeasts were also estimated.

Scanning electron microscopy

The microstructures of the kefir grains were observed by scanning electron microscope (SEM) according to the methods of Lin et al. (1999) and Chen et al. (2005). In brief, pieces of samples were fixed in 30 g/L glutaraldehyde in 0.1 M phosphate buffer (pH 7.0) at 25°C for 4 h. Samples were washed with 3 changes of buffer and then post-fixed with 10 g/L osmium tetroxide in the same buffer at 25°C for 1 h. After washing in distilled water, samples were dehydrated in an ethanol series: 15, 30, 50 and 70% for 10 min each; 85 and 95% for 15 min each and 100% for 1 h. The resulting specimens were critical-point dried with CO_2 using a Critical Point Dryer Samdri-PVT-3B (Tousimis, Rockville, MD., USA). The samples were fixed in stubs on a double-faced metallic tape and covered with a fine layer of gold (Ion Coater JJFC1100E; JEOL Ltd., Japan) while applying a current of 40 mA. Observations were made using an SEM (JSM-6300, JEOL Ltd., Japan).

Statistical analysis

Statistical analysis was performed using the ANOVA general linear models (GLM) procedure and Duncan's multiple range test with Statistical Analysis Systems software (SAS Institute Inc., 2001). Statistical significance was judged at the level $p<0.05$. Experiments were performed three times.

RESULTS AND DISCUSSION

Effect of freeze drying process on the survival and structure of microorganism in kefir

Kefir was subjected to freeze drying process to evaluate the survival of lactic acid bacteria and yeasts. The loss in viability of microorganisms in kefir was found after freeze-drying (Figure 1). The survival of lactic acid bacteria and

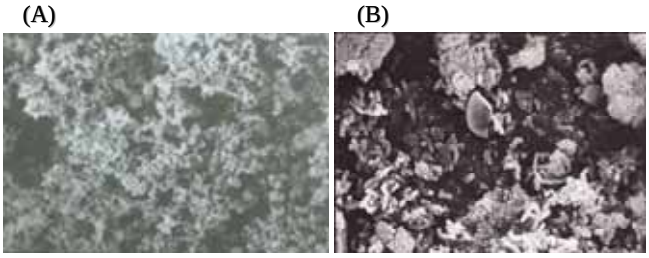

Figure 2. Scanning electron micrographs of fresh and freeze-dried kefir. (A) fresh kefir (×3,000), (B) freeze-dried kefir (×3,000).

yeasts without lyoprotectants was less than 10%. Furthermore, studying the microorganism structure in freeze-dried kefir found that cell-surface damage with extensive cell wall pitting, wrinkling and cell disruption were observed (Figure 2B). Whereas, the cell surface in fresh kefir appeared smoother without any cell-wall disruption (Figure 2A). Champagne et al. (1991) explained that the ice formation and increase in salt concentrations within and around cells as dehydration progresses might be responsible for damage of cell membranes. Brennan et al. (1986) reported that freeze drying of *L. acidophilus* removed the bound water, destabilizing the structural integrity of macromolecules present in the cell wall.

Effect of lyoprotectants

Five different carbohydrates were applied individually as lyoprotectants to compare their protective abilities for microorganisms in freeze dried kefir. Results indicated that addition of 10% lyoprotectants including galactose, maltose, sucrose and trehalose significantly improved the viabilities of lactic acid bacteria and yeasts after freeze-drying, with galactose and sucrose providing the best protection ($p<0.05$) (Figure 1).

Many studies (Champagne et al., 1991; Leslie et al., 1995; Carvalho et al., 2002) reported that the carbohydrates are able to lower the transition temperature of dry membranes, via replacement of water between the lipid headgroups. This phenomenon prevents phase transition and its accompanying leakage during rehydration. Heckley and Quay (1983) reported that sugars such as sucrose inhibited free radical production that was associated with loss of viability of lyophilized bacteria exposed to air. Zayed and Roos (2004) studied the influence of trehalose on survival of *Lactobacillus salivarius* and found that trehalose greatly enhanced survival rate when used alone or together with other protective materials. The disaccharide trehalose acts as a critical membrane-protecting agent for yeast cells during environmental stress conditions such as dehydration (Diniz-Mendes et al., 1999).

In our own findings, addition of 10% sucrose or 10% galactose increased the survival rate for both lactic acid bacteria and yeasts by more than 50%, while addition of

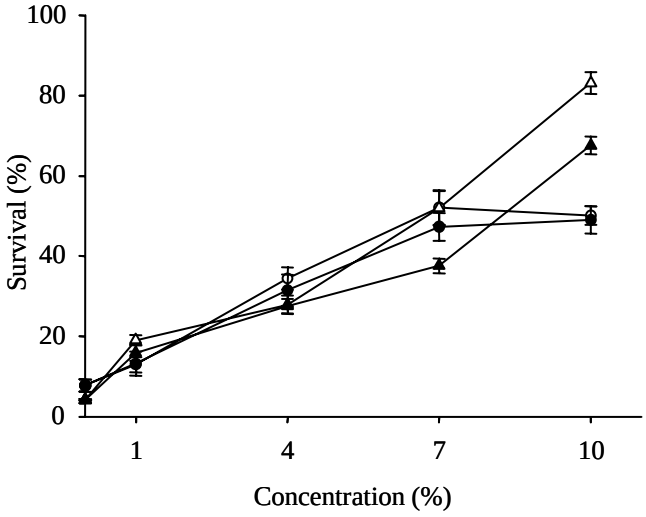

Figure 3. Survival of lactic acid bacteria and yeasts in kefir after freeze-drying using different concentrations of lyoprotectants. ○: lactic acid bacteria with galactose; ●: lactic acid bacteria with sucrose; △: yeasts with galactose; ▲: yeasts with sucrose.

10% trehalose only increased survival by 20% (Figure 1). Therefore, galactose and sucrose were chosen for further study.

The effect of lyoprotectant concentrations on the viabilities of microorganisms after freeze drying was studied. The results given in Figure 3 showed that the survivals of lactic acid bacteria and yeasts were increased with increasing lyoprotectant concentrations. Addition of 10% galactose or sucrose had the best survival for yeast, while addition of 7-10% showed the highest surivival for lactic acid bacteria. Conrad et al. (2000) studied the stabilization of *L. acidophilus* in saccharide matrices and demonstrated increasing in amounts of carbohydrate improves their stability during storage. Ten percentage of galactose and 10% sucrose were used for the following study.

Effect of rehydration conditions

Rehydration is an important step in the recovery of microorganisms from dried products. A microorganism which survives as freezing, drying and storage, may lose its viability during rehydration (Fry and Greaves, 1966). Poor recovery of organisms may be attributed to inadequate rehydration procedure. Table 1 shows the influence of rehydration temperature and medium on the survival of organisms in kefir. Results indicate that yeasts showed a better survival than lactic acid bacteria after rehydration. This may be due to cell wall damage of lactic acid bacteria during freeze drying process of kefir. This phenomena increased cell linkage of nucleic acid and decreased the survival of microorganisms in freeze dried kefir. Furthermore, differences in cell wall and membrane

Table 1. Survival of microorganism (%) in different combinations of lyoprotectants and rehydration conditions for freeze dried keifr

	Survival of bacteria (%)			Survival of yeasts (%)		
	None	Galactose	Sucrose	None	Galactose	Sucrose
25°C						
water	7.42^{ay}	49.63^{bx}	48.99^{bx}	4.42^{ax}	73.33^{cx}	58.90^{bx}
10% SM	7.00^{ay}	46.67^{bx}	49.63^{bx}	4.79^{axy}	75.94^{cxy}	63.83^{bxy}
4°C						
water	6.24^{ax}	42.67^{bx}	49.03^{cx}	5.57^{ay}	84.15^{cy}	68.43^{byz}
10%SM	6.84^{axy}	44.68^{bx}	49.55^{cx}	5.62^{ay}	81.30^{cxy}	71.66^{bz}

Values in the same column with different letter $^{x, y, z}$ and the same row with different letters $^{a, b, c}$ were significantly different by Duncan's multiple range test ($p<0.05$). SM: Skim milk.

composition, with different melting points of its phospholipids, caused differences between strains and species.

Other workers (Champagne et al., 1991; Carvalho et al., 2004) suggested that a rehydration medium composed of 10% reconstituted skim milk substantially increased recovery of cells. In our research, however, there were no significant differences between water and 10% skim milk as rehydration medium ($p>0.05$) for the viabilities of microorganisms in freeze dried kefir (Table 1). This is probably because kefir was made from raw milk. Thus, it already contained the milk components that protect to recover the microorganisms during rehydration.

Rehydration of freeze-dried kefir at 4°C showed a higher viability for yeasts, however, rehydration temperature did not affect the viabilities of lactic acid bacteria in freeze dried kefir (Table 1). Several investigators have reported that rehydration at the refrigeration temperature may cause leakage of intracellular substances from the cells, thereby resulting in low viability. Our own finding was somewhat contradictory for yeast results.

CONCLUSIONS

In summary, addition of 10% galactose or 10% sucrose as lyoprotectants significantly improved the viabilities of lactic acid bacteria and yeasts ($p<0.05$) for freeze-dried kefir. Blending freeze dried kefir with water at 4°C increased viability for yeasts.

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