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Effect of protective agents, freezing temperature, rehydration media on viability of malolactic bacteria subjected to freeze-drying

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ABSTRACT

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Aims: The effects of protective agents, rehydration media and freezing temperature on the viabilities of *Lactobacillus brevis* and *Oenococcus oeni* H-2 when subjected to freeze-drying were investigated.

Methods and Results: Several protectants and rehydration media were tested to improve the survival after freeze-drying. The cells were also frozen at −65 and −20°C to check the effect of freezing temperature on the viability.

Conclusions: The best protectant and rehydration medium to obtain the highest viability after freeze-drying varied with the species of bacteria. Yeast extract (4·0%) and sodium glutamate (2·5%) gave maximum viability of *L. brevis* and *O. oeni* (67·8% and 53·6% respectively). The highest survival of *L. brevis* and *O. oeni* were obtained when rehydrated with 10% sucrose and MGY medium respectively. When the bacterial cells were frozen quickly (−65°C) than slowly (−20°C), *L. brevis* and *O. oeni* both showed increased viability after freeze-drying.

Significance and Impact of the Study: The viabilities of *L. brevis* and *O. oeni* after freeze-drying were shown to be strain specific and dependent on protective agents, rehydration media and freezing temperature.

Keywords: freeze-drying, lactic acid bacteria, malolactic fermentation, viability.

INTRODUCTION

Malolactic fermentation (MLF) in wine production is an important process. MLF is the bioconversion of L-malic acid to L-lactic acid and CO_2, causing a decrease in acidity of the wine. MLF results from the metabolism of certain lactic acid bacteria (LAB) in wines, mainly *Oenococcus oeni* (Kunkee 1967; Davis *et al.* 1985; Maicas *et al.* 1999a,b). MLF is essential for nearly all red wines and some white wines. As well as decreasing wine acidity, MLF can improve the flavour of wines and increase the microbiological stability of wines (Gao and Fleet 1994; Henick-Kling 1995; Maicas *et al.* 1999c). Traditionally, MLF is allowed to develop spontaneously by the growth of LAB naturally present in wine. However, this natural process is slow and unreliable. It may take weeks or months, and the results are unpredictable because MLF is working in extreme environments of high alcohol and SO_2, low pH such that growth is marginal. In recent years starter culture technology, involving the inoculation of *O. oeni* into wines, has been developed for inducing MLF (Edwards *et al.* 1991; Nielsen *et al.* 1996).

Freeze-drying is widely used in production of starter cultures of LAB. However, during such a treatment bacterial cells are exposed to freezing and drying processes that subject the cells to the stresses of high concentration of solutes including extremes of pH, to low temperature, to the formation of ice crystals and to the remove of water from within the cell. Freeze-drying might cause cell membrane damage, protein and DNA denaturation, and decreased cell survival (Panoff *et al.* 1998; Wolfe and Bryant 1999). In order to use the starter cultures of malolactic bacteria as commercial products it is necessary to optimize the process of freeze-drying and obtain maximum survival.

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Bacterial cell survival during freeze-drying process is dependent on many factors, including the growth conditions (Palmfeldt and Hahn-Hägerdal 2000), the protective medium (Font de Valdez *et al.* 1983), the initial cell concentration (Bozoglu *et al.* 1987; Costa *et al.* 2000), the freezing temperature (Sanders *et al.* 1999), and the rehydration conditions (Sinha *et al.* 1982; Font de Valdez *et al.* 1985a,b). Protective agents have an important role in the conservation of viability. A good protectant should provide cryoprotection to the cells during the freezing process, be easily dried, and provide a good matrix to allow stability and ease of rehydration. Various groups of substances have been tested for their protective action, including polyols, polysaccharides, disaccharides, amino acids, proteins, minerals, salts of organic acids and vitamins-complex media (Champagne *et al.* 1991; Hubálek 2003). However, protection afforded by a given additive during these processes will vary with the species of micro-organism (Font de Valdez *et al.* 1983).

Freezing temperature (cooling rate) is also a critical factor in freeze-drying process. If cooling is slow enough, water will have time to flow out of the cell by osmosis, ice crystals form extracellularly. As ice forms, water is removed from the extracellular environment, and the concentration of solutes increase and an osmotic imbalance occurs. If cooling is very fast, the cell does not loose water quickly enough to maintain equilibrium and ice crystals eventually form intracellularly. Ice formation inside the cell may cause lethal damage. The optimum rate of freezing varies from one genus to another (Champagne *et al.* 1991; Sanders *et al.* 1999).

Rehydration is a critical step in the recovery of freeze-dried bacteria. Direct rehydration of *O. oeni* in wine for the purpose of MLF, however, can result in mortality of 95% of the freeze-dried culture (Davis *et al.* 1985). Cells that are subjected to sublethal injury may not be able to repair the damage that has occurred if they are rehydrated under inappropriate conditions (Font de Valdez *et al.* 1985a; Theunissen *et al.* 1993). The rehydration medium itself, its molarity and the rehydration conditions can significantly affect the rate of recovery (Ray *et al.* 1971; Font de Valdez *et al.* 1983).

The aim of this paper was to evaluate the effect of protective agents, freezing temperature and rehydration media on the viabilities of two malolactic bacteria, *O. oeni* H-2 and *Lactobacillus brevis*, when subjected to a freeze-drying process.

MATERIALS AND METHODS

Bacterial strains

Lactobacillus brevis and *O. oeni* H-2 were obtained from Alcoholic Beverage Research Institute, Hebei University of Science and Technology, Hebei, China. They were isolated from the red wines produced in Great Wall Wine Company and used to carry out MLF.

Cell preparation

The medium (MGY) used comprise (g l^{-1} in distilled water): glucose, 10·0; fructose, 8·0; yeast extract (YE), 10·0; $(NH_4)_2SO_4$, 2·0; KH_2PO_4, 2·0; Tween-80, 1·0; $MgSO_4 \cdot 7H_2O$, 0·2; $MnSO_4 \cdot 4H_2O$, 0·05; $CaCl_2 \cdot 4H_2O$, 0·3, and pH of the medium was about 5·8.

The bacteria were cultured in MGY media at 25°C. When the cells had grown up to early stationary phase, the bacterial cells were harvested by centrifugation (13 000 *g* for 6 min). The cell paste was resuspended in 0·05 mol l^{-1} phosphate buffer (pH 6·5) and a volume of resuspended cells was dispersed into the protective medium. This suspension was incubated for 20 min at room temperature (about 25°C) to allow cell adaptation.

Freeze-drying

Serum bottles (50 ml) were filled with 10 ml of bacterial suspension produced as described above and frozen at −20°C in a static state for 24 h. After overnight storage in the freezer, samples were desiccated in an Edwards freeze dryer at a condenser temperature −45°C, and at a chamber pressure <0·06 mbar for 24 h. The end temperature of samples is 28°C. After freeze-drying, the serum bottles were sealed under vacuum.

Rehydration

After freeze-drying, samples were immediately brought to their original volume (10 ml) with each rehydration medium at 25°C. Then, samples were homogenized for 1 min with a Vortex mixer (SA-5, Stuart Scientific, Redhill, UK) and incubated at room temperature for 15 min. Serial dilutions were spread-plated onto the surface of Petri plates containing MGY medium agar (MGY medium and 2·0% agar). These plates were incubated at 27°C in an anaerobic system for about 72 h, and the viability was then determined. Survival levels were expressed as the quotient of colony-forming units per millilitre (CFU ml^{-1}) on MGY medium before (N_0) and after (N_f) freeze-drying. Viability = $(N_f/N_0) \times 100$.

Protectants used in assays

Suspensions of protectants were prepared in distilled water. The additives tested as protective agents against freeze-drying injury were divided into four groups: (i) sugars (10·0%): trehalose, lactose, maltose, sucrose, fructose and glucose; (ii) amino acids: sodium glutamate (2·5%) and yeast extract (4·0%); (iii) polyols (5·0%): sorbitol and mannitol;

(iv) others: MGY medium and phosphate buffer (pH 6·5), as control. These protectants were selected on the basis of previous studies on other micro-organisms (Font de Valdez *et al.* 1983; Champagne *et al.* 1991). Another factor to be considered is that protectants used cannot influence the quality of wines such as taste and colour when the starter cultures with protectants are inoculated into wines. Protectant solutions were sterilized at 121°C for 10 min before mixing with a volume of washed cells of the malolactic bacteria to obtain an initial concentration of 1·5–$4{\cdot}0 \times 10^9$ CFU ml^{-1}. The general procedure for cell preparation, freeze-drying and rehydration was described above. After freeze-drying, all samples were rehydrated with fresh MGY medium to the original volume and the level of survival was evaluated.

Effect of freezing temperature on survival of the bacteria after freeze-drying

The bacterial suspension that had an initial concentration $2{\cdot}6 \times 10^9$ CFU ml^{-1} with 10·0% lactose solution was equally dispensed into two sterile serum bottles. One bottle was directly frozen without shaking at −20°C for 24 h. Another was frozen fast by shaking it in the mixture of dry ice and methanol (−65°C), and kept it at −20°C for 24 h. The general methodology of cell preparation, freeze-drying and rehydration was as previously described.

Rehydration assay media

In this assay, the initial concentration used was $3{\cdot}0 \times 10^9$ CFU ml^{-1} and 10% lactose was used as the protectant. Freeze-dried samples were rehydrated with the following rehydration media: sucrose (10·0%), lactose (10·0%), maltose (10·0%), glutamate (5·0 g l^{-1}), $CaCl_2$ (2·0 g l^{-1}), $MnCl_2$ (2·0 g l^{-1}), MGY medium, distilled water and phosphate buffer (pH 6·5, as control). Each rehydration medium was also used for serial dilution plating to assess viability of cells. The general procedure of cell preparation, freeze-drying and rehydration was as described above.

Reproducibility

All results presented in this paper are the average of three independent replicate assays. The variations were <5%.

RESULTS

Assay of protective agents

Using a range of protectants, significant differences in the viabilities of cells of *L. brevis* and *O. oeni* after freeze-drying were observed, depending on the protectant used (Table 1). The best protection was given by amino acids. Yeast extract (4·0%) and sodium glutamate (2·5%) were the best protectants for *L. brevis* and *O. oeni*, giving cell viability 67·8 and 53·6% respectively.

Table 1 Comparative effect of different protective agents on viability (%) of *Lactobacillus brevis* and *Oenococcus oeni* after freeze-drying and rehydrating with phosphate buffer

Protectants	*L. brevis*	*O. oeni*
10% Sucrose	45·1	38·2
10% Lactose	47·8	32·4
10% Trehalose	56·8	40·2
10% Maltose	41·2	27·6
10% Glucose	18·6	9·1
10% Fructose	14·2	10·2
2·5% Sodium glutamate	49·2	53·6
4% Yeast extract	67·8	32·5
5% Sorbitol	10·6	9·8
5% Mannitol	11·8	8·1
MGY medium	21·3	13·4
Phosphate buffer	8·7	7·2

Sugars could be divided into two groups: disaccharides (sucrose, trehalose, maltose and lactose) and monosaccharides (glucose and fructose). Disaccharides had significant protection of *L. brevis* and *O. oeni* after freeze-drying. For example, disaccharides gave cell viability of *L. brevis* >40%. On the contrary, monosaccharides offer much less protection than disaccharides, giving cell viabilities of <20%. MGY medium also provided some protection of *L. brevis* and *O. oeni* from freeze-drying. Polyols tested showed very low viabilities, and there was no significant difference from the control (phosphate buffer).

Effect of freezing temperature on the survival after freeze-drying

Different freezing temperature causes different cooling rate as heat transfer rate is changed. Certainly, the lower the temperature the faster cooling rate. Shaking increase the heat transfer. Table 2 shows the effect of freezing tempera-

Table 2 Effect of freezing temperature on the viabilities (%) of *Lactobacillus brevis* and *Oenococcus oeni* after freeze-drying and rehydrating with phosphate buffer

Freezing temperature	*L. brevis*	*O. oeni*
−65°C	65·2	42·5
−20°C	46·4	31·3

10% lactose was used as protectant.

ture on the cell viabilities of *L. brevis* and *O. oeni* after freeze-drying. *Lactobacillus brevis* and *O. oeni* both had higher survival rate when the cells were frozen quickly (−65°C) than those frozen slowly (−20°C). These results indicated that freezing temperature was an important factor that affected the cell viability, and under the condition of fast cooling rate *L. brevis* and *O. oeni* obtained higher survival rate after freeze-drying.

Assay of rehydration media

Percentage viability of the malolactic bacteria with different rehydration media, and 10% lactose as a protectant, are shown in Table 3. All samples were rehydrated to the initial volume, because a rule of thumb is to rehydrate the culture back to the volume it had prior to being freeze-dried (Champagne *et al.* 1991).

The results indicated that there were differences in viabilities of the bacteria depending on the rehydration media used. As shown in Table 3, rehydration in 10% sucrose solution gave the best recovery of *L. brevis* (57·7%) among the rehydration media tested. Lactose, maltose and $CaCl_2$ also improve the recovery of *L. brevis*. In contrast, rehydration in distilled water show the lowest survival rate (37·4%). Other rehydration media (sodium glutamate, $MnCl_2$ and MGY medium) showed no significant difference from the control (phosphate buffer).

The case with *O. oeni* was different from *L. brevis*. When the freeze-dried *O. oeni* was rehydrated in MGY medium, the cell viability of *O. oeni* was the highest (45·2%) and the cell recovery was improved significantly. Surprisingly, rehydration in 10% sugar solutions (sucrose, lactose and maltose) made the cell viability of *O. oeni* decrease obviously, compared with the control (phosphate buffer). It was also observed that sodium glutamate, $MnCl_2$, $CaCl_2$ and distilled water gave slightly lower recovery than phosphate buffer.

Table 3 Effect of rehydration media on the viabilities (%) of *Lactobacillus brevis* and *Oenococcus oeni* cells freeze-dried in 10% lactose as protectant

Rehydration media	*L. brevis*	*O. oeni*
10% Sucrose	57·7	22·5
10% Lactose	51·8	19·7
10% Maltose	52·0	23·6
5 g l^{-1} Sodium glutamate	48·3	30·6
2 g l^{-1} $MnCl_2$	44·1	28·0
2 g l^{-1} $CaCl_2$	51·6	29·8
MGY medium	44·9	45·2
Phosphate buffer	46·1	32·1
Distilled water	37·4	30·7

DISCUSSION

The industrial use of LAB as starter cultures for the food industry depends on the concentration and preservation technologies employed, which are required to guarantee long-term delivery of stable cultures in terms of viability and functional activity (Carvalho *et al.* 2003). Freeze-drying has commonly been used for this purpose, but these techniques bring about undesirable side-effects, such as denaturation of sensitive proteins and decreased viability of many cell types (Leslie *et al.* 1995). Maximization of survival of LAB cultures during drying and subsequent storage for long periods is of vital importance, both technologically and economically.

The differences exhibited in cell survival in this study indicate that certain additives are more effective than others in protecting the malolactic bacteria. Maximum protection of cells of the bacteria during freeze-drying was achieved with amino acid group, 4·0% yeast extract for *L. brevis* and 2·5% sodium glutamate for *O. oeni*. This protection by amino acids was thought to be the result of a reaction between the carboxyl groups of the bacterial proteins and the amino group of the protectant, stabilizing the proteins structure (Moriche 1970). Cysteine, however, was thought to prevent the oxidation of SH groups to intermediate SS bonds (Font de Valdez *et al.* 1983). Yeast extract contains many amino acids, mainly glutamate. According to the results, amino acids have different protective ability on the viability and are related to the species of bacteria. Sugars also were found to provide good protection on the bacteria during freeze-drying. Sugars replace structural water in membranes after dehydration (Clegg 1986; Crowe and Crowe 1986) and prevent unfolding and aggregation of proteins by hydrogen bonding with polar groups of proteins (Hanafusa 1985; Carpenter *et al.* 1990). Differences exhibited by sugars are connected with their water-binding capacity and prevention of intracellular and extracellular ice crystal formation (Baumann and Reinbold 1964; Burke 1986). Polyols (sorbitol and mannitol) tested in this study were not effective in protecting cells of the malolactic bacteria. Font de Valdez *et al.* (1983) also found that sorbitol and mannitol provided little or no protection of 12 strains of lactococci, lactobacilli and *Leuconostoc* when added to milk. Mannitol crystallizes readily when its aqueous solutions are freeze-dried (Kim *et al.* 1998). Crystallization of mannitol may decrease its ability to stabilize freeze-dried β-galactosidase (Izutsu *et al.* 1993). The relationship between crystallization of protectants and their protection of bacterial cells from freeze-drying was not examined in this study. The fresh MGY medium also contained yeast extract, but viability with MGY medium was much lower than that with 4·0% yeast extract. The reason may be that the concentration of yeast extract in the MGY medium was too low to provide enough protection.

The freezing process involves complex phenomena that, even after decades research, are not fully understood. When the bacterial cell was frozen, ice crystals form at different rates. During slow cooling, freezing occurs external to the cell before intracellular ice crystals begin to form. As ice crystals form, water is removed from the extracellular environment, and the concentration of solutes increase and an osmotic imbalance occurs across the cell membrane, leading to water migration out of the cell. The increase in solute concentration (osmotic stress) outside the cell, as well as intracellular, can be detrimental to cell survival. If too much water remains inside the cell, damage occurs due to ice crystal formation. The rate of cooling has a dramatic effect on these phenomena. Rapid cooling minimizes the solute concentration effects as ice forms uniformly, but leads to more intracellular ice crystals. Slow cooling, on the contrary, results in a greater loss of water from the cell and less internal ice crystals, but increases the solute concentration effects. Ice crystal formation and increase in solute concentration within and around the cells might be responsible for damage of cell membranes during freezing process (Mazur 1970; Fonseca *et al.* 2001). Slow cooling produces solute effects whereas rapid cooling results in more intracellular ice crystals. Both can be lethal (Heckly 1985). The optimum rate of freezing varies from one genus to another (Champagne *et al.* 1991; Sanders *et al.* 1999). In this study it was found that *L. brevis* and *O. oeni* showed increased viability after freeze-drying when the bacterial cells were frozen quickly (−65°C) than slowly (−20°C). According to this result, it can be speculated that cells of *L. brevis* and *O. oeni* might be more sensitive to osmotic stress outside the cell than formation of ice crystals inside the cell, and the viability was lower when the cells were frozen slowly.

When using freeze-dried micro-organisms, rehydration has been considered as an important step. Freeze-dried bacteria suspensions usually contain dead cells and unharmed cells as well as those sub-lethally injured. The injured cells may repair and regain normal function if they are rehydrated under appropriate conditions (Font de Valdez *et al.* 1985a,b; Theunissen *et al.* 1993). The results in this study showed that rehydration media had a significant influence on the cell viability, and the effect of rehydration media on viability varied with the species of bacteria. As shown in Table 3, *L. brevis* obtained the best recovery when the freeze-dried cells were rehydrated in 10% sucrose solution, whereas rehydration with MGY medium gave maximum viability of *O. oeni*. The results indicated that rehydration media showed different capacities for injury repair, which were species dependent. Sucrose was a good rehydration medium for cells of lactic streptococci (Sinha *et al.* 1982), with similar results reported by Record *et al.* (1962) for *Escherichia coli* and other Gram-negative organisms. However, it was also noted that cell viability of *O. oeni* decrease when the freeze-dried *O. oeni* were rehydrated with 10% sugar solution, compared with phosphate buffer, and MGY medium were the best rehydration medium for *O. oeni*. The possible reason was that there was osmotic pressure shock and *O. oeni* was very sensitive to this shock. Font de Valdez *et al.* (1985b) also found that diluent medium (1·5% peptone, 1% tryptone and 0·5% meat extract) gave the best recovery for many LAB.

In conclusion, this study has shown that the survival of malolactic bacteria, *L. brevis* and *O. oeni*, when subjected to freeze-drying is dependent on the protective medium used, freezing temperature and the rehydration medium. An appropriate selection of these factors is essential for obtaining maximum viability of cells. Because freeze-dried malolactic bacteria will be inoculated into wines, further research should focus on ethanol tolerance of freeze-dried bacteria to ensure successful MLF. It would also be important to study the stability of freeze-dried malolactic bacteria. These findings are necessary for the industrial development of the starter culture formulation.

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