Evidence of membrane damage in *Lactobacillus bulgaricus* following freeze drying

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H.P. CASTRO, P.M. TEIXEIRA AND R. KIRBY. 1997. The mechanism of inactivation of *Lactobacillus bulgaricus* due to freeze drying was investigated. Cells were freeze-dried in skim milk powder, maltodextrin, glycerol, trehalose and water. Results are presented confirming previous authors' observations regarding membrane damage during freeze drying. In an attempt to define more clearly the nature of this damage, further experiments were carried out. Results show that following freeze drying changes occur in the unsaturated : saturated fatty acid ratio, a decrease in the activity of the membrane-bound enzyme ATPase and a loss of ΔpH.

INTRODUCTION

Freeze drying is a process in which a solvent (usually water) is removed from a frozen solution or a frozen foodstuff by sublimation. Since freeze drying is a low-temperature process during which the chemical alteration of the product is minimized, it has several advantages over competitive desiccation processes, especially when used for biological materials and temperature-sensitive products (Rovero *et al.* 1991).

The addition of certain agents, like sugars, amino acids, peptides, proteins or other substances, to suspending fluids is known to improve survival through the process (Strange and Cox 1976). Many authors have reported the positive effects of using skim milk powder (SMP) as a suspending agent prior to freeze drying (Morichi and Irie 1973; Sinha *et al.* 1974). Even so, under favourable conditions the percentage of organisms that die during freeze drying might be greater than the percentage that fail during subsequent storage (Bozoglu *et al.* 1987). When bacterial cultures are lyophilized, however, the process of freezing and drying can cause cell injury. There are indications that the cell wall, cell membrane and ribonucleic acid are involved (Wagman 1960; Sinskey and Silverman 1970). Lievense *et al.* (1994) considered the cytoplasmic membrane to be the principal site of lethal damage.

The purpose of this work was to examine the behaviour of *Lactobacillus bulgaricus* during lyophilization in order to identify the reasons for loss of viability. A brief evaluation of the damage caused by freezing and how to avoid it using suitable cryoprotectants was made in order to make possible an evaluation of the damage caused during the drying stage.

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Previous authors have used chemical markers in the recovery medium to indicate sites of lethal damage. Examples of chemical markers include sodium chloride for cell membrane damage (Ray 1993), chloramphenicol for protein synthesis (Sogin and Ordal 1967), rifampicin for ribosome damage (Johnson *et al.* 1984), actinomycin D for ribosome damage (Hurwitz *et al.* 1962) and pyronin Y for DNA damage (Onishi *et al.* 1977; Asada *et al.* 1980).

Early experiments confirmed the conclusions of Lievense *et al.* (1994). Membrane damage was therefore studied in more detail. Changes in membrane permeability were studied by measuring leakage of β-galactosidase into supernatant fluid (Teixeira *et al.* 1995) and potassium : sodium ratios (Hurst *et al.* 1973). Changes in membrane function were studied by measuring changes in ΔpH (Rottenberg 1979) and ATPase activity (Belli and Marquis 1991). Changes in membrane structure were studied by measuring the unsat/sat lipid ratio (Uchida 1975).

MATERIALS AND METHODS

Organism

Lactobacillus delbrueckii ssp. *bulgaricus* NCFB 1489 (National Collection of Food Bacteria, UK) was used throughout the study. The original reference cultures were maintained in cryogenic storage at −80°C on glass beads. Working cultures were maintained as stabs on MRS agar (Lab M; MRSA) at 4°C. Stabs were prepared from cultures grown at 37°C for 24 h aerobically in a shaking water bath. Fresh stabs were prepared from working cultures every month. New working cultures were prepared from the original reference culture every 3 months.

Media

Unless otherwise stated all media were prepared and sterilized according to the manufacturers' instructions. The growth media used in this study were Man, Rogosa and Sharpe broth (De Man *et al.* 1964; MRS) and MRS agar (MRSA) from Lab M. The phosphate buffer diluent was prepared by mixing 0·01 mol l^{-1} K_2HPO_4 (Merck, Frankfurt, Germany) and 0·01 mol l^{-1} KH_2PO_4 (Merck), both dissolved in a solution of 0·15 mol l^{-1} NaCl (Merck), adjusted to pH 7·0 ± 0·1 and sterilized by autoclaving (121°C/15 min).

Culture preparation

To ensure culture homogeneity between experiments and to model industrial protocols, the following procedure was used. Ten ml of MRS broth were inoculated from the MRSA stabs and incubated for 24 h at 37°C, with gentle shaking. This first broth was subsequently subcultured into a second MRS broth (1% inoculum) and incubated as before. This broth was then used to inoculate 1 l of a third MRS broth (1% inoculum). The cultures were incubated at 37°C for 16 h and agitated using magnetic stirrer bars to produce early stationary phase cells. Cells were harvested by centrifugation at 16 000 *g* at 4°C and washed twice with cold deionized water (4°C). Cells were then suspended in deionized water and aqueous suspensions of the following additives (pH 6·3 ± 0·1): skim milk powder (SMP) (11% w/v), glycerol (1 mol l^{-1}), maltodextrin (11% w/v) and trehalose (5% w/v). Cells were suspended in the cryoprotectant at 10°C for 10 min prior to freezing to allow for equilibration between cell and cryoprotectant. About 10^9 cells ml^{-1} were present in the suspending media prior to lyophilization.

Lyophilization

Samples were first frozen at −80°C in a deep freezer and then desiccated under vacuum (50 mtorr) in the freeze-drier (Martin Christ Alpha 1–4).

Enumeration

Prior to plating, frozen samples were thawed at room temperature and freeze-dried samples were rehydrated with sterile distilled water at room temperature (20°C) and immediately agitated. Viability was determined immediately after freeze drying on solid media by the Miles and Misra (1938) technique. Plates were incubated at 37°C for 48 h before enumeration.

Selective media for the detection of sublethally injured organisms were as follows: MRSA plus NaCl (6·25 mg ml^{-1}), MRSA plus chloramphenicol (0·313 μg ml^{-1}), MRSA plus rifampicin (0·125 μg ml^{-1}), MRSA plus actinomycin D (0·125 μg ml^{-1}) and MRSA plus pyronin Y (5 μg ml^{-1}). Each agent was made up fresh and sterilized by filtration (membrane filter pore size 0·22 μm). Each selective agent was used at the minimum inhibitory concentration previously determined. MRSA was prepared as above, sterilized by autoclaving, cooled to 50°C before selective agents were added prior to distribution into Petri dishes.

Water activity measurement

The water activity (a_w) of the freeze-dried cells was measured using a Nova Sina water activity meter at 20°C. Salt solutions in deionized water of known water activities were used for calibration of the sensors. Sensors were calibrated at the same temperature at which the samples were measured.

Readings were taken until three sequential readings gave the same result. Results obtained were the average of three successive comparable determinations.

β-Galactosidase assay

Preparation of cell-free extract. Three ml of cell suspension (≅ 10^9 cells ml^{-1}) were centrifuged at 9000 *g* for 12 min at 1°C and the supernatant fluid removed for assay of released enzyme. The supernatant liquid was diluted 10-fold with buffer and assayed for enzyme according with the method described by Citti *et al.* (1965).

Preparation of toluene-treated cell suspension. Two ml from the same previous cell suspension (≅ 10^9 cells ml^{-1}) were treated with 0·1 ml of toluene–acetone (1 : 9) solution, and incubated for 5 min at 25°C with vigorous agitation. The treated suspension was diluted 100-fold with buffer and assayed for the enzyme as described below.

The chromogenic substrate *o*-nitrophenyl-β-D-galactopyranoside (ONPG) was used to measure enzyme activity (Lederberg 1950). A solution of 0·005 mol l^{-1} ONPG was prepared in sodium phosphate 0·05 mol l^{-1}. Two hundred μl of cell-free extract and toluene-treated cell suspension were incubated with 0·8 ml of ONPG solution at 37°C. Colour development was stopped by adding 1 ml of cold 0·5 mol l^{-1} sodium carbonate to the reaction mixture. The absorbance of the supernatant liquid was measured at 420 nm. The μmol of *o*-nitrophenol liberated from ONPG were determined from a standard curve, which was prepared by measuring the change in absorbance of different concentrations of *o*-nitrophenol. The reaction was linear over the entire time period. The activity of β-galactosidase was expressed as μmol of *o*-nitrophenol liberated from ONPG per min in 1 ml of reaction mixture.

Atomic absorption

K^+ and Na^+ content of the bacteria was determined by flame photometry as described by Rabaste *et al.* (1992). For this assay 2 ml of cell suspension (*ca* 10^9 cells ml^{-1} in deionized water) were added to 18 ml of MRS and incubated at 25°C for 15 min. After incubation, bacteria were separated by centrifugation. The pellet was washed twice with bi-deionized water and then aspirated. Finally 300 μl of pellet was digested in 5 ml of formic acid for 48 h at 37°C and analysed.

ATPase assay

The proton-stimulated ATPase was assayed using permeabilized cells as described by Belli and Marquis (1991) in the presence of 100 μmol l^{-1} *N,N'*-dicyclohexylcarbodiimide (DCCD).

Cell permeabilization. Cells from 3 ml samples (*ca* 10^9 cells ml^{-1}, in sterile deionized water) were centrifuged at 4°C and resuspended in 3·75 ml of Tris-HCl buffer 75 mmol l^{-1} (pH 7·0) containing 10 mmol l^{-1} $MgSO_4$. Toluene (375 μl) was added to the cell suspension prior to vigorous vortex mixing and incubation for 5 min at 37°C. Each cell suspension was then subjected to two cycles of freezing at −80°C and thawing at 37°C. Permeabilized cells were harvested by centrifugation. They were then resuspended in 1·2 ml of Tris-HCl buffer 75 mmol l^{-1} (pH 7·0) containing 10 mmol l^{-1} $MgSO_4$.

A 50 μl sample of permeabilized cell suspension was added to 2 ml of Tris-maleate buffer 50 mmol l^{-1} (pH 6·0) containing 10 mmol l^{-1} $MgSO_4$, and the mixture was warmed to 37°C. The ATPase reaction was initiated by the addition of 20 μl of ATP 0·5 mol l^{-1} (pH 6·0) and stopped by addition of 480 μl of 26·7% ice-cold trichloroacetic acid with the tubes being immediately transferred to an ice bath. Three hundred μl samples were then removed and assayed for inorganic phosphate liberated from cleavage of ATP by the method of Rathbun and Betlach (1969). The values obtained were corrected for the spontaneous decomposition of the substrate and for the endogenous phosphate content of the respective preparations. ATPase activities were expressed as μmol of phosphate released from ATP per g of cell dry weight per min.

Dry weight determination. Samples of 200 μl of permeabilized cells were harvested by centrifugation and washed with cold deionized water. The tubes containing the washed permeabilized cells were dried overnight in pre-tarred Eppendorf tubes at 105°C and weighed.

Lipid composition

Membrane lipids were analysed by gas–liquid chromatography by the method of Rozès *et al.* (1993). Methanolysis of cell materials was carried out with sodium methoxide (1 mol l^{-1}) in methanol. Fatty acid methyl esters (FAME) were extracted with hexane. Retention times of the FAME were compared with known standards obtained from Sigma. The total areas of the main fatty acids present, lactobacillic, palmitoleic, stearic, palmitic and oleic acids, were used to determine the relative per cent of each fatty acid present, and calculation of the ratio of unsaturated to saturated fatty acids (unsat/sat index).

Internal pH measurement

The internal pH (pH_{in}) and cell volume were determined by using radioactive probes (isotopes were purchased from Du Pont Co., USA with the exception of tritiated water purchased from Amersham, UK), as described by Rottenberg (1979). Cells were centrifuged at 22°C, at 9000 *g* for 10 min, suspended in MRS broth, and re-centrifuged as above. For determination of pH_{in}, washed cells were suspended to an optical density at 650 nm of 1·0 in the test medium (MRS at different pHs). A 1 ml aliquot was placed into each of four Eppendorf tubes. To each tube, ^3H_2O (5 μCi) was added to determine the total volume. For extracellular volume determination, [^{14}C] sorbitol (3 μCi) was added to two of the tubes. For pH_{in} determination, [^{14}C] salicylic acid (3 μCi) was added to the remaining two tubes. Following a 15 min incubation, the tubes were centrifuged in an Eppendorf microcentrifuge (Picofuge ™-Stratagene) for 5 min. A 60 μl sample of supernatant fluid was pipetted into a scintillation vial containing 3 ml of scintillation liquid (Insta-Gel II Packard), previously diluted with deionized water (1 : 7, water *vs* scintillation liquid) and 0·3 ml of 0·6 mol l^{-1} perchloric acid. The remaining fluid was aspirated from the tube, and the entire pellet was immediately cut off quantitatively with a razor through the polyethylene tube and mixed vigorously with the scintillation liquid and the perchloric acid into a scintillation vial. The vials were placed into a scintillation counter (Beckman Instruments, USA) for determining counts per min. Both pH_{in} and the intracellular volume were calculated on the basis of the counts per min in the pellet and the supernatant fluid (Rottenberg 1979).

RESULTS

By dividing the process of freeze drying into its two components of freezing and drying it was possible to study the effects of each process in the bacterial population. Results in

Fig.1 show that freezing with suitable cryoprotectors results in no loss of viability. Freezing, however, in poor cryoprotectors does result in a loss of viability ($P \leqslant 0{\cdot}05$).

The effect of additives on survival following freeze drying is shown in Fig. 2. Results show that freeze drying was responsible for killing over 70% of the population for all the tested additives.

The percentage of the population suspended in water and maltodextrin killed by drying from the frozen stage was much smaller ($P \leqslant 0{\cdot}05$), compared to the other additives. It should be remembered, however, that most of the sensitive population was already eliminated by freezing.

The effect of incorporating sodium chloride into the recovery medium following freezing and freeze drying in various suspending liquids, is shown in Table 1.

Results show that cells frozen in maltodextrin showed an increased sensitivity to sodium chloride post stress. The same effect was not observed in milk, water, glycerol and trehalose ($P \leqslant 0{\cdot}05$). Following freeze drying, cells suspended in water and maltodextrin become sensitive. Cells dried in glycerol and trehalose did not show any sensitivity to salt after the drying stage ($P \leqslant 0{\cdot}05$) (salt sensitivity involving cells freeze-dried in milk is described in Fig. 3).

Incorporation of chloramphenicol, actinomycin D, rifampicin and pyronin Y into the recovery medium, following freezing and freeze drying had no effect (data not shown).

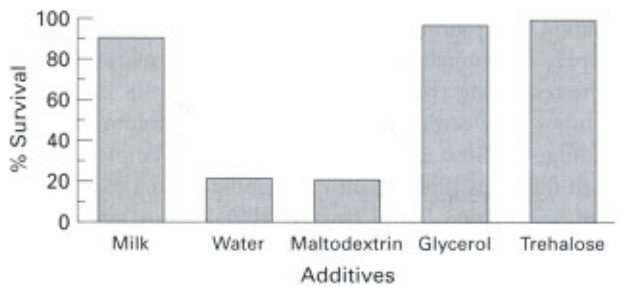

Fig. 1 Survival (%) of *Lactobacillus bulgaricus* after freezing in different additives, determined on Man, Rogosa and Sharpe agar (MRSA)

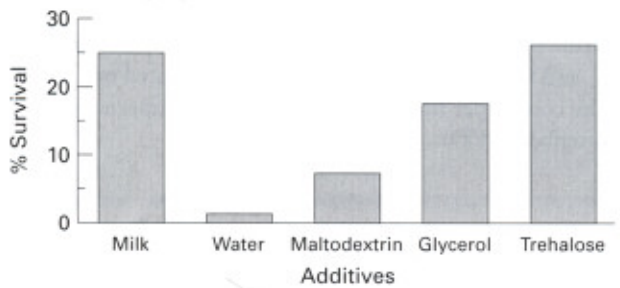

Fig. 2 Survival (%) of *Lactobacillus bulgaricus* after freeze drying in different additives, determined on Man, Rogosa and Sharpe agar (MRSA)

Table 1 Survival of *Lactobacillus bulgaricus* in Man, Rogosa and Sharpe agar (MRSA) and $MRSA_{NaCl}$ following freezing and freeze drying

	Freezing		Drying	
Suspending medium	MRS	MRS_{NaCl}	MRS	MRS_{NaCl}
Milk	9·4	9·4	—	—
Water	8·5	8·4	8·0	7·5
Maltodextrin	9·0	8·7	8·2	7·8
Glycerol	9·4	9·4	8·2	8·2
Trehalose	9·4	9·4	8·8	8·9

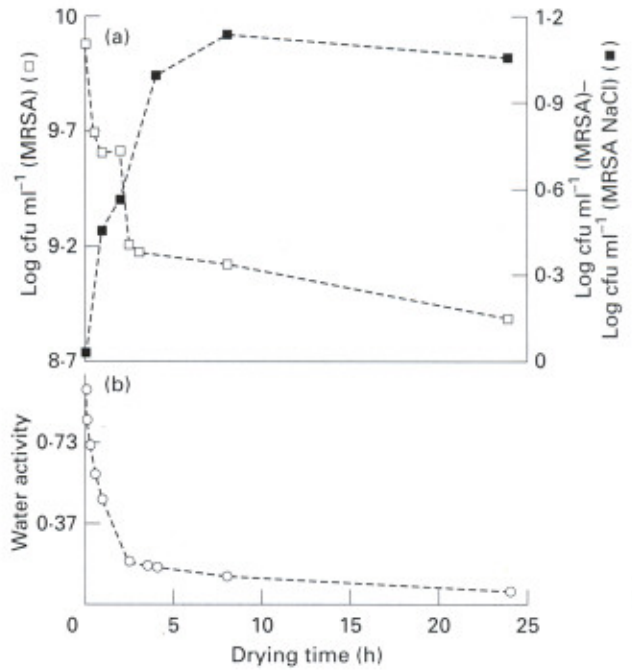

Fig. 3 (a) Survival (□) and sensitivity (■) of *Lactobacillus bulgaricus* to sodium chloride, during freeze drying on SMP, determined on Man, Rogosa and Sharpe agar (MRSA) and $MRSA_{NaCl}$. (b) Change in water activity (○) of the bacterial suspension, during the process

The effect of the drying time (water removal) from the frozen stage of cells suspended in SMP on survival is shown in Fig. 3. Results show that survival decreased with increasing drying time. The decrease was more evident in the first 4 h of drying and then decreased slowly. An increased sensitivity to sodium chloride was observed in parallel with the decrease in viability, during the first 5 h of lyophilization.

Further experiments to determine the exact nature of the membrane damage showed an increase in membrane permeability to β-galactosidase (Table 2) and a reduced capability to maintain the ΔpH across the cell membrane (Fig. 4),

Table 2 Effects on membrane function and permeability of freeze drying *Lactobacillus bulgaricus*

Manifestations	Normal	Freeze-dried cell
β-Galactosidase activity*		
in cell supernatant fluid	0	0·11 ± 0·04
in permeabilized cells	—	0·71 ± 0·16
K/Na relationship in cell suspension**	17·88 ± 0·02	0·34 ± 0·01
ATPase***	156 ± 3	139 ± 1
U/S index***	14·6 ± 0·8	6·0 ± 0·4

ATPase activities are expressed as μmol of phosphate released from ATP per g of cell dry weight per min. β-Galactosidase activity is expressed as μmol of *o*-nitrophenol liberated from *o*-nitrophenyl-β-D-galactopyranoside (ONPG) per min, in 1 ml of reaction mixture. Cells suspended in SMP*, water** and maltodextrin***. Results were expressed as the mean of three replicates ± standard deviation.

especially noticeable at lowest pH tested (pH 3 and 4). Before lyophilization, *Lact. bulgaricus* did not maintain a constant internal pH (pH_{in}) over the range of external pHs (pH_{out}) studied. As the pH_{out} decreased, the pH_{in} decreased, but was always higher than the external pH. Freeze drying caused the collapse of the pH gradient ($P \leqslant 0{\cdot}05$).

Due to technical difficulties it was not possible to study the effect of freeze drying in SMP on ATPase activity and internal ion concentrations. However, cells freeze-dried in maltodextrin showed a small decrease of the ATPase activity.

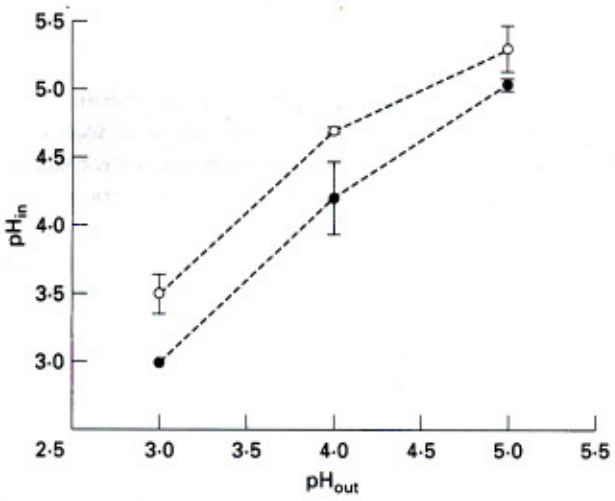

Fig. 4 Effect of pH_{out} on pH_{in} of washed cells of *Lactobacillus bulgaricus*. ○, Control; ●, freeze dried (in skim milk powder). The bars represent the standard deviation of the mean of three experiments

Furthermore, they also presented a decrease in the unsaturated/saturated index. Cells lyophilized in water revealed an inversion of the Na/K relationship following freeze drying.

DISCUSSION

The purpose of this study was to investigate the consequences of freeze drying cultures of *Lact. bulgaricus* and, if possible, to determine a target site responsible for cell death.

The benefits of using glycerol, SMP and sugars (trehalose) in the protection of bacterial cells during freezing are in agreement with previously published reports (Moss and Speck 1963; Barbour and Priest 1986; Berny and Hennebert 1991). The exact mechanisms by which these compounds and other cryoprotectors provide protection against freeze injury in bacteria are not all clearly understood. Reduction of the lethal effect of solute concentration, prevention of intracellular ice formation or reduction of its lethal consequences, through hydrogen bonding with water and cell structures, were some of the explanations advanced. The percentage survival of *Lact. bulgaricus* suspended in maltodextrin was similar to survival in the absence of any protective agent, in spite of the percentage of maltodextrin (11% w/v) being the same as SMP in the suspending media.

Freezing *Lact. bulgaricus* suspended in maltodextrin caused cells to develop a sensitivity to sodium chloride post stress. These results indicate membrane damage (Sogin and Ordal 1967) and are in agreement with previous publications (Mazur 1970). Cells suspended in water did not develop any sensitivity to salt following freezing. More damage might have been caused to the cells, hindering recovery in the MRS medium.

Drying from the frozen state (lyophilization) caused an additional decrease in viability, especially pronounced in cells suspended in more effective cryoprotectors (milk, glycerol and trehalose). This effect was smaller in poorly or non-protected cells (cells suspended in maltodextrin and water) probably as a result of the elimination of the most sensitive part of the population during the freezing stage.

Cells freeze-dried in SMP showed a decrease in viability that was followed by an increase in the sensitivity to sodium chloride, with the time of drying. The exact mechanism by which sodium salt inhibits cell recovery is not known. Pure lipid (non-dried) bilayers are characterized by an extremely low permeability to the transverse diffusion of ions (Corvera *et al.* 1992), but freeze drying produces an increase in membrane permeability (Sinskey and Silverman 1970). It is possible that with the increase in the time of drying, sodium chloride entered more easily through the cell membrane. The necessity for the removal of this extra quantity of sodium chloride might constitute a problem to the cell, further aggravated if the enzyme catalysed extruding mechanisms are dam-

aged. The excess of sodium salt may eventually interfere with recovery of other cell structures affected by drying, although not detected by the use of cell inhibitors. Salt sensitivity was verified in the case of cells freeze-dried in water and maltodextrin. Cells lyophilized in glycerol and trehalose did not develop sensitivity to the sodium salt. This might be related to a different type of protection conferred by these two additives, probably at the cell membrane level. Trehalose in particular was described as capable of stabilizing dry biological membranes by hydrogen bonding to the polar groups of the membrane phospholipids (Crowe and Crowe 1984).

Following rehydration the cell membrane of *Lact. bulgaricus* becomes not only incapable of controlling the entry of sodium chloride, but also preventing the exit of internal constituents like β-galactosidase. The change in the K/Na relationship of lyophilized cells incubated in normal (non-supplemented) MRS medium is an indication of the increase in membrane permeability, proving a change in the internal ionic environment of the cell.

Results of changes in lipid profile present further evidence for membrane damage. Previously oxidation has been reported as a mechanism of causing loss of viability in freeze-dried cells (Strange and Cox 1976). To avoid this damage the use of nitrogen is recommended in breaking the vacuum following freeze drying (Anon. 1961). This was not done in this work and may be an explanation for an increased loss of viability. In lipid membranes a result of the decrease in unsat/sat fatty acid index would be an increase in the phase transition temperature (Watson *et al.* 1973). Following the model of Crowe *et al.* (1989), this would result in a further increase in membrane permeability.

The study of an enzyme function associated with the membrane also indicated damage to this system, following freeze drying. A reduction in the activity of the proton-translocating ATPase was observed. In most aerobic, respiring micro-organisms, H^+-ATPase can generate ATP. ATP synthase enzyme uses the proton gradient to provide the necessary energy to drive the endergonic ATP synthesis (Senior 1988). ATP synthase activity may also occur in lactic acid bacteria (Maloney 1982). But under physiological conditions, H^+-ATPase functions primarily as a proton pump (Konings *et al.* 1987). ATPase activity seems to be independent of the magnitude of the proton motive force, being regulated by the internal pH (Kobayashi *et al.* 1984; Sturr and Marquis 1992). Experiments with gramicidin D, a protonophore that allows free diffusion of protons across the membrane of *Enterococcus faecalis* (lactic acid bacteria), increased the H^+-ATPase extrusion activity fivefold (Kobayashi *et al.* 1984). In the experiments reported here, to measure H^+-ATPase activity, before and after freeze drying, the pH, Na^+ and K^+ gradients were destroyed as permeabilized cells were used. The observed decrease in activity following freeze drying is therefore concluded to be a result of either indirect (change in membrane binding necessary for enzyme function) or direct damage to the enzyme itself.

Loss of membrane structure and function was also indicated by the results obtained for ΔpH. The inability of the cell to maintain a pH gradient following freeze drying is a direct consequence of the increase in membrane permeability.

The cytoplasmic membrane is generally considered to be the main site of dehydration damage (Lievense *et al.* 1994). The increase in membrane permeability is just one of the expressions of damage upon freeze drying (Sinskey and Silverman 1970). According to Crowe *et al.* (1989), the increase in permeability is due to a transition between the gel and liquid crystal states of the membrane that occurs during rehydration. The amount of interface formed between the gel and fluid domains in the bilayer in the neighbourhood of the main phase transition has already been theoretically related with the enhancement of the passive ion permeability (Papahadjopoulos *et al.* 1973; Cruzeiro *et al.* 1988). According to the model of Crowe *et al.* (1989), the protective effects of trehalose and other carbohydrates on the membrane could come from a decrease of the transition temperature, thus avoiding the gel state even in dehydrated, cold membranes (Crowe *et al.* 1987). The inability of SMP to protect the cell from the entry of sodium chloride, might be a question of the amount of lactose present in SMP.

Membrane injury and stabilization has been widely studied in the case of liposomes, in view of their potentially important role in the delivery of water-soluble drugs to cells in whole organisms (Crowe *et al.* 1987). Concerning starter cultures, this subject has generally been approached from a practical point of view. The understanding of the mechanisms responsible for causing cell death will be very important, since at the industrial level the percentage of the population surviving freeze drying is still insufficient to allow for direct inoculation of fermentation tanks (Stanley 1983). From the results presented here, it can be concluded that freezing and particularly drying can cause membrane injury. The increase in membrane permeability can be responsible for, or at least contribute to cell death particularly in environments containing excessive amounts of sodium salt. It will be important to increase research on suitable additives, particularly those that decrease membrane permeability. The absence of oxygen during the freeze drying process also is important in order to reduce lipid oxidation, that can contribute to a further increase in permeability and also affect enzymatic activities associated with the membrane.

ACKNOWLEDGEMENT

The authors thank Graça Lobo for her technical assistance with the Beckman scintillation counter.

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