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ORIGINAL ARTICLE

Volume recovery, surface properties and membrane integrity of Lactobacillus delbrueckii subsp. bulgaricus dehydrated in the presence of trehalose or sucrose

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Abstract

Aims: Although the practical importance of adding sugars before drying is well known, the mechanism of protection of bacteria by sugars is not clear. The response of the dehydrated micro-organisms to rehydration is analysed in terms of structural and functional changes, and correlated with their potentiality to grow in rich media. These aspects are related with the membrane integrity and the metabolic state of the rehydrated bacteria, measured by means of surface properties and permeability. To attain this objective, *Lactobacillus delbrueckii* subsp. *bulgaricus* was dehydrated in the presence and in the absence of sucrose and trehalose. The bacterial response upon rehydration was investigated by determining: (i) the lag time of the bacterial growing in rich media, (ii) the restoration of the surface properties and the cellular volume and (iii) the membrane integrity.

Methods and Results: *Lactobacillus delbrueckii* subsp. *bulgaricus* was grown in MRS at 37°C overnight [De Man *et al.* (1960) *J Appl Bacteriol* 23, 130] and then dehydrated for 10, 20 and 30 min at 70°C in a vacuum centrifuge. The lag time of micro-organisms was determined by optical density changes after rehydration. The surface properties were determined by measuring the zeta potential of the bacteria suspended in aqueous solution. The cellular volume recovery was measured, after stabilization in saline solution, by light scattering and by the haematocrit method [Alemohammad and Knowles (1974) *J Gen Microbiol* 82, 125]. Finally, the membrane integrity has been determined by using specific fluorescent probes [SYTO 9 and propidium iodide, (PI)] that bind differentially depending on the integrity of the bacterial membrane. The lag time of *Lact. delbrueckii* subsp *bulgaricus*, dehydrated by heat in the presence of sucrose or trehalose and after that rehydrated, was significantly shortened, when compared with that obtained for bacteria dried in the absence of sugars. In these conditions, trehalose and sucrose maintained the zeta potential and the cell volume close to the control (nondried) cells. However, the membrane integrity, measured with fluorescent probes, was maintained only when cells were dehydrated for 10 min in the presence of sugars. For larger times of dehydration, the membrane integrity was not preserved, even in the presence of sugars.

Conclusions: When the micro-organisms are dehydrated in the absence of protectants, the membrane damage occurs with a decrease in the absolute value of the zeta potential and a decrease in the cellular volume recovered after rehydration. In contrast, when the zeta potential and the cellular volume are restored after rehydration to that corresponding to nondried cells, the micro-organisms are able to recover and grow with a reduced lag time. This can only be

achieved when the dehydration is carried out in the presence of sugars. At short dehydration times, the response is associated with the preservation of the membrane integrity. However, for longer times of dehydration the zeta potential and volume recovery occurs in the presence of sugars in spite of a severe damage at membrane level. In this condition, cells are also recovered. In conclusion, to predict the ability of growing after dehydration, other bacterial structural parameters besides membrane integrity, such as zeta potential and cellular volume, should be taken into account.

Significance and Impact of the Study: The correlation of the lag time with the surface and permeability properties is of practical importance because the correlation of these two parameters with cell viability, allow to determine the potential bacterial capacity to grow in a rich medium after the preservation procedure, without necessity of performing a kinetic curve of growth, which is certainly time-consuming.

Introduction

The preservation processes of bacteria by freeze-drying (lyophilization) or heat-drying induces a decrease in the number of viable micro-organisms. These preservation procedures involve a combination of two factors: temperature and dehydration. Both, simultaneously or independently, can affect severely the structure and properties of cell components.

It has been proposed that bacterial death results from the inactivation of critical sites in the cells (Teixeira *et al.* 1997). Membranes, nucleic acids and certain enzymes have been identified as cellular targets of damage caused by dehydration (Tomlins and Ordal 1976; Gould 1989).

Polyhydroxylated compounds such as sucrose and trehalose have extensively been reported to preserve bacterial cells (De Antoni *et al.* 1989; Leslie *et al.* 1995; Linders *et al.* 1997; Gouffi *et al.* 1999; Lodato *et al.* 1999; Oldenhof *et al.* 2005). Although the practical importance of these sugars as protectants is well known, the mechanism of protection is not clear and several proposals have been performed.

One of them explains the protective effect of biomolecules by the *water-replacement hypothesis*. According to it, sugars can substitute water molecules upon dehydration, by forming hydrogen bonds around the polar and charged groups present in phospholipid membranes and proteins, thereby stabilizing their native structure in the absence of water (Crowe *et al.* 1984, 1992, 1998).

Another possibility considers that, in the presence of water, sugars are excluded from the surface and, it may concentrate residual water molecules close to the biomolecular surface, thus preserving to a large extent its solvation and native properties (Arakawa and Timasheff 1982; Xie and Timasheff 1997).

It is possible that both mechanisms can be operating during the dehydration rehydration processes.

Starters of different types of micro-organism for the milk industry are usually preserved by freeze-thawing, lyophilization and spray drying. However, little is known about the affectivity and the mechanism of action of the sugars when they are used during heat-drying processes.

In this regard, it has been reported that the recovery of *Lactobacillus delbrueckii* subsp. *bulgaricus* air-dried at 70°C in vacuum during 30 min is considerably improved when the bacteria are dried in the presence of trehalose or sucrose (Gómez-Zavaglia *et al.* 2003; Tymczyszyn *et al.* 2007).

Lactobacillus delbrueckii subsp. *bulgaricus* are widely used in the elaboration of milk products, and, in particular, it is affected by different kinds of stress (Teixeira *et al.* 1997). Thus, it appears of interest to study the effect of drying on cell recovery in relation to membrane properties, such as integrity and permeability. For these reasons, in this work, the response of the dehydrated bacteria in the presence of different sugars to the rehydration is analysed in terms of structural and functional changes in correlation to their potentiality to grow in rich media. With this purpose, the membrane integrity was correlated with the metabolic state of the rehydrated bacteria, the surface properties and the permeability.

The metabolic state is determined by the ionic distribution across the membrane and it changes the surface charge distribution. Hence, the surface properties can be followed by the variation in the zeta potential of the rehydrated bacteria and it is indicative of the metabolic state (Fernández-Murga *et al.* 2000).

In this work, the membrane integrity of *Lact. delbrueckii* subsp. *bulgaricus* dried in the presence or absence of trehalose or sucrose was investigated by determining

the volume recovery of the rehydrated cells and the permeation of fluorescent probes.

Materials and methods

Bacterial strains and growth conditions

Lactobacillus delbrueckii subsp. *bulgaricus* CIDCA 333 was isolated from a fermented product (Gómez-Zavaglia *et al.* 1999). The strain was maintained frozen at −80°C in 120 g l^{-1} nonfat milk solids. Cultures were grown in MRS broth (De Man *et al.* 1960).

Dehydration procedure

Cultures in the stationary phase were harvested by centrifugation at 10 000 ***g*** for 10 min. Volumes of cell culture of 1·2 ml were washed with sucrose (Merck, Darmstadt, Germany) or trehalose (Fluka, Buchs, Switzerland) 250 mmol l^{-1} or maintained in water as control, and incubated in that media during 15 min. In all cases, samples were centrifuged at 10 000 ***g*** and the pellets were dehydrated simultaneously at 70°C for 10, 20 and 30 min in a vacuum centrifuge (Integrated Speed Vac System ISS 100; Savant Instrument Inc., Farmerdale, NY, USA). The powder obtained in all cases was immediately rehydrated in an equal volume of distilled water.

After rehydration during 15 min, growth kinetics was followed by measuring the absorbance at 600 nm (A600) during incubation at 37°C in MRS broth.

Water activity

Water activity was measured in samples before drying and after drying them for 10, 20 and 30 min at 70°C in all the conditions analysed using a Novasina water activity instrument (Pfaeffikon, Switzerland). The water activity was measured at different times of dehydration in the presence and the absence of sugars.

Fluorescence assays

Measure of membrane fluidity of intact cells was determined using 1,6-diphenyl hexatriene (DPH) as a probe (Trevors 2003; Tymczyszyn *et al.* 2005). DPH was dissolved in tetrahydrofuran as a stock solution of 146 mg l^{-1}. One millilitre of the cultures was centrifuged (5 min, 10 000 ***g***) and washed twice in Milli Q water; and the pellet (10^9 CFU) resuspended in 2·5 ml water. A volume of 13·5 μl of the stock solution of DPH was added to cells to reach a final concentration of $4{\cdot}6 \times 10^{-5}$ mol l^{-1}. The tubes were shaken for 20 min in darkness. The suspension was then centrifuged at 10 000 ***g*** and the pellet was resuspended in 2·5 ml of water.

Steady state fluorescence anisotropy measurements with DPH

Measurements were carried out in a Perkin Elmer Luminescence spectrometer Model LS 55 (Perkin Elmer, Norwalk, CT), equipped with excitation and emission polarizers and a circulating water bath. Experiments were performed using intact cells doped with DPH (excitation 350 nm and emission 452 nm). The temperature was controlled inside the cuvette with a thermocouple within ±0·2°C. Steady state anisotropy (r) was calculated by using the equation

$$r = I_{vv} - GI_{vh}/I_{vv} + 2GI_{vh},$$

where Ivv and Ivh represent the fluorescence intensity (IF) obtained with the vertical and horizontal orientations of the excitation and emission polarizers. $G = I_{hv}/I_{hh}$ is a correction factor accounting for the polarization bias in the detection system (Trevors 2003).

Zeta potential

The zeta potential of whole cells was determined in a Zeta-Meter System 3·0 (Zeta-Meter Inc., Staunton, VA, USA) by measuring the rate of migration of the cells in the stationary layer when a constant electric field was applied (Fernández-Murga *et al.* 2000). The effective electrical distance of the cell was calculated by using KCl solutions of known conductivity at 25°C. The rate of migration was determined by microscopic observation of the displacement of individual cells in a rectilinear and uniform movement along a reticular lattice. The potential was fixed at 40 V. At least 20 determinations were made for each sample.

Measures of cell volume

The optical density at 600 nm of aliquots of the cell suspension ($1{\cdot}10^8$ CFU ml^{-1}) was read. Changes in cell volume were calculated from the measured variations in absorbance with respect to the control (nondried cells) by assuming that the absorbance is inversely proportional to the volume at the 3/2 power (Alemohammad and Knowles 1974; Koch 1984; Poirier *et al.* 1998).

Packed cell volumes were measured by the haematocrit method (Alemohammad and Knowles 1974). Cell suspensions (about 50 mg dry wt/ml) were drawn into 7·5 cm capillary tube to a height of about 6 cm. Tubes were centrifuged at 2000 ***g*** for 5 min in a horizontal head fitted to a Microcentrifuge. The packed cell volumes were determined

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F[illegible]mbrane integ[illegible]

A[illegible] μl of the [illegible]xture containing[illegible]TO 9® and [illegible]: 1 (Kit B[illegible] Molecular Prob[illegible]L-7012; Mol[illegible]r Probes In[illegible]ne, OR) were ad[illegible]d to 1 ml of [illegible] dried unde[illegible]nt conditions and rehydrated ([illegible]scribed abo[illegible]ncubated for 15 min at room [illegible]perature. Bo[illegible]ro[illegible] are nucleic acid stains: SYTO [illegible]s a green fl[illegible]esc[illegible] probe and PI is a red-fluoresent probe. T[illegible] dy[illegible] differ in their spectral characteristics and i[illegible]eir [illegible]ity to penetrate healthy bacterial cells: SYTO[illegible]gen[illegible]ly labels all bacteria in a population (tho[illegible]ith [illegible]ct and those with damaged membranes), [illegible]ereas PI penetrate[illegible]only when bacterial membr[illegible] are [illegible]maged, cau[illegible] a reduction in the SYTO [illegible]luores[illegible]ce when bo[illegible]yes are present. Thus, with [illegible] appr[illegible]ate mixtu[illegible]t the SYTO 9 and PI t[illegible]bacteri[illegible]ith intact [illegible] membranes stain fluoresgreen, [illegible]ereas bact[illegible] with damaged membranes [illegible] fluores[illegible]t red.

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For [illegible]crosco[illegible]examination, 5 μl of the cell suspensions, [illegible]cubat[illegible]with the dye mixture, were trapped betw[illegible] a sli[illegible]nd 18 mm square coverslip. Cells were view[illegible] wit[illegible] Eclipse 800 epifluorescence microscope (Ni[illegible], T[illegible], Japan) equipped with a 100W HBO lamp, a [illegible]da[illegible]uorescein iso[illegible]yanate filter set, and a 40× o[illegible]ctiv[illegible]mages were[illegible]d with a Nikon Coolpix E [illegible] d[illegible] camera ([illegible]

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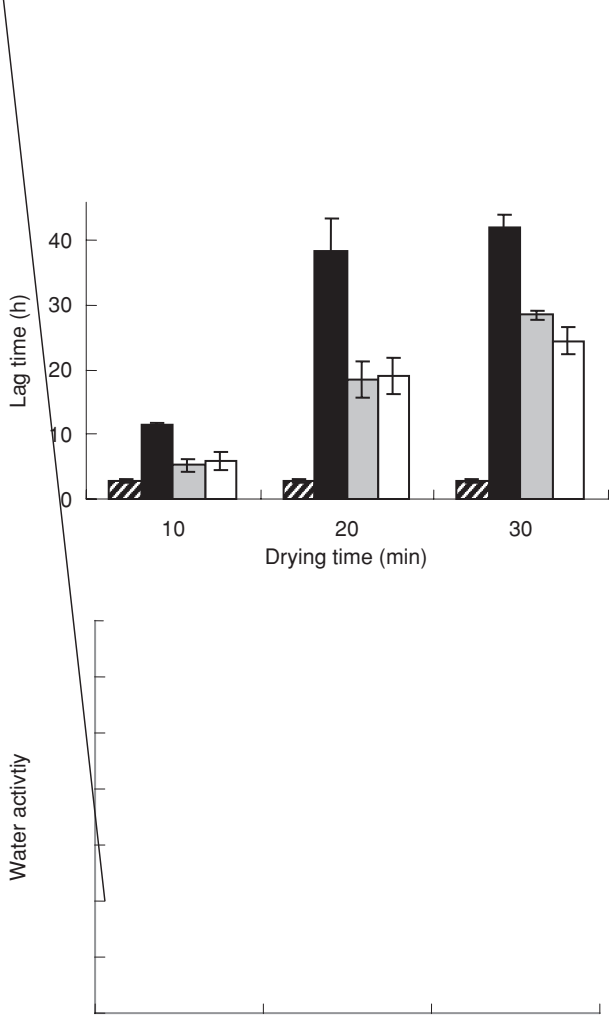
Resul[illegible]

A n[illegible]able increase in t[illegible]ag time was observed in cells dri[illegible]n the absence of s[illegible]rs during 10 min. In contrast, w[illegible] cells were dried [illegible]ng the same time but in the [illegible]ence of trehalose or [illegible]crose, the lag time of the rehyted cells remained [illegible] close to that corresponding to [illegible]ondehydrated micro[illegible]ganisms (Fig. 1a). In the same [illegible]gure, it can be ob[illegible]ved that at lon[illegible]r drying times,



sugars also reduce the lag time, although the values are higher in comparison to nondried cells.

The water activity of cells dried at different times was determined in the presence and the absence of sugars. The water activity of the cellular pellet (aw ~1) decreases independently of the presence or the absence of sugars (Fig. 1b). According to these results, after 20 min of dehydration, the water activity decreases and remains constant at a value of aw ~0·45. This is much lower than that corresponding to saturated solutions of sucrose (aw = 0·86 at 25°C (Starzak and Mathlouthi 2006). Thus, the water activity measures correspond to the water in the sample that may exchange with the vapour phase.

In the presence of sugars, the lag times remain near those corresponding to nondried bacteria when the water activity is around 0·60. In the same conditions, the lag

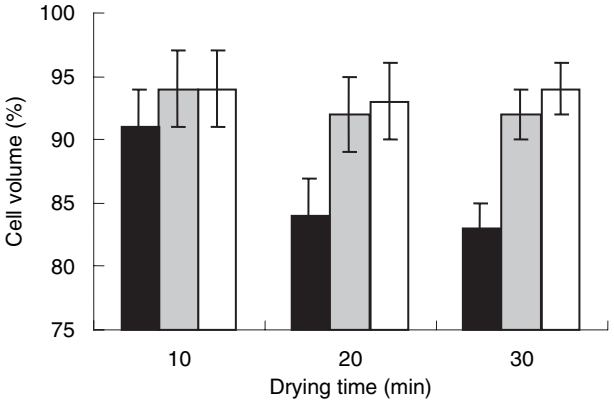


Figure 2 Volume of cells recovered after drying in the presence and in the absence of sugars. Changes in cell volume were calculated from variations in absorbance at 600 nm with respect to the control (nondried cells). Absorbance was assumed to be inversely proportional to the volume at the 3/2 power (Alemohammad and Knowles 1974; Koch 1984; Poirier *et al.* 1998). A total of 100% corresponds to the volume of nondehydrated cells. Cells dried without sugar (black bars), in sucrose 250 mmol l^{-1} (gray bars) and in trehalose 250 mmol l^{-1} (white bars).

time is higher when the bacteria reach water activities lower than 0·45 (at times longer than 20 min). However, in these cases, the lag times are much lower than that obtained in the absence of sugars.

At both water activities, sucrose and trehalose are able to protect cells during dehydration. To investigate the possible reasons for this response the surface properties and the volume recovery after rehydration of the cells dried before growing, in the same conditions were determined.

The recovery of the cell volume was calculated from variations in absorbance of the cells at 600 nm rehydrated after different times of drying (Fig. 2). The absorbance in the presence of sugars was close to those corresponding to nondehydrated cells for which the cell volume is the highest (100%).

In order to determine whether the changes in the optical density correspond to volume changes, the effect of sugars on the cell volume recovery after rehydration was checked by measuring the volume of packed cells (Table 1). The results obtained by both methods correlate well with the decrease of the lag time after rehydration as shown in Fig. 1a. The more pronounced the volume recovery the shorter is the lag time.

Table 1 Effect of thermoprotectants on the packed cell of *Lactobacillus bulgaricus* dried after 30 min dehydration measured by the haematocrit method*

Condition	Cell volume (%)
Nondehydrated	100 ± 1
Dehydrated without sugars	82 ± 1
Dried in 250 mmol l^{-1} trehalose	91 ± 1
Dried in 250 mmol l^{-1} sucrose	89 ± 1

*Alemohammad and Knowles (1974).

The zeta potential variation of cells has been observed along with the bacterial kinetic of growth, thus demonstrating the relation between zeta potential and metabolic changes (Fernández-Murga *et al.* 2000). The zeta potential is defined as the difference in electrical potential between the surface of the bacterium and the bulk-surrounding medium. It is a measure of the net distribution of electrical charges on the bacterial surface. The zeta potential reached less negative values after freezing and thawing lactobacilli without cryoprotectants (Fernandez Murga *et al.* 2000).

In this work, the zeta potential of nondehydrated cells was about −30 mV and shifted to −24 mV in the rehydrated cells after 30 min of dehydration in the absence of sugars (Fig. 3). In contrast, the values of zeta potential for cells dehydrated during 30 min in the presence of trehalose or sucrose remained at −28 mV, which is only slightly lower than the zeta potential of the nondehydrated cells (Fig. 3). Thus, the restoration of the zeta potential corresponds to short lag times during cell recovery.

It appears from the results of Figs 1, 2 and 3 that the recovery of cells is correlated with the restoration of membrane properties such as permeability to water and ions. These two properties may be related to the phase state of the hydrocarbon chains of the lipid membrane. In order to correlate the volume recovery and the cell surface potential with the phase and stability properties of the membrane, the fluorescence anisotropy and the mem-

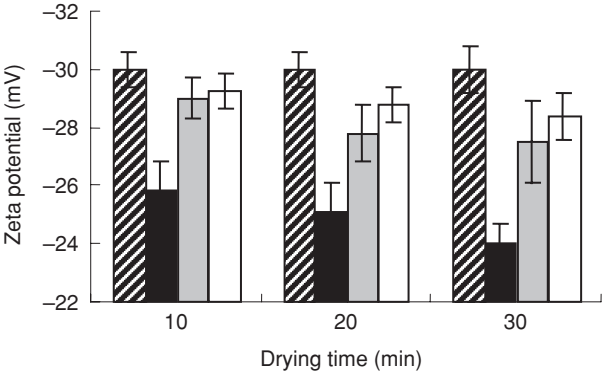


Figure 3 Zeta potential of recovered cells dried in the presence of thermoprotectants as a function of the drying time. Control nondried (dashed bars), cells dried without sugar (black bars), cells dried with sucrose 250 mmol l^{-1} (gray bars) and cells dried with trehalose 250 mmol l^{-1} (white bars).

Table 2 Effect of 250 mmol l^{-1} trehalose or sucrose on the DPH anisotropy after 30 min dehydration

Condition	Anisotropy (*r*)	Intensity DPH (%)
Nondried	0·273 ± 0·005	100
Dried	0·278 ± 0·010	10
Dried with trehalose	0·277 ± 0·007	20
Dried with sucrose	0·279 ± 0·005	28

DPH, 1,6-diphenyl hexatriene; *r*, steady state anisotropy.

brane integrity were measured after rehydrating cells that were previously dehydrated in the presence and in the absence of sugars. In the first case, the anisotropy of the membrane was analysed by the incorporation of DPH, a fluorophore that partitions in the hydrocarbon core of the membranes. Dehydration of cells with or without sucrose or trehalose does not affect the anisotropy relatively to nondehydrated cells (Table 2). Thus, the effects observed on volume and zeta potential recovery are not apparently related with the fatty acid composition of the cell membrane. However, it must be noticed that the intensity of DPH fluorescence is markedly decreased in cells recovered after drying 30 min in the absence of sugars (Table 2) in comparison with the nondehydrated cells. This decrease is slightly altered when the dehydration is carried out in the presence of trehalose or sucrose suggesting a low incorporation of the probe in all the conditions of dehydration. This might be related to changes in the membrane properties.

A direct observation of the membrane integrity, after dehydration, can be obtained using SYTO 9 and PI as fluorescent probes. SYTO 9 can penetrate undamaged cells. When the cells are not damaged, a green fluorescence is observed due to the entrance of SYTO 9. The green fluorescence observed in Fig. 4a indicates that in nondried *Lact. bulgaricus* almost all micro-organisms were not damaged.

PI gives a red fluorescence when penetrates the cell. However, this probe can only enter when the membrane is damaged. The entrance of PI to the cell causes a reduction in the SYTO 9 fluorescence when both dyes are present. Thus, the red fluorescence observed in Fig. 4c indicates that, when cells were dried without sugars, only damaged membranes were observed.

When the micro-organisms were dried in the presence of sugars, only some of the cells were damaged (red ones) and the others remained unchanged (green ones) (Fig. 4b). Thus, the relative shift from green to red fluorescence when both probes are present is related to the magnitude of the penetration of PI, that is, to membrane damages.

These results were quantified by performing a spectral scan within the range 470–670 nm. Nondehydrated cells displayed an intense peak at 500 nm, corresponding to SYTO 9 probe. Figure 5a depicts *Lact. bulgaricus* dehydrated in the presence of trehalose after 10, 20 and 30 min. The intensity of the peak centered at 500 nm decreased due to the entrance of PI. The same behaviour was observed when cells were dehydrated in the presence of sucrose (Fig. 5b). Dehydration in the absence of thermoprotectants induces a drastic decrease of the intensity even after 10 min dehydration (Fig. 5c).

The decrease of IF at 500 nm was correlated with the membrane damage for each dehydration condition as:

$$\%\ \text{membrane damage} = \frac{\text{IF control} - \text{IF sample}}{\text{IF control}} \times 100,$$

where IF = fluorescence intensity at 500 nm. Control corresponds to nondried cells.

According to this formula, the membrane damages observed by fluorescence after drying 10 min in the presence of trehalose or sucrose were only (30–40%) of those occurring in control (nondehydrated cells) in comparison with (90%) obtained for cells dehydrated in the absence of sugars (Fig. 6). However, when dehydration in the presence of sugars is longer (20–30 min), the membrane is practically not protected for PI penetration. When plots corresponding to cells dehydrated in the presence of trehalose and sucrose for 20 min are compared (Figs 5 and 6) no differences were observed between cells dehydrated in the presence or in the absence of sugars. These results suggest that both sucrose and trehalose strongly prevented the membrane damage if dehydration is carried out at times shorter than 10 min.

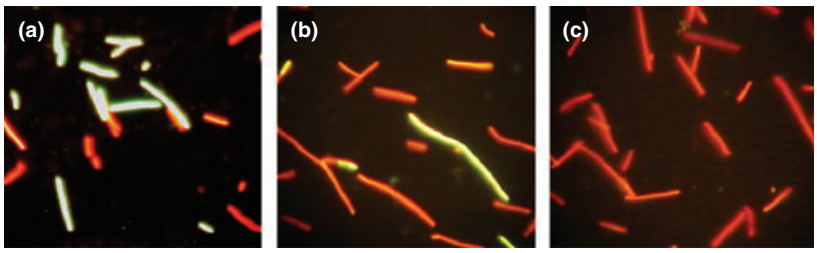


Figure 4 Fluorescence image of *Lactobacillus bulgaricus* dehydrated 30 min and stained with SYTO 9 and propidium iodide. (a) Control: Nondried cells, (b) dried in the presence of trehalose 250 mmol l^{-1} and (c) dried without protectants.

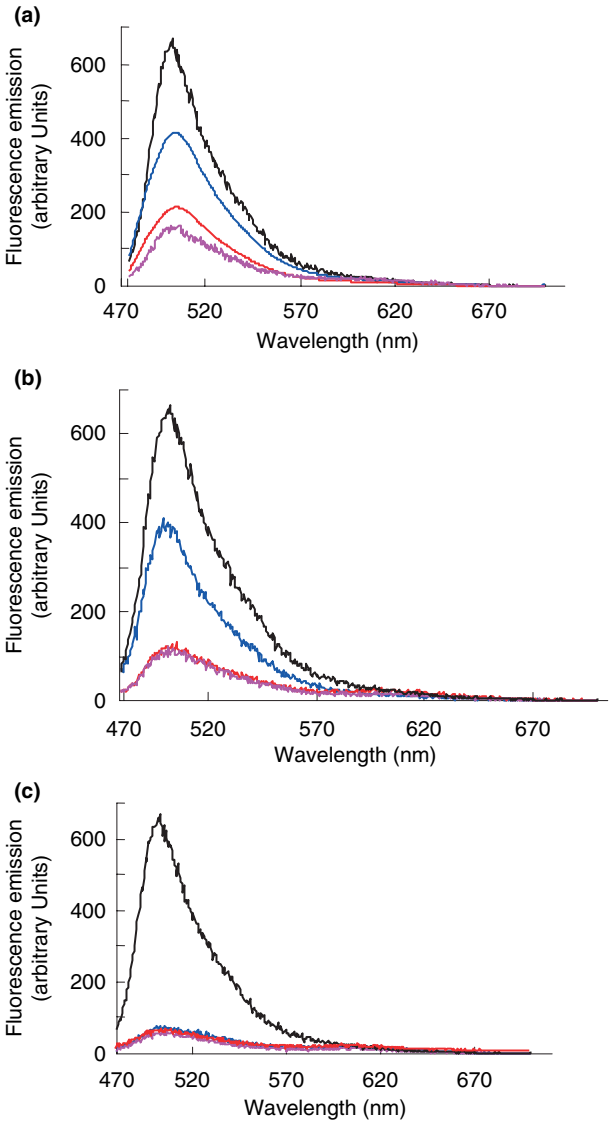


Figure 5 Fluorescence scan of SYTO 9 and propidium iodide incorporated in *Lactobacillus bulgaricus* dried in the presence of (a) trehalose 250 mmol l^{-1}, (b) sucrose 250 mmol l^{-1} and (c) in the absence of protectants within the range 470–670 nm. Nondried cells (top line), dried 10 min (upper middle line), dried 20 min (lower middle line) and dried 30 min (bottom line).

Discussion

In this paper, it is shown that the lag time increases with the drying time and is correlated with the decrease in the water activity of the cells (Fig. 1b). However, the presence of sugars does not affect the changes in water activity. The decrease of the lag time denotes that for the same amount of water eliminated, sugars counterbalance the dehydration and consequently preserve the cell structures.

The decrease of the lag time of bacteria dried in the presence of sugars is correlated with the maintenance of the zeta potential and the cell volume near those corresponding to nondehydrated cells.

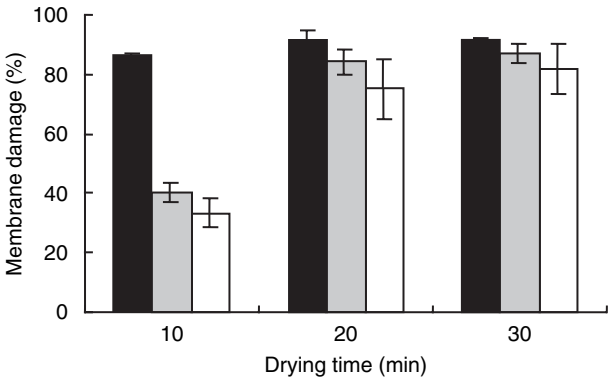


Figure 6 Membrane damage expressed as percentage of the SYTO 9 fluorescence decay.

$$\%\ \text{membrane damage} = \frac{\text{IF control} - \text{IF sample}}{\text{IF control}} \times 100$$

IF = fluorescence intensity at 500 nm. Control corresponds to non-dried cells. (a) Membrane damage of 0% corresponds to nondehydrated micro-organisms. Dried without sugar (black bars), in sucrose 250 mmol l^{-1} (gray bars) and in trehalose 250 mmol l^{-1} (white bars).

The zeta potential is related to the membrane potential and reflects the metabolic state of the bacteria (Fernandez Murga *et al.* 2000).

The cell volume recovery is related to the permeability of water and reflects the membrane integrity of the cell as selective barrier for ions (Alemohammad and Knowles 1974). In addition, volume recovery may be due to the restoration of the membrane potential and hence linked to the metabolic properties as mentioned above.

The correlation of the lag time with the surface and permeability properties is of practical importance because both determinations show the potential bacterial capacity to grow in a rich medium after the preservation procedure, without the necessity of performing a kinetic curve of growth.

In addition to this projection of the present results, the correlation of these responses with the phase membrane properties and membrane integrity opens a discussion on the basic mechanism of dehydration and preservation in the presence of sugars. The effect of drying on fluorescence indicates that the protective effect of both sugars is noticeable for dehydration no longer than 10 min (Figs 5 and 6).

The presence of sugars does not affect the anisotropy of the membrane after drying during 30 min (Table 2). Trehalose and sucrose are effective in decreasing the damage produced by dehydration, by preserving the volume recovery (osmotic response) and the metabolic state (zeta potential) for dehydration processes shorter than 10 min. Longer drying times (20 and 30 min) produce

severe changes in bacteria dried without the sugars. However, the presence of sugars during dehydration for 20 or 30 min preserves the cell volume and the zeta potential, though the membrane integrity is strongly affected.

It is important to point out that since the lag time strongly decreases when cells are dehydrated in the presence of sugars (Fig. 1), the evaluation of cell recovery through changes in the membrane could be misleading. In contrast, cell volume and zeta potential determinations could be more accurate as criteria to determine the potential recovery.

In addition, the preservation of the membrane integrity together with the volume and zeta potential recovery is attained at a period of time at which the water activity remains around 0·60 (Figs 1, 2 and 3). Longer times decrease the water activity to 0·45 and, in this condition, the volume and zeta potential are recovered if the drying is carried out in the presence of sugars but the membrane damage cannot be avoided. This means that membrane is damaged when the water activity drops below 0·60. However, this damage is not irreversible for cell recovery, since the lag time is reduced together with a restoration of the cell volume and zeta potential.

In the presence of sucrose or trehalose, the zeta potential decreases only slightly in comparison with nondried cells. This can be interpreted considering that sugars hinder the exposure of hydrophobic bacterial surface groups to the air by preserving a hydrophilic environment. This behaviour has been previously observed in liposomes (Anchordoguy *et al.* 1987; Leslie *et al.* 1995; Luzardo *et al.* 2000). This means that in spite of the fact that trehalose and sucrose do not preserve the membrane integrity; bacterial cells are able to repair damages and grow (Fig. 1). The repair mechanism is more efficient when cells are dehydrated in the presence of sugars.

These results suggest that the role of sugars is not restrained to the protection of the bacterial membrane. As bacteria can repair damages and growth, the effect of both trehalose and sucrose is addressed to more critical targets such as proteins. Different authors have referred that it is protein denaturation as the rate-limiting step of cell killing (Lepock *et al.* 1990; Teixeira *et al.* 1997). In fact, Teixeira *et al.* have found by DSC (Differential Scanning Calorimetry) methodologies that damage produced in membrane lipids, ribosomes and DNA are reversible, whereas damages produced in proteins are not (Teixeira *et al.* 1997).

Leslie *et al.* have reported that trehalose and sucrose are able to protect not only cell membranes but also proteins from dehydration (Leslie *et al.* 1995). In fact, by using FTIR (Fourier Transform Infrared Spectroscopy) spectroscopy these authors were able to observe that the band corresponding to Amide II protein structures of *Escherichia coli* and *Bacillus thuringiensis* shifts 10 cm^{-1} towards the dried state. They have also found that both sucrose and trehalose protect proteins in the dried state because when these sugars are present, no shifts in the Amide II bands are observed (Leslie *et al.* 1995). The decrease in the vibration frequency represents a change in protein structure in the dried cells, which can be related with the decrease in viability (Carpenter and Crowe 1989; Leslie *et al.* 1995). In this sense, it has been reported that the protein–sugar interaction may maintain proteins in a conformation similar to that of the hydrated protein, perhaps by binding to the hydrophilic domains of the proteins and preventing inter- and intraprotein hydrogen bonding during drying and rehydration (Carpenter *et al.* 1990, 1992).

In order to explain this possible mechanism of protection it would be required that trehalose crosses the membrane, as it occurs in other types of cells (Diniz-Mendes *et al.* 1999; Duong *et al.* 2006). However, to our knowledge no study has been reported in these bacteria.

The heat resistance of *Lact. bulgaricus* has been also analysed by Teixeira *et al.* (1997). They have determined that there exists a critical temperature for the repair of the damage produced after heating.

In the light of the present results, the preservation procedures in which heat is involved (spray drying, ultra high temperature treatments, sterilization, etc) must be considered as a combination of two factors: time and temperature. This may explain that membrane damage occurs only after 20 and 30 min dehydration. Even when the dehydration conditions used in this work are not equivalent to the ones used by Teixeira *et al.* 1997; it is important to point out the agreement showing that the membrane damage can be repaired if cells are dehydrated in the presence of sugars (see Figs 5, 6 and 1 together).

According to these results, cell volume recovery (meaning no loss of intracellular material) and maintenance of zeta potential are responsible for the maintenance of the cellular volume and the preservation of the surface properties. Only if these two parameters remain constant, the micro-organisms are able to repair the membrane damage and grow.

Conclusion

The damage produced by dehydration in the absence of sugars induces a disruption of the membrane integrity.

When cells are dehydrated at short times in the presence of sugars, cell recover and cell volume and the zeta potential are preserved upon rehydration with a low effect on membrane integrity.

However, when dehydration in the presence of trehalose and sucrose occurs for a longer period of time (20 and 30 min), the maintenance of the zeta potential and the cellular volume recovery are congruent with the preservation of the cells, but membrane damage cannot be avoided.

Taking into account these facts, it can be concluded that the evaluation of the membrane damage is not a conclusive parameter to predict the ability to grow after rehydration of dehydrated cells. In contrast, zeta potential and cellular volume are more relevant to predict cell growing after the dehydration processes.

The cell recovery has already been reported and ascribed to the protection of other structures such as DNA, ribosomes and proteins (Teixeira *et al.* 1997).

In this work, two supramolecular parameters have been tested in order to determine cell viability, independent on the membrane damage. This result is of great practical importance because the determination of these parameters does not require performing growing curves, which are certainly time-consuming.

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References

Alemohammad, M.M. and Knowles, C.J. (1974) Osmotically induced volume and turbidity changes of *Escherichia coli* due to salts, sucrose and glycerol, with particular references to the rapid permeation of glycerol into the cell. *J Gen Microbiol* **82**, 125–142.

Anchordoguy, R.J., Rudolph, A.S., Carpenter, J.F. and Crowe, J.H. (1987) Modes of interaction of cryoprotectans with membrane phospholipids during freezing. *Cryobiology* **24**, 324–331.

Arakawa, T. and Timasheff, S.N. (1982) Stabilization of protein structure by sugars. *Biochemistry* **21**, 6536–6544.

Carpenter, J.F. and Crowe, J.H. (1989) An infrared spectroscopic study of the interactions of carbohydrates with dried proteins. *Biochemistry* **28**, 3916–3922.

Carpenter, J.F., Crowe, J.H. and Arakawa, T. (1990) Comparison of solute-induced protein stabilization in aqueous solution and in the frozen and dried states. *J Dairy Sci* **73**, 3627–3636.

Carpenter, J.F., Arakawa, T. and Crowe, J.H. (1992) Interactions of stabilizing additives with proteins during freeze-thawing and freeze-drying. *Dev Biol Stand* **74**, 225–239.

Crowe, J.H., Crowe, L.M. and Chapman, D. (1984) Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **223**, 701–703.

Crowe, J.H., Hoekstra, F.A. and Crowe, L.M. (1992) Anhydrobiosis. *Annu Rev Physiol* **54**, 579–599.

Crowe, J.H., Carpeneter, J.F. and Crowe, L.M. (1998) The role of vitrification in anhydrobiosis. *Annu Rev Physiol* **60**, 73–103.

De Antoni, G.L., Pérez., P., Abraham, A. and Añón, M. (1989) Trehalose, a cryoprotectant for *Lactobacillus bulgaricus*. *Cryobiology* **26**, 149–153.

De Man, J.O., Rogosa, M. and Sharpe, M.E. (1960) A medium for the cultivation of *Lactobacilli*. *J Appl Bacteriol* **23**, 130–135.

Diniz-Mendes, L., Bernardes, E., de Araujo, P.S., Panek, A.D. and Paschoalin, V.M.F. (1999) Preservation of frozen yeast cells by trehalose. *Biotechnol Bioeng* **65**, 572–578.

Duong, T., Barrangou, R, Russell, W.M. and Klaenhammer, T.R. (2006) Characterization of the tre locus and analysis of trehalose cryoprotection in *Lactobacillus acidophilus* NCFM. *Appl Env Microbiol* **77**, 1218–1225.

Fernández-Murga, L., Font de Valdez, G. and Disalvo, E.A. (2000) Changes in the surface potential of *Lactobacillus acidophilus* under freeze/thawing stress. *Cryobiology* **41**, 10–16.

Gómez-Zavaglia, A., Abraham, A., Giorgieri, S. and De Antoni, G. (1999) Application of polyacrylamide gel electrophoresis and capillary gel electrophoresis to the analysis of *Lactobacillus delbrueckii* whole-cell proteins. *J Dairy Sci* **82**, 870–877.

Gómez-Zavaglia, A., Tymczyszyn, E.E., De Antoni, G.L. and Disalvo, A. (2003) Action of trehalose on the preservation of *Lactobacillus delbrueckii ssp. bulgaricus* by heat and osmotic dehydration. *J Appl Microbiol* **95**, 1315–1320.

Gouffi, K., Pica, N., Pichereau, V. and Blanco, C. (1999) Disccharides as a new class of nonaccumulated osmmoprotectants for *Sinorhizobium meliloti*. *Appl Env Microbiol* **65**, 1491–1500.

Gould, G.W. (1989) Heat-induced injury and inactivation. In *Mechanisms of Action of Food Preservation Procedures* ed. Gould, G.W. pp. 11–42. London, UK: Elsevier Applied Science.

Koch, A.L. (1984) Shrinkage of growing cells of *Escherichia coli* by osmotic challenge. *J Bacteriol* **159**, 919–924.

Lepock, J.R., Frey, H.E. and Inniss, W.E. (1990) Thermal analysis of bacteria by differential scanning calorimetry: relationship of protein denaturation in situ to maximum growth temperature. *Biochim Biophys Acta* **1055**, 19–26.

Leslie, S., Israeli, E., Lighthart, B., Crowe, J.H. and Crowe, L.M. (1995) Trehalose and sucrose protect both membranes and proteins in intact bacteria during drying. *Appl Environ Microbiol* **61**, 3592–3597.

Linders, L., Wolkers, W.F., Hoekstra, F.A. and van't Riet, K. (1997) Effect of added carbohydrates on membrane phase behavior and survival on dried *Lactobacillus plantarum*. *Cryobiology* **35**, 31–40.

Lodato, P., Segovia de Huergo, M. and Buera, M.P. (1999) Viability and thermal stability of a strain of *Saccharomyces cerevisiae* freeze-dried in different sugar and polymer matrices. *Appl Microbiol Biotechnol* **52**, 215–220.

Luzardo, M., Amalfa, F., Nuñez, A., Díaz, S., Biondi de López, A. and Disalvo, E.A. (2000) Effect of trehalose and sucrose on the hydration and dipole potential of lipid bilayers. *Biophys J* **78**, 2452–2458.

Oldenhof, H., Wolkers, W., Fonseca, F., Passot, S. and Marin, M. (2005) Effect of sucrose and maltodextrin on the physical properties and survival of air-dried *Lactobacillus bulgaricus*: an *in situ* Fourier transform infrared spectroscopy study. *Biotechnol Prog* **21**, 885–892.

Poirier, I., Marechal, P.A., Evrard, C. and Gervais, P. (1998) *Escherichia coli* and *Lactobacillus plantarum* responses to osmotic stress. *Appl Microbiol Biotechnol* **50**, 704–709.

Starzak, M. and Mathlouthi, M. (2006) Temperature dependence of water activity in aqueous solutions of sucrose. *Food Chem* **96**, 346–370.

Teixeira, P.M., Castro, H., Mohácsi-Farkas, C. and Kirby, R. (1997) Identification of sites of injury in *Lactobacillus bulgaricus* during heat stress. *J Appl Microbiol* **83**, 219–226.

Tomlins, R.I. and Ordal, Z.J. (1976) Thermal injury and inactivation of vegetative bacteria. In *Inactivation of Vegetative Bacteria* ed. Skinner, F.A. and Hugo, W.B. pp. 153–185. London UK: Academic Press.

Trevors, J.T. (2003) Fluorescent probes for bacterial cytoplasmic membrane research. *J Biochem Biophys Methods* **57**, 87–103.

Tymczyszyn, E.E., Gómez-Zavaglia, A. and Disalvo, E.A. (2005) Influence of the growth at high osmolality on the lipid composition, water permeability and osmotic response of *Lactobacillus bulgaricus*. *Arch Biochem Biophys* **443**, 66–73.

Tymczyszyn, E.E., Gómez-Zavaglia, A. and Disalvo, E.A. (2007) Effect of sugars and growth media on the dehydration of *Lactobacillus delbrueckii* ssp. *bulgaricus*. *J Appl Microbiol* **102**, 845–851.

Xie, G.F. and Timasheff, S.N. (1997) The thermodynamic mechanism of protein stabilization by trehalose. *Biophys Chem* **64**, 25–43.