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Survival of *Lactobacillus helveticus* entrapped in Ca-alginate in relation to water content, storage and rehydration

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Lactobacillus helveticus **CNRZ 303 entrapped in Ca-alginate gel beads was investigated for improved survival and stability during fluidized-bed drying, storage and rehydration. Addition of protective solutes was very important. Studies of the conditions showed that inactivation of entrapped *L. helveticus* started when the water content exceeded 0.3–0.4 g H_2O (g dry wt)$^{-1}$ for adonitol, glycerol and reconstituted non fat milk solids (NFSM). With Ringer's solution (control) and betaine, the fall in viability was evident above 1 g H_2O (g dry wt)$^{-1}$. Drying down to 0.2 g H_2O (g dry wt)$^{-1}$ required the removal of 98.5–98.9% of the water. The best survival rate with the least injured cells among survivors was experienced with adonitol and NFMS, respectively, 71% and 57% (compared to the initial) immediately after dehydration. Adonitol and NFMS were also best for survival during storage. The highest cell recovery was obtained by rehydrating the cells in cheese whey permeate between 20–30°C done at pH 6.0–7.0, satisfying the demands for cell survival, repair and slow swelling (adaptions).**

Keywords: Ca-alginate entrapped lactobacilli; dehydration; water content; protective solutes; survival

Introduction

Lactic acid bacteria (LAB) have important roles in the production of fermented foods and feeds. Also the use of immobilized LAB cultures has found some interest during recent years. Thus the preservation of immobilized cultures has become an aspect of concern for the technological use of LAB.

Convective drying of immobilized LAB is expected to be a supplement to other frequently used preservation methods like freezing and freeze drying. With such methods the bacteria are brought to an anabiosis state, where the metabolism is reversibly reduced to an extremely low level. Inactivation during drying and poor shelf-life of the product under uncontrolled storage conditions are the main disadvantages. Among the convective drying methods for the preservation of bacteria, the three most promising are: spray drying, fluidized-bed drying and a combination spray granulation [14].

Inactivation during drying may be caused both by dehydration and high temperature leading to injury or death of bacterial cells by damage to: DNA/RNA, proteins, and cell envelopes, and by unfavourable oxidation reactions, and increased concentration of acids and other small molecules [15–17,19–21].

Damage to the cell envelope and cell membrane in particular is expected to lead to leakage of intracellular components. The organization of the polymorphic mixtures of phospholipids and water in cell envelopes is mainly dependent on the composition, temperature and hydration state of the lipid bilayer, and on the ionic strength and pH of the surrounding medium. During de- and rehydration, the phospholipid-water system passes the phase boundary between the liquid crystalline and the gel phase which can lead to damage to the bilayer [5,7,15].

During the period of a constant drying rate the free and intracellular water are evaporated. When the bound water starts to evaporate, the drying rate decreases [2]. Dehydration rate and final water concentration are both parameters which are expected to influence the survival of bacteria during convective drying [14].

Changes in cell water content in bacteria occur as a result of changes in the osmolality of the surroundings, or of the cell cytoplasm, or as a result of changes in tension in the cell envelope [7]. Protective solutes will influence this. Acceptable viability during dehydration occurs with a slow water potential variation at optimal temperature and a low residence time of cells in the critical range of water potential. Physiological adaption to the increased osmotic pressure in the cell is probably the reason [15,19,26,27].

The thermophilic *L. helveticus* with a significant negative relationship between leakage of intracellular lactate dehydrogenase (LDH) and survival after dehydration was chosen for the present studies. For comparison mesophilic strains of lactobacilli showed only negligible leakage (unpublished results). The dehydration operations in this study were performed at 5°C where injury (membrane damage) of *L. helveticus* will occur if a proper protective solute is not provided.

The aim of this study was to describe a procedure for producing storage-stable immobilized LAB. Thus we studied the influence of the removal of water and the effect of protective solutes on the survival and injury of *L. helveticus* immobilized in Ca-alginate gel during fluidized-bed drying. Inactivation during short-time storage and the effects of temperature and pH on rehydration survival were also determined.

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Materials and methods

Organisms, media and cultivation

The thermophilic, homofermentative *Lactobacillus helveticus* CNRZ 303 obtained from Centre National de Recherches Zootechniques, Jouy-en-Josas, France, was studied due to its high sensitivity to dehydration stress (unpublished results). The stock culture was stored at –80°C in MRS broth (Difco, Detroit, MI, USA) supplemented with 15% (v/v) glycerol. MRS broth was autoclaved for 20 min at 121°C prior to use. The strain was incubated at 43°C and an exponentially growing culture was used as inoculum (1%).

Biomass production of strain CNRZ 303 was in MRS medium in a 2-L batch fermentor at 43°C, pH was maintained above 6.0 by titration with 1.5 N NaOH in a Radiometer Titrator system (Radiometer, ETS822 system, Copenhagen, Denmark) and stirring was with a gentle impeller at 250 rpm. Cultivation was terminated after 20 h. Growth was monitored by recording OD at 600 nm with a Shimadzu spectrophotometer (UV-1601, Shimadzu Europa, GMBH, Germany), dilution of samples was in growth medium. Light transmission microscopy was used to control the uniformity of the culture prior to harvesting them by centrifugation at 10 000 × *g* for 5 min at <5°C. Cells were washed twice in Ringer's solution (quarter-strength, Oxoid, Basingstoke, Hampshire, UK) and resuspended in Ringer's solution.

Cell immobilization

The cells in suspension were mixed with an equal amount of previously heat treated (80°C, 1 min) 4% (w/v) Na-alginate solution (Protanal, LF 10/60, Pronova Biopolymer A/S, Drammen, Norway) to a final concentration of 20 g dry cell mass per liter 2% (w/v) alginate solution. Ca-alginate gel beads were then formed by the dropwise addition of this suspension to a gently stirred 0.05 M Ca-lactate solution at pH 6.9 and 5°C [30]. After stirring the beads for 30 min, they were rinsed with sterile Ringer's solution to remove excess calcium ions and free cells, transferred to Ringer's solution and kept at 2–3°C. The bead diameter was 3.0 ± 0.1 mm determined according to Øyaas *et al* [25].

Dehydration, rehydration and storage

Immobilized cells in Ca-alginate gel beads were dried in a home-made laboratory fluidized-bed dryer. Simultaneous drying of five separate samples was conducted under controlled air flow, temperature (5°C), and relative humidity (55% ± 2.5%) of entering air. The drying time was either 6 h (Figure 1) or 3 h (Figure 6), with frequent removal of samples during dehydration. Mean value of all dehydrated preparations with, respectively: Ringer's solution; adonitol; betaine; glycerol; and reconstituted non-fat milk solids (NFMS), was 0.17 g H_2O (g dry wt)$^{-1}$ ± 0.05 (SD). The incorporation of the drying solutes, dehydration and storage were done at temperatures <5°C to keep metabolic activity at a low level.

Storage of dehydrated beads containing cells was in ampoules of polypropylene in a dark glass flask at 2–3°C. The Ca-alginate gel beads shrank remarkedly during dehydration and the diffusion of oxygen was considered to be limited.

An appropriate energy source in the rehydration medium may also be important for cell recovery. Ultrafiltered cheese whey (CW) permeate previously heat-treated at 80°C for 1 min was used. pH was maintained at 6.0, 6.5 and 7.0 during rehydration by titration with 0.25 N NaOH. The temperature was 5, 20 or 30°C. These conditions were chosen to determine eventually optimal values for cell repair after stress and sublethal damage. The rehydration was stopped when the gel bead volume was identical to freshly made beads. This was achieved within 2–3 h, however, it was somewhat dependent on the protective additive used. The gel bead volume was verified by determining the length of 30 beads giving the actual mean diameter of beads. The mechanical strength of the beads was measured by 'back extrusion' according to Steffe and Osorio [32].

Cell leakage from the Ca-alginate gel beads was determined during the steps in this procedure. Dehydration and rehydration experiments were run twice.

Desorption isotherms

Desorption isotherms were determined for the Ca-alginate gel beads with respective drying solutions (Ringer's, adonitol, betaine, glycerol, and NFMS). The moisture content (X) (g H_2O (g dry wt)$^{-1}$) of the samples was found by weighing them before and after drying in an atmospheric oven at 100°C for 4 h. The a_w was determined by placing the sample of known water content in a sealed glass tube at constant temperature (20°C). The equilibrium relative humidity (RH_{eq}) (%) surrounding the beads was measured with an HM 34 C humidity meter (Vaisala, Helsinki, Finland) and the a_w was calculated ($a_w = RH_{eq}/100$).

Drying solutes

Ca-alginate gel beads containing cells were suspended in twice the volume of beads of the protective solutes and kept on ice with mixing 10 times during 1 h prior to drying, to allow for equilibration of solute throughout the system. The additives incorporated and tested for protective effect were: 0.5 M adonitol (Sigma Chemical Co, St Louis, MO, USA); 0.5 M betaine (glycine betaine, Sigma Chemical Co); 0.5 M glycerol (BDH Laboratory Supplies, Poole, UK); 10% (w/v) NFMS (Merck, Darmstadt, Germany), and the control was quarter strength Ringer's solution (Oxoid).

The following drying solutes were also tested, but not in relation to water properties: MRS broth (Difco); 0.5 M sucrose (BDH Laboratory Supplies); 0.5 M maltose (Difco); 0.5 M d-(+) trehalose (Sigma Chemical Co); and 0.5 M d-sorbitol (Sigma Chemical Co).

pH was adjusted to 6.9 for all additives and osmolality of the protective solutes was 534, 567, 531, and 267 mOsm, for adonitol, betaine, glycerol and NFMS, respectively. The osmolality of quarter-strength Ringer's solution was 107 mOsm. Osmolality was measured using an osmometer (The Advanced™ Osmometer Model 3D3, Advanced Instruments, Norwood, MA, USA). The solutions were heat treated at 80°C for 1 min and cooled (2–3°C) prior to use.

Activity and survival determination

The specific lactic acid-producing activity reflecting directly the rate of lactose utilization was determined by assessing lactic acid production in CW permeate for a

known amount of Ca-alginate gel beads (cell mass). The experiments were conducted in a stirred (500 rpm), batch reactor of 300-ml working volume, at 43°C for 2 h. The pH was maintained at 5.5 by titration with 0.25 N NaOH.

Survival rates expressed as colony-forming units (CFU) were determined by plating serial dilutions on MRS-agar (MRSA) and on MRSOA containing 0.15% (w/v) oxgall (Difco) to assess sublethal cellular injury [12]. The beads were first dissolved in 0.2 M NaH_2PO_4, pH 6.9. Plates were incubated at 43°C for 48 h. Survival was defined as the ratio of the viable counts after (N) and before drying (N_0). The difference in CFU on MRSA and MRSOA was due to sublethally injured cells among the survivors.

Results

Figure 1 shows the drying curves of *L. helveticus*-alginate gel beads in a fluidized-bed dryer (5°C and ≈ 55% *RH*). The initial moisture content (X_0) was either ≈ 18 or ≈ 13 g H_2O (g dry wt)$^{-1}$ for: (i) Ringer's and glycerol; and (ii) adonitol, betaine and NFMS, respectively (Figure 1). The drying rate could be expressed as the characteristic drying time (τ_d), which was defined by Linders *et al* [20] as the time needed to reach a certain X at a given a_w or a certain drying efficiency [16,20]. In our study the time to reach an X of 1 g H_2O (g dry wt)$^{-1}$ (a_w = 0.85–0.9) varied between 2 and 2.5 h depending on the protective solute used and the initial X_0. Adaption was expected to occur when the X decreased from 1.5 to 0.5 g H_2O (g dry wt)$^{-1}$ [20].

The relation between X and a_w is given by the moisture sorption isotherm (MSI) curve for the ternary mixtures composed of *L. helveticus*, Ca-alginate gel and a drying solute (Figure 2). The classic shape for biological products is sigmoidal. During the first 60 min of drying, an almost linear decrease in X was observed, indicating a constant drying rate of about 10 and 6 g H_2O (g dry wt · h)$^{-1}$ for group (i) and (ii), respectively (Figure 1). Then, water evaporation slowed gradually.

The shrinking and swelling *vs* time, are given as the ratio between the volume of the beads during respectively de-

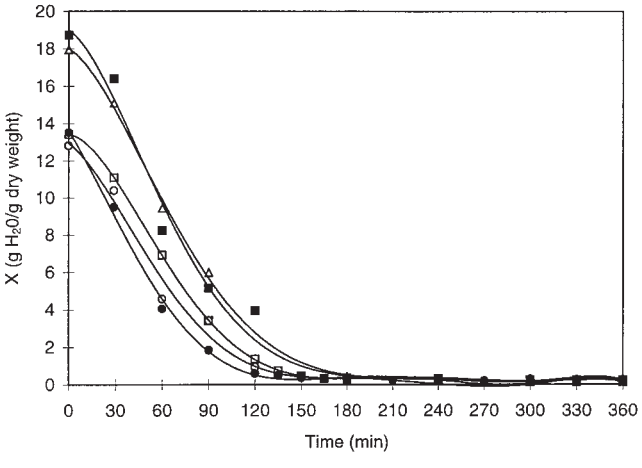

Figure 1 Drying of Ca-alginate gel beads with entrapped *Lactobacillus helveticus* CNRZ 303 at 5°C and ≈ 55% RH in a fluidized-bed dryer. Solutes used: ■, Ringer's; □, adonitol; ○, betaine; △, glycerol; ●, non fat milk solids (NFMS).

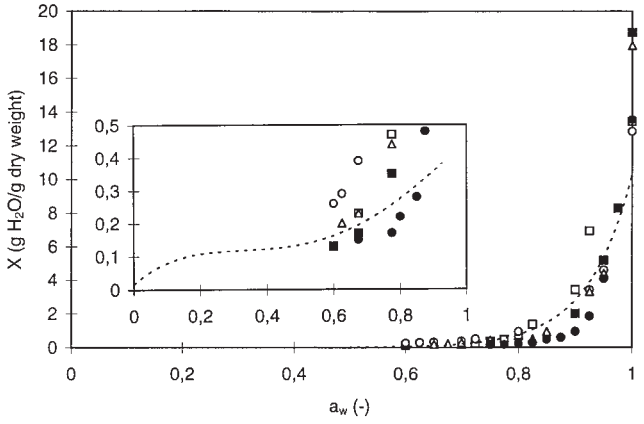

Figure 2 Desorption isotherms of Ca-alginate gel beads with entrapped *Lactobacillus helveticus* CNRZ 303. The line represents the estimated fit for beads dried in quarter strength Ringer's solution. The inserted figure represents the typical lapse for low values. Symbols for drying solutes; ■, Ringer's; □, adonitol; ○, betaine; △, glycerol; ●, NFMS.

and rehydration in CW permeate (V), and the volume of the beads after gel formation in Ca-lactate solution (V_0) (Figure 3). The rehydration curves for all drying solutes almost reached V_0 after 3 h.

The water and viability losses were further characterized by plotting the residual colony counts as the fractions of survivor cells (N/N_0) *vs* the water content X (Figure 4). The fall in viability was not uniform either regarding time or water loss. From the initial X_0 to approx. 0.2 g H_2O (g dry wt)$^{-1}$, 98.5–98.9% of the water was evaporated. The corresponding viability varied in accordance with the chosen drying solute as shown for survivors and survivors injured (Table 1).

Colony counts were used as an indicator of survival during dehydration. A comparison has been performed for six strains of *Lactobacillus*, to verify whether there was a linear relation between measurement of survival and activity. The correlation coefficient (r) for survival (viable counts) *vs*

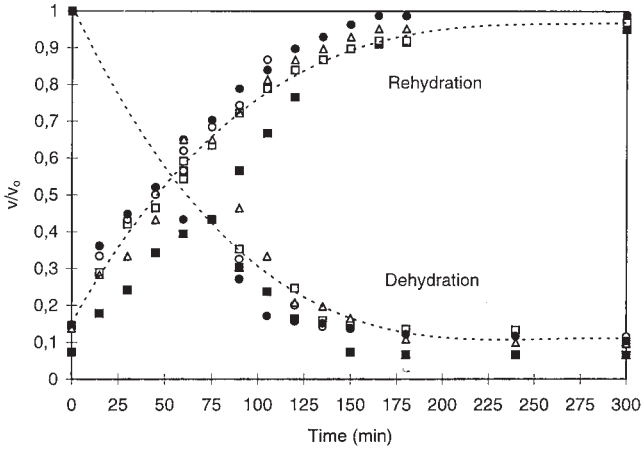

Figure 3 Dehydration and rehydration curve of Ca-alginate gel beads with entrapped *Lactobacillus helveticus* CNRZ 303. Dehydration in a fluidized-bed dryer was to a mean level of 0.17 g H_2O (g dry weight)$^{-1}$. Rehydration was in CW permeate at 20°C and pH 6.9 as a function of time. The line represents the estimated fit for beads with adonitol. Symbols for drying solutes; ■, Ringer's; □, adonitol; ○, betaine; △, glycerol; ●, NFMS.

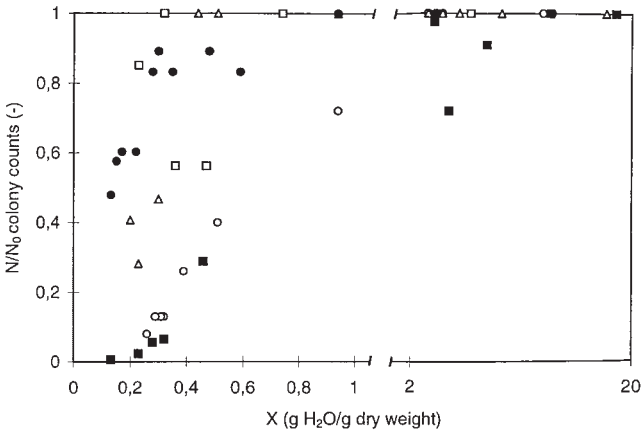

Figure 4 Residual survival of *Lactobacillus helveticus* CNRZ 303 entrapped in Ca-alginate gel beads with different solutes after fluidized-bed drying as a function of overall moisture content. Symbols for drying solutes; ■, Ringer's; □, adonitol; ○, betaine; △, glycerol; ●, NFMS.

Table 1 Results with Ca-alginate gel beads and *Lactobacillus helveticus* CNRZ 303 entrapped in beads after fluidized-bed drying to comparable water contents: residual water, survival rates, injured cells among survivors. Use of protective solutes is compared to the use of quarter-strength Ringer's solution

Solute	*X* (g H_2O (g dry weight)$^{-1}$)	*n* survivors cells (%)[a]	*n* survivors injured cells (%)[b]
Ringer's	0.13–0.32	3.7 ± 2.7 (SD)	32.0 ± 6.1 (SD)
Adonitol	0.23–0.32	70.7 ± 20.4 (SD)	58.4 ± 12.0 (SD)
Betaine	0.26–0.32	11.8 ± 2.5 (SD)	30.7 ± 4.5 (SD)
Glycerol	0.20–0.30	38.6 ± 9.5 (SD)	30.8 ± 4.6 (SD)
NFMS	0.15–0.28	56.5 ± 5.9 (SD)	61.9 ± 7.5 (SD)

[a]Ratio of number of viable cells after drying and rehydration relative to number of viable cells of recently-made Ca-alginate beads before drying. [b]Injured cells among survivors, ie percent survivors injured.

residual lactose fermenting activity after drying with adonitol, betaine, glycerol or NFMS, was determined to be, respectively, 0.87, 0.66, 0.76, 0.68 ($n \geq 16$ for each protective solute). The experimental error is influenced by the potential for cell repair in MRSA during incubation.

High recovery of immobilized and dried *L. helveticus* was obtained by rehydrating the cells in CW permeate between 20–30°C at pH 6.0–7.0 (Figure 5). Rehydration at 5°C gave significantly less survival compared to 20–30°C, which is in accordance with the membrane phase transition theory [6,15].

In addition to drying and storage, rehydration of immobilized cells may be highly important for the success of the present strategy for producing and using entrapped and dried LAB. The introduction of drying solutes may result in a higher residual activity (*A*) after drying. The differences in initial lactic acid productivity (A_0) observed with several drying solutes and storage times are shown in Figure 6. In these experiments the results, especially for betaine, differ considerably from those shown in Figure 4. The explanation can be due to a difference in rest moisture content in Figures 4 and 6 and the low *r* of 0.66 for survival *vs* residual lactose fermenting activity after drying. Based

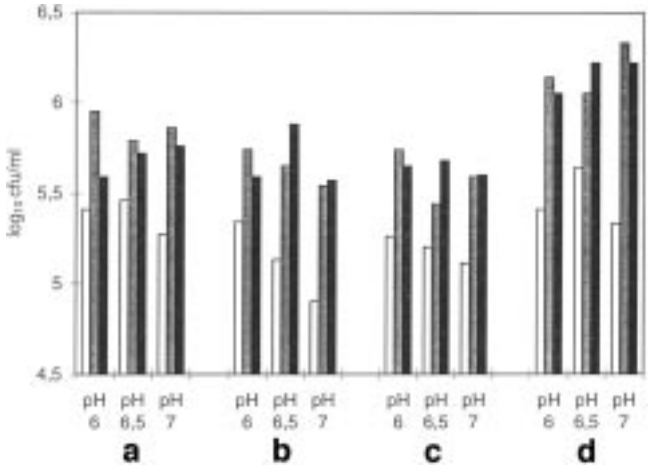

Figure 5 Survival after rehydration of *Lactobacillus helveticus* CNRZ 303 entrapped in Ca-alginate gel beads in cheese whey permeate at pH 6.0, 6.5, and 7.0 and □, 5°C; ▨, 20°C; ■, 30°C; as influenced by (a) adonitol; (b) betaine; (c) glycerol; (d) NFMS.

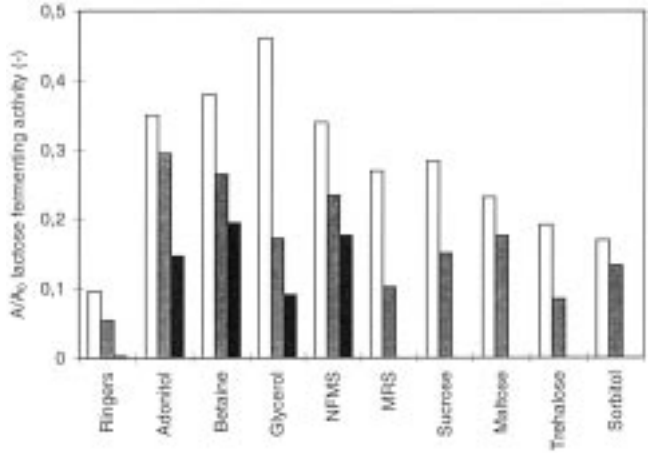

Figure 6 Influence of drying solutes and storage time on lactose fermenting activity of *Lactobacillus helveticus* CNRZ 303 entrapped in Ca-alginate gel beads. Entrapped cells were dehydrated in a fluidized-bed dryer to a mean level of 0.34 g H_2O (g dry weight)$^{-1}$. □, Storage time 0 days; ▨, 7 days; ■ 28 days. Rehydration was in cheese whey permeate at 20°C and pH 6.9. Beads with drying solutes: MRS; sucrose; maltose; trehalose; and sorbitol were stored for only 7 days.

on Figure 6, adonitol, NFMS and betaine showed the best protective properties during both drying and storage of dried *L. helveticus*, while the protective effect of glycerol was better during dehydration than during storage.

Considering all the steps in the procedure used, leakage and loss of cells were $< 1 \times 10^3$ CFU ml^{-1} in both the solution of divalent cation (Ca-lactate, 2 L) and in the Ringer's solution (500 ml) used for removing excess calcium ions prior to dehydration. During activity measurements in CW permeate (250 ml) for 2 h the cell leakage was $< 1 \times 10^4$ CFU ml^{-1}, $< 1 \times 10^3$ CFU ml^{-1} in the solution of protective agents (50 ml) and $< 1 \times 10^4$ CFU ml^{-1} in the rehydration solution (50–100 ml). Zhou *et al* [39] found a cell release of 1×10^5 CFU ml^{-1} after 6 h fermentation using *Lactococcus lactis* subsp *cremoris*.

Discussion

Mechanisms of inactivation during dehydration

For *L. plantarum* the drying temperature should be as high as 30°C, both for physiological adaption and to maintain an intact cell envelope [20]. Fluidized-bed drying in the present study involved limited drying time and minimal metabolic activity (5°C) of the cells during dehydration. Since we observed only minor differences in survival of *L. helveticus* when rehydrated at 20 or 30°C in the presence of protective solutes, we consider that leakage had been minimized compared to observations at 5°C. This supports the theory of greater leakage through the membrane in the gel phase than in the liquid crystalline phase [6,15].

In another study without protectants, conducted at 30°C and 20% *RH* to a level of 0.11 g H_2O (g dry wt)$^{-1}$, we found that three mesophilic lactobacilli, *L. plantarum*, *L. casei* subsp *casei* and *L. casei*, had survival rates of, respectively, 8, 12 and 45%, after 100 days of storage. In comparison survival of the thermophilic *L. helveticus* was $< 0.1\%$.

Lievense *et al* [17] showed that dehydration inactivation of *L. plantarum* was associated with clear evidence of membrane damage and inactivation was dependent on the final moisture concentration [16,17]. Lievense and van't Riet [15] found that dehydration inactivation of *L. plantarum* immobilized in starch started at a water concentration of 0.35 g H_2O (g dry wt)$^{-1}$. Paul *et al* [26] observed the same tendency for dehydrated alginate-entrapped cells of *Azospirillum lipoferum*. The loss of viability increased down to $X = 0.25$ g H_2O (g dry wt)$^{-1}$ and then stopped until the end of the drying operation. In the present experiments with *L. helveticus* the same phenomenon was observed at 0.3–0.4 g H_2O (g dry wt)$^{-1}$ with adonitol, glycerol and NFMS, somewhat depending on drying solute (Table 1 and Figure 4). Table 1 also shows that injured cells among survivors were high for adonitol and NFMS, respectively, 58% and 62% measured immediately after drying.

a_w may play an important role in survival of dehydrated bacteria [3,18,20,24]. Cassidy *et al* [3] expressed that in an a_w range between 0.069 and 0.83 depending somewhat on temperature, solute concentration and mobility allow water (solvent) to reach the cell surface and destroy cells by an osmotic effect. The rates of survival may depend on the composition of the growth medium, thus larger molecular weight compounds (C_6–C_{12}) tend to be protective [3,24].

Factors influencing survival

A number of factors influence the survival of dried bacteria: bacterial species and even strains; the age of the culture; growth conditions; cell concentration; pH; protective agents; drying gas, rate, and extent; storage and rehydration conditions [9,10,15,19–21,31,37].

Differences in cell survival show that certain additives are more effective than others in protecting the thermophilic *L. helveticus* subjected to convective drying (Figures 4 and 6). Desorption isotherms measured for Ca-alginate gel beads with the respective drying solutes indicate changes in moisture distribution and a_w (Figure 2). The residual survival *vs* overall moisture content (Figure 4) shows an improved residual survival after dehydration on the addition of adonitol, glycerol and NFMS. The overall moisture content we measured, is expected to be somewhat different from the moisture content (desorption isotherm) of entrapped cells in Ca-alginate gel beads. According to Linders *et al* [19], the X in the cell is related to the a_w through the sorption isotherm, if no interaction occurs.

The effect of a protectant is thought to increase with concentration until a maximum protection level is reached. For added lactose we observed this phenomenon [28], however, lactose did not improve survival during storage, probably caused by its reducing sugar properties leading to Maillard reactions under the conditions studied.

Bacteria may respond to high medium osmotic pressure by accumulating protective low-molecular-weight solutes to high intracellular concentrations [8,11,21,22]. Betaine and proline are well-known osmoprotectants and compatible solutes with a growth-stimulating effect. Some bacteria do not synthesize betaine *de novo* and are dependent on supplied betaine or its precursor, choline [22]. However, the lactobacilli can not synthesise and are probably not able to accumulate compatible solutes to any great extent during the rather short drying process. Natural polymers added before dehydration may provide increased survival after desiccation and during storage [3]. Several hypotheses for the mechanisms have been proposed. Crowe *et al* [6] reported that the stabilizing effect of saccharides is probably based on direct hydrogen bonding interactions with the phosphate of the phospholipid heads lowering the membrane phase transition temperature (T_m) between the gel and liquid crystalline phase. The lower T_m permits the membrane to exist in the liquid crystalline phase during de- and rehydration [6,15]. Linders *et al* [19] suggested that carbohydrates act through their free radical scavenging activity and not by direct interaction with the polar lipid headgroups. Among others adonitol, betaine, glycerol and NFMS have been used as osmoprotectants during freeze- or convective drying [1,3,10,28,29,33,35].

Adonitol, the sugar alcohol derived from ribose, a constituent of the teichoic acids of bacterial cell walls, cannot be metabolized by most lactobacilli. Adonitol has a protective action towards bacterial cells [10,35]. The effect of sugar alcohols and saccharides may be due to the esteric structure of their hydroxyl groups which may be capable of replacing water molecules in protein structures [34]. Skim milk stabilizes cell membrane constituents during preservation procedures [4,10]. The protective solute glycerol which, unlike salts and sugars, rapidly permeates the cell membrane causes no, or only transient, plasmolysis. Sorbitol, a non-permeant solute, and sucrose do not readily permeate the cell membrane, thus they effect plasmolysis of the cell by osmosis [7]. Trehalose preserves model membranes of *Saccharomyces cerevisiae*, *Escherichia coli* and *Bacillus thuringiensis* [13,15,19], but no protection was observed for *L. helveticus* (Figure 6). Sucrose, maltose, trehalose and sorbitol all have protective effects for freeze-dried cells, and sorbitol, maltose and to a lesser extent sucrose protected *L. plantarum* during fluidized-bed drying and storage. This was found in this study and in another work involving a mixture of starch and carbohydrate [18,19].

Valdez *et al* [34,35] showed that adonitol produced a

small change in X at various times during freeze drying and allowed a higher rate of survival than NFMS. This was also found for convectively dried Ca-alginate gel beads with adonitol or NFMS (Figure 4). These protectants have high water-binding potential together with stabilization effects towards proteins and membrane structures. However, the most important factor is probably that the cell membrane exists in the liquid crystalline phase under conditions at which it normally would be in the gel phase [13,15]. It is important to be aware that the best protective agents during dehydration are not necessarily best for protection during storage of the cells [6].

The initial cell concentration is found to influence the residual activity of air dried *L. plantarum* [18]. Therefore we used the same initial cell concentration (C_0 = 0.02 g dry cell (g alginate)$^{-1}$ throughout this study. The cell concentration might be increased to obtain higher numbers of surviving cells.

Kearney *et al* [10] suggested that protection during de- and rehydration of immobilized cells may occur because the diffusional properties and limited volume of gel beads control the rate and volume of fluids entering and exiting the beads. The chance of osmotic shock by an instantaneous removal or influx of water is reduced. The composition of the rehydration fluid, its a_w and the rate of rehydration will determine cell repair and survival during rehydration. The sensitivity of Ca-alginate to chelating compounds such as phosphate, citrate and lactate (competing with alginate for binding Ca^{2+}) or anti-gelling cations such as Na^+ or Mg^{2+} could be used with a view to rehydration. We tested several rehydration solutes but only CW permeate fermented by LAB satisfied our demands for cell survival, repair and slow swelling conditions (adaption) for LAB in shrunken Ca-alginate gel beads made from sodium alginate with high guluronate content. A recalcification step with the addition of Ca^{2+} giving 3–5% reduced volume of the beads could also be used. The mechanical strength of Ca-alginate gel beads was measured by 'back extrusion' and found slightly weaker than for the starting material (η_l; 0.46 Pa·s *vs* 0.67 Pa·s, ± 5%) [28].

Survival and repair of injured cells are strongly influenced by the rehydration conditions: rehydration solution, osmolality, temperature and pH; and rate of rehydration [15,36]. We found high survival rates with rehydration between 20–30°C and pH 6.0–7.0 (Figure 5). Valdez *et al* [36] studied the effect of rehydration conditions on viability of freeze-dried LAB; rehydration at 32°C and pH 7.0 in a rich medium gave the best recovery.

Linders [18] found that the membrane phase transition temperature (T_m) between the gel and liquid crystalline phase was 4°C for hydrated and 20°C for dried *L. plantarum*. Cellular ribonucleotides leaked from dried cells during rehydration, and rehydration in the presence of RNase or DNase caused a marked loss of viability and an increased leakage of hydrolysed nucleotides. These results suggest that the cell surface had been damaged by drying and its selective permeability was to some extent lost [17,23]. Castro *et al* [5] observed an increase in membrane permeability to β-galactosidase for freeze-dried *L. bulgaricus* and a reduced capability to maintain the ΔpH across the cell membrane.

A negative relationship was found between leakage of LDH and survival for thermophilic lactobacilli, when dehydrated at 30°C and rehydrated at 20°C, whereas mesophilic lactobacilli showed only negligible leakage (unpublished results). Cell membrane damage is expected to occur during rehydration, as a result of phase transitions in the phospholipids, but different drying solutes (sugars) may change the T_m thus preventing leakage through the cell envelope [15,19].

Stability during storage

Dehydrated alginate-entrapped cells of *Azospirillum lipoferum* at a_w below 0.55 survived well during prolonged storage [26]. The present dehydration experiments using a selection of drying solutes (Figure 6) showed decreasing survival with storage time, which can probably be explained by the limited removal of water during dehydration, even though the chosen suspending medium affects the water properties within the environment of the bacteria. Survival of dried LAB is strongly dependent on the storage RH_{eq} and temperature. Entrapped cells can exist in different states depending on the composition of the matrix, temperature and water concentration; a rubbery and a glassy state can be distinguished. The diffusion coefficients in the glassy state together with reaction rates are extremely low and a stable product is obtained. To reach the glassy state, low temperature or low water concentration is needed, but it may also be obtained by additives which change the position of the glass transition curve to higher temperatures, thus stabilizing the product [7,15].

It is an advantage to operate with membrane lipids in the glassy state, dependent on the glass transition temperature (T_g), since the diffusion rate in the glassy state is very low. Thus deleterious reaction rates are expected to decrease, proteins are stabilized and oxygen is depleted. Cultures stored at 11% RH_{eq} and low temperature obtained a small decrease in viability during storage. The effect of RH_{eq} on the fatty acid profile showed that at high RH lipid oxidation occurs more rapidly [4].

Cell injury and inactivation proceeds not only during dehydration, but also when dried preparations are stored. Storage stability increases with decreasing temperature [15,29]. Inactivation during storage is related to formation of radicals in the presence of oxygen, fatty acid oxidation and DNA damage. Lipid oxidation can contribute to a further increase in permeability and also affect enzymatic activities associated with the membrane [5]. A positive effect of anti-oxidants on survival has been shown, and the lipid composition of the cell membrane showed that the unsaturated/saturated fatty acid index may change with time during storage [4].

Storage under vacuum or inert gases has also been reported to improve survival of bacterial cells [29,38] and should be investigated with the intention to further improve survival of LAB. Improvement of the drying procedure, the choice of protective solutes both for protection during dehydration and during storage, and optimal rehydration conditions, are all expected to be important to achieve high survival rates for dried LAB.

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