Effects of Addition of Sucrose and Salt, and of Starvation upon Thermotolerance and Survival During Storage of Freeze-dried *Lactobacillus delbrueckii* ssp. *bulgaricus*

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ABSTRACT: Increased survival of freeze-dried cells of *Lactobacillus bulgaricus* was observed when the drying medium was supplemented with sucrose; however, the magnitude of such protection was dependent on the growth medium used. Supplementing the growth medium with NaCl markedly increased survival of dried cells, and only a small effect was exerted by the composition of the drying medium or prior to starvation of cells. The D_{57} values of *Lactobacillus bulgaricus* cells grown in MRS were about half of those of cells grown in MRS supplemented with sucrose, with sucrose plus NaCl, or with NaCl.

Keywords: starter culture, preservation, growth medium, viability

Introduction

Culture concentrates of lactic acid bacteria are widely used in the food industry for fermentation of milk, meat, fruit, vegetables, and bread products. The preparation of those concentrates requires manufacture and preservation techniques that will eventually maximize storage stability, viability, and activity of the bacterial cells (Desmons and others 1998).

It is well known (Bâati and others 2000) that lactic acid bacteria are exposed to a number of stress conditions in industrial environments, such as low temperature, low pH, and low water activity (a_w), which bring about membrane and cell wall damage, inhibition of active transport, retention of nutrients, morphological changes, and loss of viability. Bacteria have developed adaptive strategies to face the challenges of changing environments and to survive under conditions of stress (Abee and Wouters 1999). Adaptations to a given stress may result in increased resistance to that specific stress, (for example, sublethal heating and increase in D-value) or development of multitolerance to several different stresses (Pichereau and others 2000). Osmotic stress can lead to accumulation of the humectant, (for example, a sugar) or synthesis of osmoregulatory compounds to maintain osmotic balance (Bayles and Wilkinson 2000). Because both freezing and drying processes expose cells to low a_w stress conditions, mechanisms of adaptation involving accumulation of osmotic stress compounds (for example, sucrose) may enhance survival during the aforementioned processes. Compatible solutes may play a role in osmoprotection; for such compounds as betaine, carnitine, and mannitol, a protective effect during drying has already been reported (Kets and others 1996). The mechanism behind this effect remains to be fully elucidated, but increased levels of compatible solutes play positive roles in cell survival and enzyme activity (Abee and Wouters 1999).

The present work was undertaken with the following objectives: (1) to assess the influence of sucrose and/or NaCl in the growth medium (de Man, Rogosa, Sharp [MRS] broth), of sucrose in the drying medium (skim milk), and of starvation conditions before drying upon survival of *Lactobacillus bulgaricus* during freeze-drying and subsequent storage; and (2) to study the effect of such conditions on the thermal tolerance of bacterial cells to determine whether treatments that enhance resistance to freeze-drying and subsequent storage also promote cell survival during heating.

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Materials and Methods

Organism and media

Lactobacillus delbrueckii ssp. *bulgaricus* (hereafter termed *L. bulgaricus*) was obtained from the culture collection held at Escola Superior de Biotecnologia (Portugal). The original reference cultures were maintained in cryogenic storage at –80 °C on glass beads. Working cultures were grown on MRS broth (LAB M, Bury, U.K.) containing 1.5% (wt/wt) agar as slope cultures (at 37 °C for 24 h). Slopes were stored at 4 °C and subcultured every month. MRS broth was inoculated from the MRS agar slopes, and incubated at 37 °C for 24 h. This culture was then inoculated, at the 1% (vol/vol) level, into a standard MRS broth (MRS A containing 20 g/L glucose) and into modified MRS broths (prepared from individual ingredients in our laboratory) with the following composition: MRS B, with the 20 g/L glucose of MRS A replaced by 10 g/L glucose + 10 g/L sucrose (note that *L. bulgaricus* does not ferment sucrose); MRS C, as MRS B further supplemented with 5 g/L NaCl; and MRS D, as MRS A supplemented with 5 g/L NaCl. All inoculated broths were incubated at 37 °C for 24 h. The minimum inhibitory concentration (MIC) of NaCl for *L. bulgaricus* in MRS was calculated to be 6.25 g/L (data not shown); 5 g/L NaCl was thus chosen because this would permit good growth at 37 °C within 24 h.

Culture preparation

Cells were harvested by centrifugation at

7000 × *g* for 10 min, and washed twice with sterile Ringer's solution (LAB M). Selected samples from each growth medium were deliberately starved for 20 min in water at room temperature (20 °C) and then resuspended (to a volume equal to the original culture) in sterile skim milk containing 11% (wt/vol) solids (LAB M); the other samples were immediately resuspended in skim milk and skim milk with sucrose added at 10 g/L. Cell suspensions were maintained for 1 h at room temperature (20 °C) to allow for equilibration between cells and the compound added before freezing at –80 °C for 24 h. The experiments were repeated twice.

Freeze-drying and storage

Samples of 20 mL were desiccated under vacuum (50 mtorr for 48 h), in a freeze-drier (Martin Christ, Osterad am Harz, Germany) at room temperature (20 °C); the condenser was cooled at –55 °C. Dried cells were stored in closed containers at 20 °C in air and kept in darkness.

Enumeration of survivors

Immediately after freeze-drying and at regular time intervals during storage, samples taken at random were rehydrated to the original volume with deionized water, and suitable dilutions were plated on MRS agar by the drop count technique (Miles and Misra 1938). Three drops (20 μL each) of suitable dilutions were placed on each of 3 separate plates; these plates were examined after incubation at 37 °C for 48 h.

Heat resistance assessment

Aliquots (2 mL) of cell suspension immediately before freeze-drying were transferred to 48 mL of Ringer's solution previously equilibrated at 57 °C (Teixeira and others 1994). At appropriate intervals, samples (1 mL) were taken from the heating medium and added to 9 mL of Ringer's solution. After serial decimal dilutions in Ringer's solution, survivors were enumerated on MRS agar by the drop count technique; plates were examined after incubation at 37 °C for 48 h.

Statistical analysis

Analysis of variance (ANOVA) of viable counts after freeze-drying and at regular time intervals during storage was carried out using the statistical program R (Ihaka and Gentleman 1996) at the 5% level of significance. Multiple comparison of treatment means (2 replicates × 2 experiments), using 95% confidence intervals, was performed via Tukey's honestly significant difference (HSD); the results were plotted using Trellis display (Becker and others 1996).

Results and Discussion

A number of factors influence the resistance of bacteria to freeze-drying, storage in the dried state, and heat stress. In this study, the impacts of the following factors upon survival of *L. bulgaricus* during heat stress, freeze-drying, and subsequent storage were investigated: (1) nature of the drying medium, (2) nature of the growth medium, and (3) exposure to starvation. Three initial replicated experiments were performed, which varied in (1) time of sampling and (2) levels of survivors after freeze-drying and during storage in the dried state (different lethalities were indeed observed between experiments, as a result of different freeze-drying batches and uncontrolled storage conditions). Although direct comparisons could not be made between the aforementioned initial experiments, similar observations to those currently reported were obtained.

Effect of addition of sucrose to the drying medium (skim milk)

Freeze drying and storage. Statistical analysis of the viable count data was performed considering 3 factors: experimental replication, storage time, and drying medium. Experimental replication and its 2-way interactions with the other 2 factors were not significant, as expected; however, the drying medium and the storage time, as well as the 2-way interactions, were statistically significant ($P < 0.05$). The experimental results pertaining to survival of freeze-dried *L. bulgaricus* during storage at room temperature in the presence of sucrose, after growth in MRS with modified concentrations of sucrose and NaCl, are shown in Figure 1. This figure indicates that addition of sucrose to the drying medium significantly increases survival of *L. bulgaricus* during storage, after growth in MRS A, MRS B, and MRS C. However, in the case of *L. bulgaricus* grown in MRS D, good survival was attained under all conditions of drying tested; sucrose in the drying medium seems to be largely ineffective in further increasing the survival of dried cells throughout storage (Figure 1).

Leslie and others (1995) have shown that sucrose can protect liposomes, isolated biological membranes, and some intact cells from the adverse effects of freezing and drying. Increase in survival has been attributed to protection of protein functionality, owing to formation of a glassy matrix during freeze-drying that possesses high viscosity and low mobility (Franks and others 1991; Bell and Hageman 1996), and/or to solute binding to the dried protein, thus serving as a water substitute when the hydration shell of the proteins is removed (Carpenter and others 1991). In addition to protecting both the structure and the function of proteins during drying, sucrose and other carbohydrates lower the transition temperature of dry membranes via replacement of water molecules between the lipid headgroups; this phenomenon prevents phase transition and consequent leakage of cell contents upon rehydration (Leslie and others 1995; Castro and others 1997). Despite all these features, sucrose was not found to be effective in protecting freeze-dried *L. bulgaricus* that had been grown in MRS D, probably because the positive effect of the disaccharide was balanced by the positive effect of the growth medium in question. Longer storage times may have shown a further protective effect of sucrose in the drying medium.

Effect of the growth medium

Freeze drying and storage. Our results have provided evidence for the impact of the growth medium on viability throughout storage of dried *L. bulgaricus*. Several studies (Leslie and others 1995; Linders and others 1997a, 1997b; Abadias and others 2001; Carvalho and others 2002) have been carried out in attempts to evaluate the efficacy of various components added to the drying medium upon survival of microorganisms during drying and subsequent storage; the current results reported here have shown that, in addition, the growth medium is a critical parameter that plays a role in survival after freeze-drying.

Statistical analysis of the viable count data was performed considering 3 factors: experimental replication, storage time, and growth medium. Experimental replication and its 2-way interactions with the other 2 factors were again found to be not significant; however, the growth medium and the storage time, as well as its 2-way interaction, were statistically significant ($P < 0.05$). The experimental results pertaining to survival of freeze-dried *L. bulgaricus* during storage at room temperature, after growth was performed in MRS with increased concentration of sucrose or of NaCl (or of both), are shown in Figure 1. Survival of freeze-dried *L. bulgaricus* in the control drying medium (skim milk) during storage (but not during freeze-drying) was proved to depend on the growth medium composition. During the period tested, *L. bulgaricus* exhibited significantly higher survival rates during storage in the dried form when cells had been grown in MRS supplemented with NaCl than in the absence thereof. There were no significant differences between survival during storage when growth was performed on standard MRS (MRS A) or MRS with sucrose (MRS B).

By the end of the storage period tested, freeze-dried *L. bulgaricus* exhibited significantly higher survival rates when grown in MRS supplemented with both sucrose and NaCl (MRS C), or with NaCl alone (MRS D).

Addition of many osmotically active compounds, such as salts and sugars, lowers a_w; a universal response to the temporary loss of turgor after a hyperosmotic shock is the cytoplasmatic accumulation of a certain class of solutes, the so-called compatible solutes (Abee and Wouters 1999). These compounds have been claimed to be beneficial for LAB, not only during osmotic stress but also during drying (Kets and de Bont 1994; Kets and others 1996; Desmond and others 2001), owing to their ability to stabilize proteins under unfavorable conditions (Roeβler and Müller 2001). In our study, the rise of the medium osmolarity via addition of an electrolyte (NaCl) and of a nonelectrolyte (sucrose) had distinct outcomes on the stability of *L. bulgaricus* during storage of freeze-dried cells. Other studies (Glaasker and others 1998; Gouesbet and others 2001) have indeed shown that sucrose-stressed and NaCl-stressed *Lactobacillus plantarum* and *L. bulgaricus* cells do not accumulate the same compounds in response to osmotic stress. Such differences could partially account for the distinct survival behavior during storage that was observed with the various growth media experimented.

Thermotolerance. The effect of addition of sucrose and/or NaCl to MRS medium on the resistance of stationary phase cells of *L. bulgaricus* was investigated. The data in Table 1 indicated that a higher thermotolerance was exhibited as a result of increased sugar and/or salt concentrations in the growth medium. The heat resistance of a microorganism may be measured by its D_T value, that is, the time required to kill 90% of its population in a sample at a specified temperature T (in °C) (Desmond and others 2001). The D_{57} values for the stationary phase, control cells of *L. bulgaricus* (52 ± 2.8 min) was about half that found for cells grown in the presence of sucrose (115 ± 5.7 min), sucrose and NaCl (100 ± 6.4 min), and NaCl (92 ± 2.7 min) (Table 1). Protection against heat may be achieved via accumulation of osmolytes, which enhance protein stability and protect enzymes against heat inactivation (Abee and Wouters 1999). In agreement with the results published by Desmond and others (2001) and Gouesbet and others (2001), our data indicate that the heat resistance of *L. bulgaricus* was improved by growing the cells in the presence of increased concentrations of sugar, salt, or both. The addition of sucrose to the growth medium, however, causes different effects on the resistance of *L. bulgaricus* to storage in the dried form and on its thermotolerance. Our findings may thus unfold an alternative explanation: the compounds produced during growth triggered by a higher concentration of sucrose might be responsible for the protection afforded against heat, but have virtually no effect on long-term storage of freeze-dried *L. bulgaricus*.

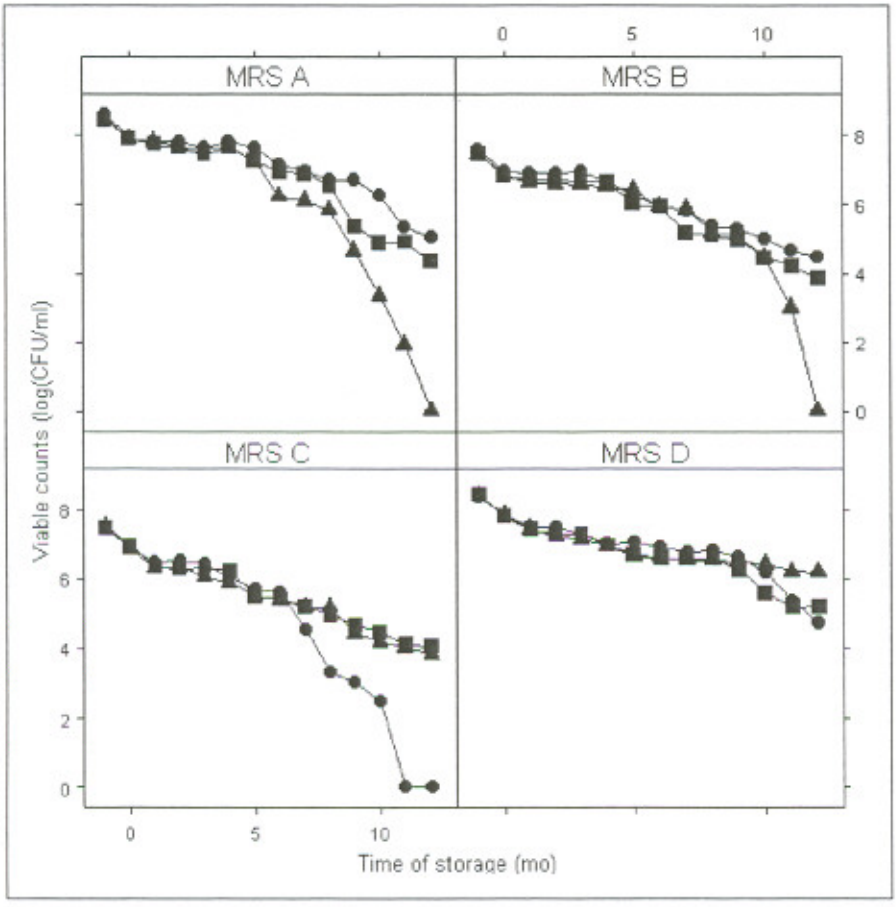

Figure 1—Effect of various growth media: MRS A (20 g/L glucose), MRS B (10 g/L glucose + 10 g/L sucrose), MRS C (10 g/L glucose + 10 g/L sucrose + 5 g/L NaCl), and MRS D (5 g/L NaCl) on survival during freeze-drying and subsequent storage of *L. bulgaricus* subject to different treatments: (▲) skim milk (control), (■) skim milk with 10 g/L sucrose, and (●) starvation. Each symbol represents the mean of 3 replicates from 2 freeze-drying experiments (6 values in all); Tukey's HSD, based on 95% confidence intervals of treatment means, is equal to 0.29 for skim milk, 0.26 for skim milk with 10 g/L sucrose, and 0.26 for starvation. The initial point in each graph represents the viable count in that cell suspension before freeze-drying.

Effect of starvation

Freeze drying and storage. The ANOVA results encompassing 3 factors (experimental replication, storage time, and starvation) indicated that 2-way interactions between starvation and storage time were statistically significant. Careful examination of Figure 1 indicates that when *L. bulgaricus* had been grown in MRS C and MRS D, starvation was rather inadequate in protecting dried cells during subsequent storage; however, a positive effect of starvation was observed when cells had been grown in MRS A and MRS B. The accumulation of compatible compounds at different levels or of different types in the various growth media may produce cells in distinct physiological states and, thus, which potentially possess distinct tolerances to a secondary stress imposed by starvation. Therefore, it is possible that starvation for 20 min was not enough to produce any stress response in the cells that had been grown in NaCl-enriched MRS broth because of the production of compatible solutes. Various studies (Teixeira and others 1994; Kim and others 2002) suggested that only the exponential phase cells are capable of adaptive response; however, starvation seems to improve resistance of stationary phase cells that had been grown in the presence of an increased concentration of sucrose. Other studies (Beney and

Table 1—Effect of various growth media[a]

	Growth medium											
	MRS A			MRS B			MRS C			MRS D		
Conditions	Lower	Mean	Upper	Lower	Mean	Upper	Lower	Mean	Upper	Lower	Mean	Upper
Without starvation	49.2	52.0	54.8	109.3	115.0	120.7	93.6	100.0	106.4	91.3	92.0	92.7
With starvation	38.8	48.0	57.2	113.3	114.0	114.7	91.5	107.0	122.5	84.0	96.0	108.0

[a]MRS A (20 g/L glucose), MRS B (10 g/L glucose + 10 g/L sucrose), MRS C (10 g/L glucose + 10 g/L sucrose + 5 g/L NaCl), and MRS D (5 g/L NaCl), and of starvation on thermotolerance of *L. bulgaricus* were measured by the D_{57} values (min). Each value represents the mean of 3 replicates from 2 individual experiments (6 values in all).

Gervais 2001) have shown that heat shock proteins may, in turn, be involved in a feedback process via their stabilizing effects on membrane proteins and lipids. As suggested by Broadbent and Lin (1999) for the effect of heat and cold-shock on *Lactococcus lactis*, the positive effect of starvation upon resistance of *L. bulgaricus* cells that is observed during storage in the freeze-dried state could be attributed to (1) stress-induced membrane changes, which may contribute to enhanced freeze and freeze-drying resistance, and (2) stress protein synthesis, which could act as macromolecular stabilizers and strengthen the hydrogen-bonded structure of water, hence increasing the level of nonfreezable water that surrounds macromolecules. It has been reported (Beney and Gervais 2001) that cells are able to resist changes in membrane fluidity only to a limited extent, which is related to the membrane fluidity prevailing during growth of cells in culture; this could explain (at least to a certain degree) why different growth media lead to cells with different membrane fluidity and consequently different resistance to the change in membrane fluidity imposed by other stresses, such as starvation.

Thermotolerance. As observed in Table 1, the thermotolerance of *L. bulgaricus* was not affected by starvation for 20 min in water. It is well known that when cells are adapted to a mild stress, they may exhibit cross-resistance against different stresses. Giard and others (1996) have indeed shown that starving *Enterococcus faecalis* caused by glucose depletion results in development of multiresistance, which protects the starved cells from other stresses. However, our results suggest that starvation of stationary phase cells of *L. bulgaricus* does not lead to increases in their heat resistance and are thus consistent with the observations reported by Carmelo and others (1998), who could not observe cross-stress-tolerance acquisition under particular conditions of acid stress. Alternatively, the set of conditions used in this study to starve the cells (time and starvation medium) may not have been optimal toward promotion (or induction) of thermotolerance.

Conclusions

Our research has shown that suitable selection of composition of both the growth and the drying media is essential to afford protection during storage of freeze-dried cells, and that treatments that enhance resistance to freeze-drying (and subsequent storage) and acquisition of thermotolerance are not necessarily coincident.

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