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Induction of stress tolerance in *Lactobacillus delbrueckii* ssp. *bulgaricus* by the addition of sucrose to the growth medium

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Introduction

The lactic acid bacteria (LAB) play an important role in the production of fermented foods. The development of concentrated cultures of LAB, for inoculating the production vat directly (bulk starters), has eliminated many problems traditionally involved in their preparation and maintenance by the food industry. For industrial use, LAB are often preserved in a frozen or dried form, the latter preparations having lower transport and storage costs (Kets et al. 1996). Dried cultures, however, lose viability/activity during storage, especially when kept at room temperature (Champagne et al. 1991; Teixeira et al. 1995a, b; Castro et al. 1996). Attempts to improve the survival of LAB during drying have already been tried (Linders et al. 1997b; Gardiner et al. 2000). Previous results indicated a direct relationship between the presence of compatible solutes in LAB and their ability to survive drying conditions. Such solutes include amino acids, amino acid derivatives, quaternary amines, sugars and tetrahydropyrimidines (Kets & De Bont, 1994; Kets et al. 1994, 1996).

It has been reported for several strains of lactobacilli that these organisms are probably unable to accumulate compatible solutes during the very short period of the drying process, and therefore they should be accumulated prior to the drying process (Kets & De Bont, 1994; Leslie et al. 1995; Kets et al. 1996; Linders et al. 1997b).

The aim of the present work was to investigate the effect of adding sucrose to the growth medium of *Lactobacillus delbrueckii* ssp. *bulgaricus* on its survival during heating, spray drying and during the time of storage.

Materials and Methods

Bacterial cultures and media

Lactobacillus delbrueckii ssp. *bulgaricus* (*Lb. bulgaricus*) originally isolated from yoghurt, was used. The original cultures were maintained in cryogenic storage at −80 °C on glass beads. Working cultures were grown on De Man, Rogosa, Sharp (MRS, Biokar Diagnostics, Beauvais, France) plus 1·5% w/v agar slopes (37 °C for 24 h). Slopes were maintained at 4 °C and sub-cultured every month.

MRS broth was inoculated from the MRS agar slopes and incubated at 37 °C for 24 h. This culture was then used to inoculate (10 ml/l) a second MRS broth and MRS broth with 20 g sucrose/l. These cultures were also incubated at 37 °C for 24 h. Cells were harvested by centrifugation at 7000 × ***g*** at 4 °C for 15 min.

Preparation of extracts for NMR analysis

Harvested cells were washed 3 times by centrifugation with phosphate buffer at pH 7·0 (0·01 M-K_2HPO_4 and 0·01 M-KH_2PO_4). Wet cell pellets (4–5 g) obtained from cultures grown in MRS and in MRS supplemented with 20 g sucrose/l were extracted twice with boiling 80% ethanol (v/v in distilled water; chromatography purity, Pronalab, Lisbon, Portugal) by the method of Reed et al. (1984), modified as previously described by Martins & Santos (1995). The control for NMR analyses was the final supernatant from the washing procedure of cells grown in MRS with sucrose. Ethanol extracts and the supernatant were freeze-dried in an Alpha 1-4 freeze-drier (Martin Christ, Osterode am Harz, Germany) and dissolved in D_2O (99·9 atom % D enrichment, Sigma-Aldrich Corporation, St. Louis, USA). Extracts were analysed by Hydrogen-1 and Carbon-13 nuclear magnetic resonance (^{1}H- and ^{13}C-NMR). Proton spectra were recorded at 300·14 MHz on a Bruker AMX300 spectrometer (Bruker, Europe) and acquired with a 6 μs pulse width (corresponding to a 60° flip angle) with a repetition delay of 20 s at 298 K. A 5 mm diameter broad-band inverse probe head was used. 13C-NMR were recorded at 125·77 MHz on a Bruker DRX500 spectrometer and acquired with a 6 μs pulse width (corresponding to a 60° flip angle) with a repetition delay of 5 s

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at 298 K and proton decoupling. A 5 mm diameter dual $^{13}C/^{1}H$ probe head was used.

Proton resonances were relative to 3-(trimethylsilyl) propanesulfonic acid (sodium salt) and ^{13}C chemical shifts were referenced to external methanol designated at 49·3 ppm.

Spray drying and storage

The cell pellets obtained from cultures previously grown in MRS and MRS supplemented with sucrose, were re-suspended to the original volume in reconstituted skim-milk powder (110 g/l; Oxoid, Hampshire, UK). Each sample was then directly spray dried in a laboratory scale apparatus (Niro Atomizer, Gladsaxevej, Denmark). Moisture in spray droplets produced by the atomization of the feed liquid by a vaned wheel (rotary atomizer) rotating at high speed, was evaporated in a vertical cocurrent drying chamber, 0·8 m diameter and 0·6 m height. Spray drier conditions were: outlet air temperature 70 °C, inlet air temperature 200 °C and atomizing air pressure 5 Bar. Powder was collected in a single cyclone separator. Samples of the spray dried powder were stored at 18 °C in hermetically sealed glass bottles in which the equilibrium relatively humidity (ERH = 0·3 %) was controlled by equilibrium with dried silica gel.

Heat treatment

The wet cell pellets obtained from cultures previously grown on MRS and MRS supplemented with sucrose, were re-suspended to the original volume in reconstituted skim-milk powder (110 g/l; Oxoid). Aliquots (1 ml) were transferred to 49 ml sterilized Ringer's solution or to 49 ml skim milk previously equilibrated at 57 °C (Teixeira et al. 1994) and maintained at this temperature for 60 min. At regular intervals, samples were taken from the heating menstrum and immediately diluted (10-fold) in sterile Ringer's solution at room temperature.

Enumeration of survivors

Each sample of spray-dried bacteria was rehydrated to the original volume with sterile deionized water. The cells were allowed to rehydrate for 2 min with vigorous shaking.

Survivors before each treatment and at appropriate intervals during heating and before and shortly after spray drying and during storage of the dried cells, were enumerated on MRS agar by the drop count technique (Miles & Misra, 1938). Plates were incubated aerobically at 37 °C for 48 h before enumeration.

Statistical analysis

The experiments were repeated twice. Viable counts on MRS agar were converted to log cfu/ml. Statistical analysis of survival during heating and during storage in the dried state was done with the ANOVA methodology using the Statview™ Package (Abacus Concepts, Berkeley, CA, USA) using as independent variable the storage time. Differences were considered significant at $P<0·05$. The error bars on the figures indicate the mean standard deviations for the data points.

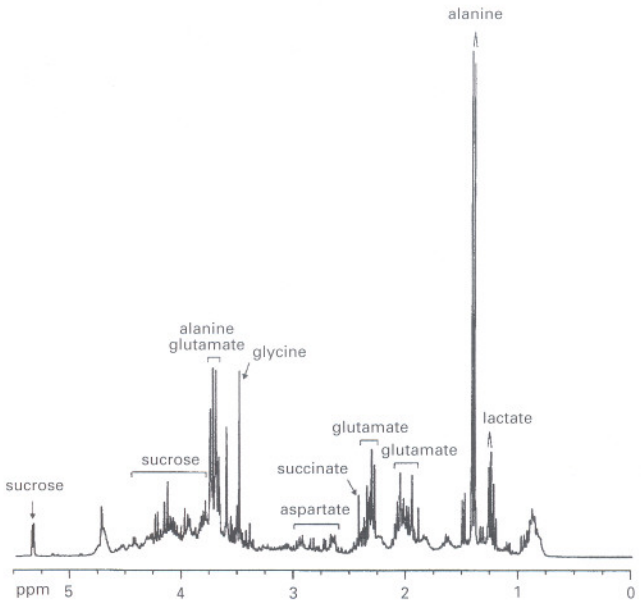

Fig. 1. ^{1}H-NMR spectrum of an ethanol extract obtained from *Lb. bulgaricus* cells grown in MRS supplemented with of sucrose.

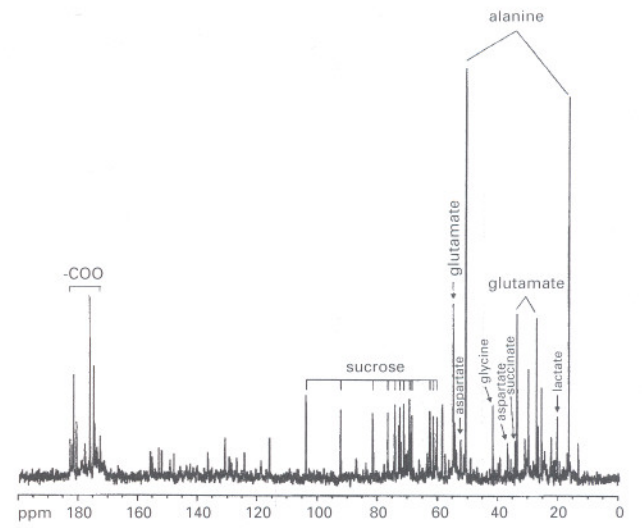

Fig. 2. ^{13}C-NMR spectrum of an ethanol extract obtained from *Lb. bulgaricus* cells grown in MRS medium supplemented with sucrose. The major solutes accumulated by the cells were identified as alanine, glutamate, sucrose, glycine, lactate, succinate and aspartate. The label $-COO^{-}$ refers to carboxylic groups of these compounds.

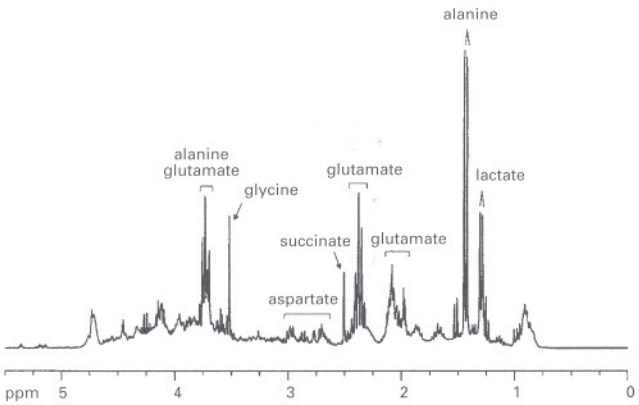

Fig. 3. ^{1}H-NMR spectrum of an ethanol extract obtained from *Lb. bulgaricus* cells grown in MRS medium.

Results

NMR analysis

The analysis of ethanol extracts, by ^{1}H- and ^{13}C-NMR, demonstrated that *Lb. bulgaricus* accumulated sucrose when this sugar was present in the growth medium (Figs 1 and 2). Other major organic solutes present in the extract and detected at the proton spectrum could be confirmed by ^{13}C-NMR, regardless of the lower carbon sensibility (^{13}C natural abundance is just 1%), and the resonances were assigned to alanine, glutamate, glycine, lactate, succinate and aspartate. Small amounts of glycerol and other minor compounds (not identified) could also be observed. Quantification, by ^{1}H-NMR, of some metabolites present in the extract revealed the presence of sucrose, lactate and alanine in a 1:4:13 ratio (7·0 μmol sucrose, 28·5 μmol lactate and 91·3 μmol alanine were extracted from 4·45 g cells wet pellet).

No sucrose was detected in the ^{1}H-NMR spectra of the control cells, grown in MRS medium without sucrose added (Fig. 3). To ensure that sucrose detected in cell extracts was intracellular and not due to contamination from the culture medium, a proton spectrum of the last washing buffer fraction was also run. The sucrose detected was negligible confirming the efficiency of the washing method (data not shown).

Effect of adding sucrose to the growth medium on the survival of Lb. bulgaricus *during heating*

Cells from either growth medium heated in skim milk had higher D_{57} values, (time required to reduce the viable count by 1 log at 57 °C) than cells heated in Ringer's solution. When heated in skim milk there were no significant differences in D_{57} values whether cells were previously grown in the presence or absence of sucrose. During heating in Ringer's solution, however, D_{57} values were strongly influenced by the presence of sucrose in the growth medium (30·9 and 51·0 min following growth in MRS and in MRS with sucrose respectively, Table 1).

Table 1. D_{57} values of *Lb. bulgaricus* grown on MRS medium and MRS supplemented with 20 g sucrose/l during heating in skim milk and sterile Ringer's solution

	D_{57} (mins) values of cells grown in		
Cells heated in	MRS	MRS+sucrose	*P*-value
Ringer's solution	30·9±0·61	51·0±0·46	<0·05
Skim milk	50·9±0·54	52·4±0·32	<0·05

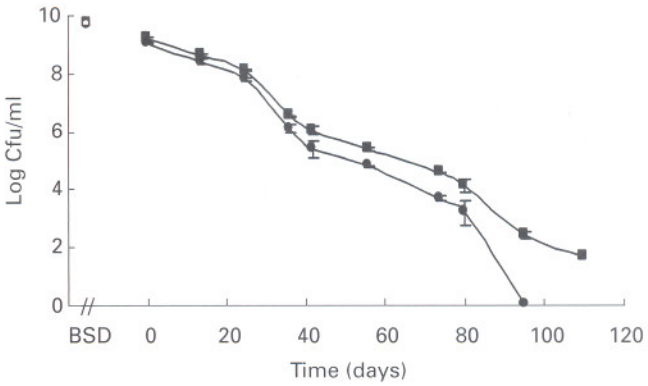

Fig. 4. Effect of the presence of sucrose in the growth medium (MRS supplemented with 20 g sucrose/l, ■; MRS, ●) on the survival of *Lb. bulgaricus* during spray drying and storage in the dried state. The error bars on the figures indicate the mean standard deviations for the data points. A loss of 0·25 log cfu/ml was recorded on spray drying of cells grown in either media.

Effect of adding sucrose to the growth medium on the survival of Lb. bulgaricus *during drying and long storage*

Figure 4 demonstrates that the presence of sucrose in the growth medium resulted in an increased survival of *Lb. bulgaricus* during long storage in the dried state but no difference was recorded as a result of the spray drying process (0·25 log cfu/ml reduction for both types of cells; $P<0·05$).

Discussion

In comparison with freeze drying, spray drying is an important technology used in the dairy industry, and can be used to produce large amounts of dairy starter cultures rapidly, relatively inexpensively, and with low costs associated with storage and transport when compared with frozen starter cultures (Gardiner et al. 2000). Spray drying of bacterial cultures involves treatments that stress the organisms in such a way that they lose some of their original activity (Teixeira et al. 1995a, b). In addition to dehydration, thermal inactivation is one of the reasons for this loss of activity.

Compatible solutes are recognised as cellular protecting agents during different drying processes and subsequent storage in the dried state (Kets et al. 1996; Linders et al. 1997a). To our knowledge, however, the effects of the use

of compatible solutes in processes for the preparation/preservation of starter cultures by spray drying have not been investigated.

In the present study, it has been demonstrated that, when present in the growth medium, sucrose, although not fermented by *Lb. bulgaricus*, is accumulated and resulted in significantly enhanced survival during heating in sterile Ringer's solution and during storage of cells in the dried state. This might suggest a relationship between solute accumulation and survival. Accumulation of compatible solutes has been shown to be associated with increased thermotolerance of various organisms (Welsh, 2000). This effect has been attributed to inhibition of the thermal denaturation of essential proteins in the presence of high concentrations of compatible solutes (Arakawa & Timasheff, 1985; Timasheff, 1998). However, the addition of sucrose to the growth medium did not affect the survival of cells during subsequent heating in skim milk, and it seems possible that the protective effect conferred by sucrose was masked by the protective effect of milk components during heating. Significantly higher survival of MRS-grown cells was observed during heating in skim milk than in sterile Ringer's solution. Similar results were previously reported for another *Lb. bulgaricus* strain and this protective effect was attributed to the milk proteins (Teixeira et al. 1994).

Sucrose and other saccharides were previously described as having the ability to protect various organisms from the adverse effects of drying (Leslie et al. 1994; Linders et al. 1997b).

According to some authors, the interaction between a carbohydrate and the polar head groups of the membrane phospholipids might be responsible for long storage stability (Crowe et al. 1984; Crowe & Crowe, 1993). Linders et al. (1997a), however, suggested that carbohydrates act through their free radical scavenging activity and not by direct interaction with polar lipid headgroups. Evidence of lipid oxidation in *Lb. bulgaricus* cells during drying and storage in the dried state was previously reported (Castro et al. 1996; Teixeira et al. 1996).

During spray drying the presence of sucrose in the growth medium did not have a significant effect on survival rates in the current study. Previously published reports, however, indicated that protection by sucrose did occur during the drying process (Leslie et al. 1995; Poolman & Glaasker, 1998). This contradictory observation was probably due to the different drying media used by the various investigators. In the current study, cells were spray dried in skim milk and the loss of viability during the process was very low. In the experiments carried out by Kets et al. (1996) the drying medium used was a phosphate buffer. It had been previously demonstrated that the choice of an appropriate drying medium is of extreme importance; cell suspensions resuspended in water and maltodextrin were more sensitive to drying and long storage in the dried state than cell suspensions in skim milk (Mazur, 1970; Castro et al. 1997). Castro et al. (1997), however, demonstrated that although a similar loss of viability was observed when cells were dried in skim milk, glycerol or trehalose, the sensitivity of the dried cells to sodium chloride varied according to the drying medium. While cells dried in glycerol did not develop sensitivity to the sodium salt, cells dried in skim milk became sensitive. Increased sensitivity to sodium chloride has been considered an indication of cell membrane damage (Teixeira et al. 1995a; Castro et al. 1997). Based on the current and previous results, we suggest that although no significant increase was observed in this study in the viability of cells grown in the presence of sucrose, through the spray drying process, it is possible that this carbohydrate might confer membrane protection against subsequent damage (possibly oxidative) when cells were stored in the dry state thereby resulting in the higher cellular viability observed during storage.

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