Journal of Food Technology 3 (4): 502-505, 2005

Effect of Various Protecting Compounds Added to the Growth Medium upon Survival of *Lactobacillus sakei* to Heating, Freezing, Freeze-drying and Storage in the Dried State

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Abstract: The aim of the present study was to investigate if the presence of sorbitol, myoinositol, xylose, mannose and Tween 80 in the growth medium increased the survival of *L. sakei* during heating, freezing, freeze drying and storage of freeze dried cells. Survival during freezing was enhanced by ca. 8% when glycerol was present in the growth medium. Viability during freeze-drying and storage in the dried state was improved by the presence of xylose in the growth medium. In addition to xylose, the presence of mannose and myoinositol protected cells during storage but not during drying. Cells grown in the presence myoinositol presented the highest D value at 55°C, 1.4 folder higher than control cells. In comparison with cells grown in MRS, total amino acids concentrations were the same order of magnitude for cells grown in the presence of mannose and myoinositol but were lower for cells grown in the presence of the other compounds.

Key words: Drying, *Lactobacillus sakei*, solutes accumulation, via

INTRODUCTION

The incidence of *Listeria monocytogenes* in dry-fermented sausages has been frequently reported[1]. The development, preservation and use of bacteriocin-producing starter cultures active against this pathogen should be considered as a potential means to improve the safety of fermented meat products by the food industry. *Lactobacillus sakei* CTC 494 is involved in the fermentation of sausages contributing to flavour development and preserving the final product by producing lactic acid. Additionally, antagonistic activity against foodborne pathogens, including *L. monocytogenes* and the capacity to decrease biogenic amine accumulation during sausage fermentations were previously described[1]. For industrial use as starter cultures, lactic acid bacteria are often preserved in a frozen or dried form, the latter preparations having lower transport and storage costs. Dried cultures, however, lose viability/activity during drying and storage in the dried state, especially at room temperature[2]. For the preservation of dried bacteria during storage at room temperature, growth, drying, rehydration and storage conditions should be optimized[3]. Indeed, it has been reported by several authors that when microbial growth occurs in the presence of protective agents, a higher viability during drying and subsequent storage in the dried state is observed[4,5]. Mechanisms behind these effects include the synthesis or accumulation of compatible solutes[5,8]. Such solutes have been demonstrated to contribute towards the enhancement of the stability of enzymes[6], the stabilization of macromolecules against denaturation through their ability to increase water structure[7] and improvement of the stability of the membrane phospholipids[8]. The degree of protection afforded by a given additive, however, was demonstrated to be species- and strain-dependent. In a previous study, we had already demonstrated that the addition of sucrose and monosodium glutamate to the growth medium increased the survival of spray-dried *L. sakei* CTC 494 during storage at room temperature. These compounds, however, did not confer any protection to the cells during heating, freeze-drying and storage in the freeze-dried state[9].

Therefore, the aim of the present study was to investigate if the addition of other compounds to the growth medium (sorbitol, myoinositol, xylose, mannose and Tween 80), increased the survival of *L. sakei* CTC 494 during heating, freezing, freeze drying and storage of freeze dried cells. To seek any underlying mechanisms of cell protection during the stresses investigated, the intracellular amino acid contents of cells grown in the presence of the added compounds were compared with that of control cells grown in MRS.

MATERIALS AND METHODS

Organism and media: *Lactobacillus sakei* CTC 494 was supplied by Dr Marta Hugas (Spain). It was grown in De Man, Rogosa, Sharpe (MRS) broth at 37 °C for 24 h and

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then inoculated (1% v/v) into MRS broth and MRS broth with the addition of 12.5 g sorbitol L^{-1} or 12.5 g myoinositol L^{-1} or 12.5 g xylose L^{-1} or 20 g mannose L^{-1} or 1 g Tween 80 L^{-1}. These cultures were incubated at 37 °C for 24 h and cells harvested by centrifugation at 7000 × g for 15 min (4°C).

Preparation of extracts for HPLC analysis: Harvested cells were washed 3 times by centrifugation with potassium phosphate buffer (0.1 M, pH 7.0). Wet cell pellets (*ca.* 4-8 g) were extracted as described by Silva *et al.*[5]. Extracts were analysed by HPLC, using a C18 column and a scanning fluorescence detector. Identification and quantification of amino acids was performed according to Souffleros *et al.* [10].

Stress treatments: Wet cell pellets were re-suspended to the original volume in 11% w/v reconstituted skim-milk powder.

- **Heating:** one milli liter of cell suspensions was transferred to 49 mL of sterile Ringer´s solution at 55° C and maintained at this temperature for 20 min. At regular intervals, samples were taken and immediately diluted in sterile Ringer´s solution at room temperature.

- **Freezing:** Cell suspensions (15 mL) were frozen at -80°C for 24 h.
- **Freeze-drying:** Cell suspensions (15 mL) were desiccated under vacuum (50 m Torr for 48 h) in a freeze-drier according to Carvalho *et al.*[4]. Dried cells were stored at room temperature in air at 20°C.

Enumeration of survivors: Survivors before each tratment and at appropriate intervals during heating and storage (dried samples were resuspended to the original volume with sterile Ringer's solution and allowed to rehydrate for 2 min with vigorous shaking), were enumerated by the drop count technique on MRS incubated aerobically at 37 °C for 24 h.

Statistical analysis: Each experiment was repeated twice. Statistical analyses of survival during heating and during storage in the dried state were performed by the ANOVA methodology using as independent variable the heating or 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.

RESULTS

Survival during freezing at -80°C was enhanced in the presence of Tween 80 (ca. 8%). With the exception of cells grown in the presence of mannose, showing a similar survival pattern to control cells, a lower survival rate was observed for all the other compounds investigated (Table1).

Viability during freeze-drying was enhanced in the presence of xylose (*ca.* 20%). With the exception of cells grown in the presence of sorbitol, showing a similar pattern to control cells, when growth was performed in the presence of the other compounds a lower survival rate was observed (Table 1). Extended survival during storage of the dried cells was observed following growth in the presence of myoinositol, xylose, or mannose. No significant differences were observed in the presence of Tween 80 and sorbitol was demonstrated to have a detrimental effect (Fig.1).

The highest thermotolerance was observed when growth occurred in the presence of myoinositol. In the presence of the other compounds lower D-values during heating at 55°C were obtained (Table 2).

As observed in Fig.2, in comparison with control cells, total amino acids concentration and in general individual concentrations of each amino acid were in the same order of magnitude for cells grown in the presence of mannose and myoinositol but were lower for cells grown in the presence of the other compounds investigated.

DISCUSSION

The effect of the presence of the protecting agents investigated (sorbitol, myoinositol, xylose, mannose and Tween 80) during growth of *L.sakei* in its subsquent

Table 1: Effect of the presence of Tween 80, mannose, myoinositol, sorbitol and xylose in the growth medium on the survival of *Lactobacillus sakei* CTC 494 during freezing at -80° C and freeze-drying

	MRS	MRST[a]	MRSM[b]	MRSMy[c]	MRSS[d]	MRSX[e]
Freezing	92.3±0.3	100±0.5	93.3±0.7	69.2±0.01	66.8±0.02	85.1±0.03
Drying	79.4±0.2	30.8±0.4	45.9±0.4	67.9±0.3	77.3±0.2	100±0.8

[a] MRS supplemented with 1 g of Tween 80 L^{-1}
[b] MRS supplemented with 20 g of mannose L^{-1}
[c] MRS supplemented with 12.5 g of myoinositol L^{-1}
[d] MRS supplemented with 12.5 g of sorbitol L^{-1}
[e] MRS supplemented with 12.5 g of xylose L^{-1}

Table 2: Effect of the presence of Tween 80, mannose, myoinositol, sorbitol and xylose in the growth medium on the heat resistance of *Lactobacillus sakei* CTC 494.

	MRS	MRST[b]	MRSM[c]	MRSMy[d]	MRSS[e]	MRSX[f]
[a]$D_{55°C}$ (min)	9.0	7.6	7.6	12.3	6.8	7.1

[a]Heat resistance of an organism may be defined by its D_T value, i.e. the time required to kill 90% of cells at a given temperature
[b] MRS supplemented with 1 g of Tween 80 L^{-1}
[c] MRS supplemented with 20 g of mannose L^{-1}
[d] MRS supplemented with 12.5 g of myoinositol L^{-1}
[e] MRS supplemented with 12.5 g of sorbitol L^{-1}
[f] MRS supplemented with 12.5 g of xylose L^{-1}

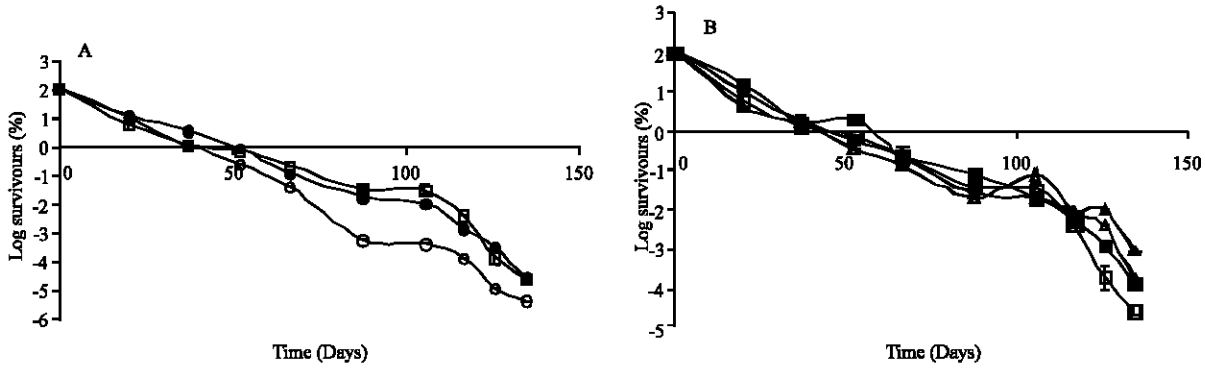

Fig.1: Effect of the presence of sorbitol, myoinositol, xylose, mannose and Tween 80 in the growth medium on the survival of *Lactobacillus sakei* CTC 494 during storage in the dried state: A) MRS, □; MRS supplemented with 1g of Tween 80 L^{-1}, ˜; MRS supplemented with 12.5 g of sorbitol L^{-1}, ™; B) MRS, □; MRS supplemented with 12.5 g of myoinositol L^{-1}, ▵MRS supplemented with 20 g mannose L^{-1}, ▵MRS supplemented with 12.5 g xylose L^{-1}, ■. The error bars on the figures indicate the mean standard deviations for the data points

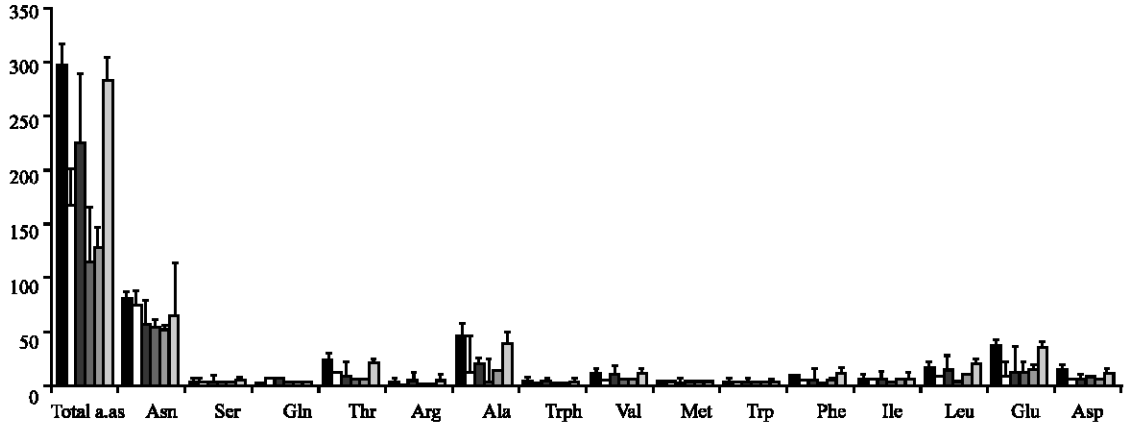

Fig. 2: Effect of the presence of sorbitol, myoinositol, xylose, mannose and Tween 80 in the growth medium on the pattern of intracellular amino acids of *Lactobacillus sakei* CTC 494: ¡ö, MRS; ¡õ, MRS supplemented with 12.5 g xylose L^{-1}; ■ MRS supplemented with 20 g mannose L^{-1}; ■ MRS supplemented with 12.5 g of myoinositol L^{-1}, ■ MRS supplemented with 12.5 g of sorbitol L^{-1}; ,MRS supplemented with 1 g of Tween 80 L^{-1}.

survival during heating, freezing, freeze-drying and storage in the dried state was dependent on the stress being imposed. The same fact was previously described. As an example, Carpenter *et al.*, [11] had already suggested that the means by which carbohydrates stabilize dried proteins is different from that observed in the aqueous and in the frozen state.

Tween 80 was the only compound conferring protection to the cells during freezing. Improved survival of other lactic acid bacteria during freezing when growth occurred in the presence of Tween 80 was previously reported and attributed to specific alterations in the cellular fatty acids[12].

Carvalho *et al.*[13] had already pointed out that in addition to glucose, the sugar present in MRS, other sugars should be used in order to extend the viability of the dried cells during storage at room temperature. The protective effect of sugar alcohols and other saccharides had been attributed to the steric structure of their hydroxyl groups that could presumably be capable of replacing water molecules in preserving the tertiary protein structure in the dried state[13].

Accumulation of compatible solutes, namely sugars, amino acids and their derivatives, is a well-known bacterial mechanism of stress adaptation. From the results obtained in this work, it is not possible to draw any relationship between increased survival to a particular stress in the presence of a particular compound and amino acids accumulation. However, it is important to stress that cells grown in the presence of mannose (confers

protection during storage) and myoinositol (confers protection during heating and storage) showed the highest total amino acid pool concentration similar to the control cells. This pattern was similar for the amino acid glutamate which is probably a counterion for K^+ to balance the intracellular charge accumulated by bacteria under osmotic stress[14].

Ferreira[9] stated, the mechanisms responsible for cellular inactivation and protection during stress treatments are complex and still not understood. Target sites of cellular damage are dependent on the stress being imposed and, consequently as demonstrated in this work, compounds that protect against one stress may not protect against a different one. Therefore, the selection of an appropriate growth medium on a case-by-case basis is essential to maximize survival of the target organisms during stress conditions.

ACKNOWLEDGMENTS

This study received partial financial support via project PRAXIS/P/BIO/12147/1998 (FCT, Portugal), co-ordinated by P. Teixeira and P. Gibbs. Financial support for author J. Silva was provided by PhD fellowships PRAXIS XXI/BD/197131/99 (FCT, Portugal).

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