Effect of the pH of growth on the survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* to stress conditions during spray-drying

J. Silva[1], A.S. Carvalho[1], R. Ferreira[2], R. Vitorino[2], F. Amado[2], P. Domingues[2], P. Teixeira[1] and P.A. Gibbs[1]

[1]Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, Porto, Portugal, and [2]Universidade de Aveiro, Departamento de Química, Campus Universitário de Santiago, Aveiro, Portugal

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ABSTRACT

J. SILVA, A.S. CARVALHO, R. FERREIRA, R. VITORINO, F. AMADO, P. DOMINGUES, P. TEIXEIRA AND P.A. GIBBS. 2005.

Aims: The aim of this study was to optimize survival of *Lactobacillus delbrueckii* subsp. *bulgaricus* during spray-drying and subsequent storage through optimizing the pH of growth conditions.

Methods and Results: Cell concentrates previously grown without or with pH controlled were spray-dried and stored at 20°C and heat treated at 57°C.

Cells grown under noncontrolled pH were more resistant to both drying and heating than cells grown under controlled pH but no significant differences were observed during storage. The intracellular proteins profile of cells grown under both conditions was studied by two-dimensional SDS-polyacrylamide gel electrophoresis. Eight proteins were identified using automated mass spectrometry (MS) and tandem mass spectrometry (MS/MS) data acquisition. Of the identified proteins, only cochaperonin GroES corresponded to a known heat shock protein (HSP). The other proteins identified are proteins involved in glycolysis.

For cells grown under noncontrolled pH the expression of the Hsp70, GroES and GroEL, measured by Western blotting, was enhanced.

Conclusions: The higher resistance of cells grown under noncontrolled pH correlates with the enhanced production of heat shock proteins.

Significance and Impact of the Study: Growth of *L. bulgaricus* under controlled pH (commonly used by the starter cultures production industry) results in cells more sensitive to stresses frequently encountered by the cells during starter cultures preparation/storage/utilization.

INTRODUCTION

Lactic acid bacteria (LAB) play an important role in the production of fermented food such as milk, meat, fruit, vegetables and bread products.

Correspondence to: Paula Teixeira, Escola Superior de Biotecnologia, Universidade Católica Portuguesa, Rua Dr. António Bernardino de Almeida, 4200-072, Porto, Portugal (e-mail: paula@esb.ucp.pt).

Within the LAB group *Lactobacillus delbrueckii* subsp. *bulgaricus* is commonly used in dairy products as a starter culture. Starters are frequently supplied to the dairy companies either as a frozen concentrate or as a powder obtained by freeze-drying or, rarely, spray-drying (SD). As freeze-drying is expensive and requires special equipment, it is a useful method for the production of small quantities only. However, SD can be used to produce large amounts of

dairy ingredients relatively inexpensively; the spray-dried powders can be transported at a low cost and can be stored in a stable form for prolonged periods (Silva *et al.* 2002). Nowadays, starter cultures have become an integral part of a successful industry; their relevance is reflected in the economic value of the end products. Although dried powders may be a more readily utilized form of preserved cultures, it has been previously demonstrated that during SD and subsequent storage in the dried state, cells of *L. bulgaricus* suffer from a variety of stresses including heat, osmotic and oxidative stress that result in the loss of cellular viability and activity, especially during storage at ambient temperatures (Teixeira *et al.* 1994, 1995a,b; Steiner and Sauer 2001; Silva *et al.* 2002). From an industrial point of view, the development of protocols for the preparation of starter cultures containing highly active, viable cells tolerant to adverse conditions, would be advantageous (Carvalho *et al.* 2004).

It is well documented that when exposed to a low level of stress most bacteria develop adaptation strategies in order to resist a subsequent exposure to a higher level of the same stress and also of a number of different stresses (O'Driscoll *et al.* 1997; Rabilloud 1998; Wang and Doyle 1998; Lorca and De Valdez 2001). The best studied stress response in bacteria is heat shock, which, like other stress responses, is characterized by the transient induction of specific proteins and physiological changes that generally enhance the ability of an organism to withstand more adverse environmental conditions (Whitaker and Batt 1991; Teixeira *et al.* 1994; Kilstrup *et al.* 1997; Glaasker *et al.* 1998; Broadbent and Lin 1999; Derré *et al.* 1999; Yan *et al.* 2000; Lorca and De Valdez 2001). Nevertheless, little is known about the potential utilization of sublethal stresses to enhance microbial resistance to SD although this had already been demonstrated to be a possibility by Desmond *et al.* (2001).

The lactic acid produced by LAB during sugar fermentation, is a weak organic acid, that can easily pass through the cell membrane in the nonionized form at low pH. In the higher pH of the cell contents, acid ionization will led to a reduction in the internal pH imposing a stress condition to the cells (Hutkins and Nannen 1993).

The purpose of this study, therefore, was to evaluate if auto-acidification occurring during growth under noncontrolled pH would result in any increase in cell survival during heating and SD by comparison with cells grown under controlled pH (6·5), condition normally used in the commercial production of starter cultures. The intracellular protein profile of cells grown under both conditions was also studied. Although the survival of *L. bulgaricus* during heating and SD had already been studied (Teixeira *et al.* 1994, 1995a,c; Gouesbet *et al.* 2001) the effect of pH control during growth on the behaviour of the organism during these processes was not known.

MATERIALS AND METHODS

Bacterial cultures and media

Lactobacillus bulgaricus previously isolated from yoghurt and maintained in ESB culture collection (ESB285), was used. The reference cultures were maintained in cryogenic storage at −80°C on glass beads. Working cultures were grown on De Man, Rogosa, Sharpe (MRS; LAB M, Bury, UK) agar as slant cultures. Slants were maintained at 4°C and subcultured every month.

To prepare the inoculum, MRS broth was inoculated from the MRS agar slants and incubated at 37°C for 24 h. This cell suspension was then used to inoculate another MRS broth (1% v/v). The cultures were incubated with agitation by a magnetic stirrer bar at 37°C without or with pH controlled to pH 6·5 (automatic addition of 1 mol l^{-1} sodium hydroxide). pH changes during growth under noncontrolled conditions were recorded. Cells were harvested by centrifugation at 7000 *g* for 15 min. (4°C) in the stationary phase of growth (20 and 24 h under controlled and uncontrolled pH respectively, according to growth curves previously determined).

Heat treatment

Cell pellets from 50 ml of the cultures grown under controlled and noncontrolled pH were resuspended to the original volume in 11% w/v reconstituted skim milk (Oxoid, Hampshire, UK) as in previous studies on survival of *L. bulgaricus* during SD and during storage in the dried state (Silva *et al.* 2002). One-millilitre aliquots were transferred with vigorous mixing to 49 ml of sterile Ringer's solution kept at 57°C and maintained at this temperature for 60 min. At defined intervals, samples (1·0 ml) were taken from the heating medium and immediately diluted in Ringer's solution (9·0 ml) at room temperature.

Spray-drying and storage

The cell pellets were resuspended to the original volume in 11% w/v reconstituted skim milk powder. Each sample was then directly spray-dried in a laboratory scale apparatus (Niro Atomizer, Gladsaxevej, Denmark) as previously described (Teixeira *et al.* 1995a,b). 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 in height. Spray drier conditions were: outlet air temperature 70°C, inlet air temperature 200°C and atomizing air pressure 5×10^5 Pa. Powder was collected in a single cyclone separator. Samples of the spray-dried powder were stored at 20°C. Immediately after drying and at regular intervals during storage, spray-dried samples were

rehydrated to the original volume with deionized water. The cells were allowed to rehydrate for 2 min with vigorous shaking.

Enumeration of survivors

Survivors before each treatment and at appropriate intervals during heating and storage in the dried state, were enumerated 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 three separate plates and incubated aerobically at 37°C for 48 h before enumeration of colonies.

Statistical analysis

Statistical analysis was performed with the ANOVA statistical test using Statview^{TM} Package (Abacus Concepts, Berkeley, CA, USA) using as independent variable the storage time.

The experiments were repeated at least twice. Viable counts on MRS agar were converted to log CFU ml^{-1}. Differences were considered significant at $P < 0{\cdot}05$. The error bars on the figures indicate the mean standard deviations for the data points.

Preparation of cell extracts and two-dimensional SDS-polyacrylamide gel electrophoresis

Stationary phase cells were prepared as described above. Harvested cells from 20 ml culture were washed twice with tris-acetate-EDTA buffer (TAE, pH 7·0) and were incubated with lysozyme (Sigma, St Louis, MO, USA) for 2·5 h at 37°C (40 mg lysozyme: 0·4 ml Tris-HCl 0·064 mol l^{-1} pH 7·0; 20 μl lysozyme solution: 20 ml TAE buffer). The cell suspension was then disrupted by grinding in a vortex with glass beads (150–212 μm diameter; Sigma). Between each disruption cycle cells were cooled on ice. The disrupted suspension was then centrifuged (15 min, 7000 *g*, 4°C) and the supernatant was stored at −80°C until use. Protein concentration was determined according to Lowry *et al.* (1951). Prior to two-dimensional electrophoretic (2DE) analysis, sample quality and protein concentration were analysed by analytical one-dimensional SDS-PAGE (Laemmli 1970) on 15% gels using the Hoefer SE600 apparatus (Pharmacia Biotech, Buckinghamshire, UK). Aliquots of 15 μg protein were used per line. 2DE analysis was performed as follows: an aliquot of the frozen thawed sample, containing 60 μg of protein, was dissolved in 250 μL of a rehydration solution containing 4% w/v CHAPS, 12·9 mmol l^{-1} DTT, 0·1% v/v IPG ampholytes 3–10, 9 mol l^{-1} urea, 2 mol l^{-1} thiourea, 0·01% bromophenol blue (Rabilloud 1998) and loaded onto an immobilized pH 4–7 gradient strip 13 cm long (Pharmacia Biotech). Rehydration occurred during 12 h applying 50 μA per IPG strip. Following rehydration, the voltage was increased to 500 V during the first hour and to 1000 V during the second hour, followed by 8000 V for an additional period of 2 h for isoelectric focusing.

After first dimension, the IPG gel strips were transferred onto vertical slab gels with a Laemmli-SDS discontinuous system (12·5% acrylamide gels of 1 mm thickness) and separated at 200 V, 10°C, Hoefer SE600 (Pharmacia Biotech). Gels were silver reversible stained, according to Yan *et al.* (2000) scanned with a laser densitometer (Bio-Rad, Hercules, CA, USA) and analysed with PDQuest Software (Bio-Rad).

Reference points (i.e. points that are visually identified as the same on both gels) were selected and marked on images to align and match gels. After gel alignment and matching, pairs (spots present in both gels) could be highlighted.

In gel digestion

About 60 protein spots of significant intensity difference (between the two protein profiles) were excised from the gels and digested with trypsin according to Shevchenko *et al.* (1996). After the gel pieces were excised and shrunk by dehydration in acetonitrile (ACN), which was then removed, they were dried in a SpeedVac (SC210A; ThermoSavant Milford, MA, USA). A volume of 10 mmol l^{-1} dithiotreitol (DTT) in 100 mmol l^{-1} NH_4HCO_3 sufficient to cover the gel pieces was added, and the proteins were reduced for 30 min at 60°C. After cooling to room temperature, the supernatant was removed and 25 μl of 55 mmol l^{-1} iodoacetamide was added to the gel pieces. After allowing the reaction to proceed in the dark for 45 min at room temperature, the supernatant was removed; gels were washed with 100 μl NH_4HCO_3 25 mmol l^{-1} and dehydrated with 25 mmol l^{-1} NH_4HCO_3 in 50% ACN. After the gel pieces were dried in the Speed Vac and sequence grade modified, porcine trypsin (Promega, Southampon, UK) was added to the gel spot and 25 mmol l^{-1} NH_4HCO_3 was added to cover the gel pieces. Samples were incubated at 37°C overnight and peptides were extracted adding 60 μl of 50% ACN/5% formic acid.

Nano-ES mass spectrometry

After digestion, all the samples were cleaned and concentrated using a ZipTip (Millipore, Madrid, Spain). Initially, the ZipTips were washed twice in 50% acetonitrile and in 0·1% trifluoroacetic acid (TFA). The samples were pipetted at least 15 times by the ZipTips and were washed twice in 0·1% TFA. Electrospray (ES) mass spectra and tandem mass spectra were acquired with a Q-TOF 2 (Micromass, Manchester, UK). The instrument resolution was set at

9500 (50% peak valley). The nanoflow capillary needle voltage was 800 V and the source temperature was maintained at 150°C. Argon was used as collision gas. Cone voltage was at 35 V for mass spectrometry (MS) and tandem mass spectrometry (MS/MS). Collision-induced decomposition mass spectra were acquired by selecting the desired ion with the quadrupole section of the mass spectrometer, and colliding it in the collision cell with argon gas (measured pressure in the penning gauge *ca* 6×10^{-4} Pa) using a collision energy of 20–25 eV. The resulting product ions were determined by the TOF analyser. Data acquisition was carried out with a Micromass MassLynx 3·4 data system (Manchester, UK). Samples were injected on a nanospray needle and proteins were identified using mass spectra or/and the tandem mass spectra data on a search on Mascot (Rabilloud 1998) and protein prospector (Clauser *et al.* 1999). Protein identification was accepted when the score reported by the Mascot search routine was higher than 40%.

Western blotting

Proteins resolved by 2DE SDS-PAGE were electroblotted onto nitrocellulose membranes for 3 h at 200 mA. The immunoblotts were probed with 1 : 5000 dilution of monoclonal anti-heat shock protein 70 (Hsp 70), clone BRM-22 (Sigma), 1 : 500 dilution of monoclonal Anti-GroES developed in rabbit IgG fraction of antiserum (Sigma), 1 : 10000 of monoclonal Anti-GroEL developed in rabbit IgG fraction of antiserum (Sigma) and 1 : 500 dilution of the secondary antibody (anti-mouse IgG peroxidase conjugate, Sigma). The bands were visualized by treating the immunoblotts with ECL chemiluminescence reagents (Amersham, Pharmacia Biotech), according to the supplier's instructions, followed by exposure to X-ray films (Kodak Biomax Light Film; Sigma).

RESULTS

Log counts of *L. bulgaricus* cells grown in MRS at 37°C under controlled and noncontrolled pH conditions and the pH changes during growth under noncontrolled conditions are presented in Fig. 1.

Heat and drying resistance

Heat resistance of stationary phase cells grown under controlled and noncontrolled pH conditions was determined in reconstituted skim milk powder at 57°C. Figure 2 shows that cells grown under controlled pH were significantly more heat sensitive ($D_{57°C} = 4{\cdot}88$ min) than cells grown under noncontrolled pH ($D_{57°C} = 78{\cdot}1$ min). $D_{57°C}$ is the time required to reduce the viable count by 1 log at 57°C.

During SD, *L. bulgaricus* cells grown under controlled pH lost almost 2 log cycles of viable cells while cells grown under noncontrolled pH conditions lost <1 log cycle in comparison with the number of viable cells before drying (Fig. 3).

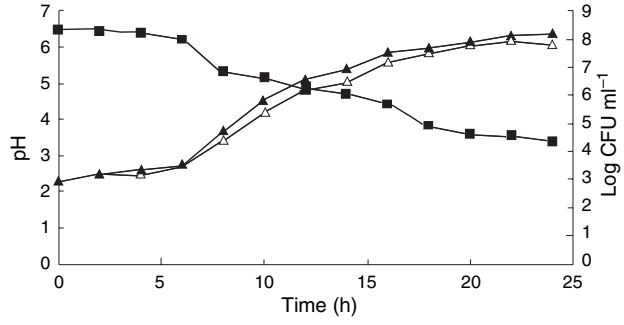

Fig. 1 Log counts of *Lactobacillus bulgaricus* during growth in MRS under controlled (▲) and noncontrolled pH (△) and pH changes (■) occurring during growth under noncontrolled pH conditions

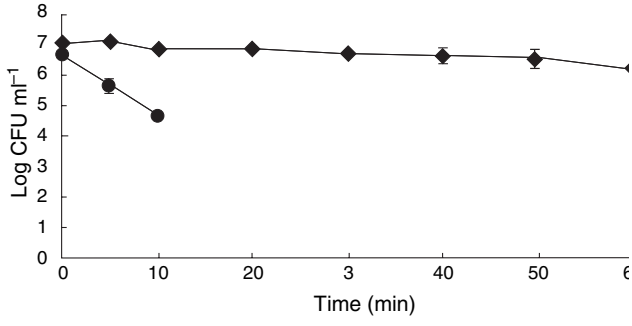

Fig. 2 Survival of *Lactobacillus bulgaricus* grown under controlled (●) and noncontrolled (♦) pH conditions during heat stress at 57°C. For cells grown under controlled pH conditions, after 20 min at 57°C counts were lower than 50 CFU ml^{-1}

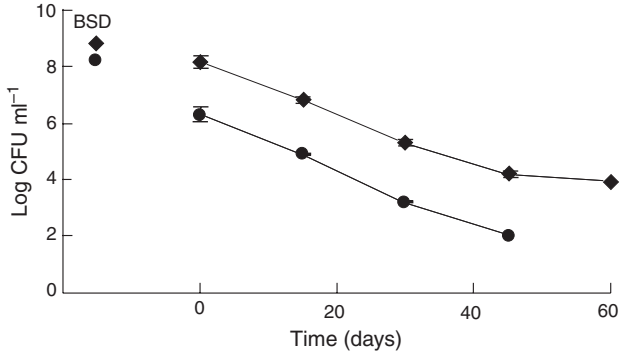

Fig. 3 Survival of *Lactobacillus bulgaricus* grown under controlled (●) and noncontrolled (♦) pH conditions to drying and during storage at 20°C (before spray-drying, BSD). For cells grown under controlled pH conditions, after 60 days of storage counts were lower than 50 CFU ml^{-1}

During the period of storage investigated, however, no significant differences in the survival were observed between cells grown under controlled and uncontrolled pH (Fig. 3).

Intracellular protein analyses

The protein profiles of *L. bulgaricus* grown under the different pH conditions were studied by 2DE. A greater diversity of proteins was observed when the cells were grown under controlled (Fig. 4b) than under noncontrolled (Fig. 4a) pH conditions. Despite important differences in expression patterns under controlled pH and noncontrolled pH, the overall 2D patterns were very similar and thus allowed us to quickly identify the same spots on different gels. It was observed that 246 spots were matched for both growth conditions. However, growth under noncontrolled pH resulted in 100 unmatched proteins relative to cells grown under pH controlled conditions and growth under controlled pH showed 241 unmatched proteins relative to cells grown under noncontrolled pH conditions. From both gels 60 protein spots were excised and analysed by mass spectra and tentatively identified by comparison on Mascot browse (Perkins *et al.* 1999) and protein prospector (Clauser *et al.* 1999). Of the 60 excised and tentatively identified protein spots, only eight were identified. Phosphoglycerate kinase, D-lactate dehydrogenase, pyruvate kinase, triose-phosphate isomerase, three isoforms of glyceraldehyde 3-phosphate dehydrogenase were identified by Mascot research (MOWSE score higher than 40%). This identification was confirmed by the isoelectric point, molecular weight, taxonomy, and sequence covered (Table 1). When no acceptable results were obtained and when the Mascot research did not recognize the spots we tried to match the amino acid sequences in a Blast search (http://www.ncbi.nlm.nih.gov/BLAST/) using a doubly charged peptide of m/z (mass to charge ratio) 869·51 which were sequenced by MS/MS [(K)LPMTVQEGDEVLYDK]. Of the spots analysed, only spot 15 was identified as being similar to GroES from *Lactobacillus acidophilus*.

To identify known heat shock proteins we used ECL Western blotting on the 2DE. Antisera against GroEL, GroES and Hsp70 proteins were used. The three proteins were detected for both cells grown under controlled and noncontrolled pH but their expression was enhanced when cells were grown under noncontrolled pH as observed in Fig. 5.

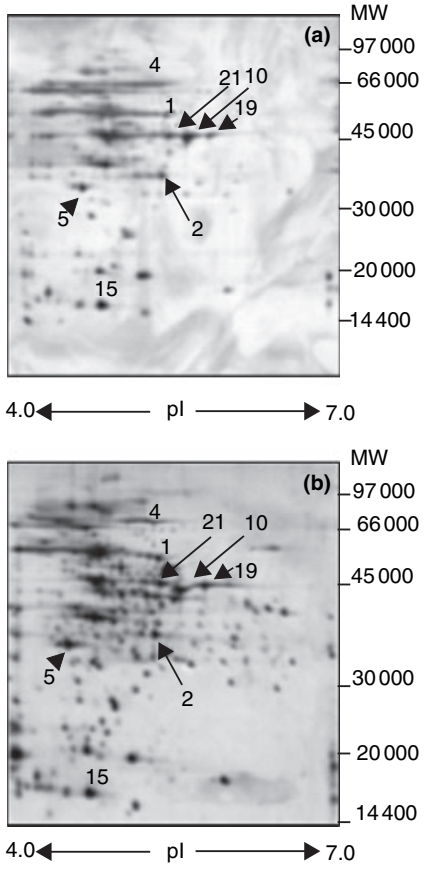

Fig. 4 Two dimensional SDS-polyacrylamide gel electrophoresis of proteins isolated from *Lactobacillus bulgaricus* grown under controlled (b) and noncontrolled (a) pH conditions. Gels have been treated using Bio-Rad PDquest software

DISCUSSION

As observed in Fig. 1, cells of *L. bulgaricus* grown under noncontrolled pH conditions were exposed to stressful pH values. When compared with cells grown at pH 6·5, cells grown under noncontrolled pH showed greater survival during heating and drying but not during storage in the dried state. This increased resistance is probably a result of the cross protection conferred by the low pH attained during growth under noncontrolled pH conditions. Previous studies had already demonstrated that acid stress induces resistance to various other stresses. However, in most studies this effect was observed when cells were subjected to an acid shock and not by growing cells under noncontrolled pH (O'Driscoll *et al.* 1996; O'Sullivan and Condon 1997; Lemay *et al.* 2000; Lim *et al.* 2000; Lorca and De Valdez 2001). Research by Lorca and De Valdez (2001) demonstrated that survival of *L. acidophilus* CRL 639 under different stress conditions (ethanol, H_2O_2, freezing, lyophilization) was higher for cells grown under free fermentation runs (noncontrolled pH conditions) than for those grown at pH 6·5. However, and in contrast with the results presented here, in their study, it was not demonstrated that heat resistance was enhanced by acid production during growth.

Table 1 Protein identification by Nano-electrospray mass spectrometry

Spot number*	Protein designation	Molecular weight (Da)	PI value	MOWSE score (ref. protein prospector)	Aminoacid sequence coverage (%)
1	Phosphoglycerate kinase	42 712	5·3	7·76E + 17	72
2	D-lactate dehydrogenase (D-LDH)	36 904	5·1	1·49E + 08	46
4	Pyruvate kinase	62 920	5·3	2·85E + 11	53
5	Triosephosphate isomerase (TIM)	27 383	4·7	2·19E + 05	47
21, 10, 19	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	36 565	5·5	4·89E + 04	44
15†	Similar to cochaperonin GroES (*Lactobacillus acidophilus*)				

Protein spots were excised from the 2-DE gels shown in Fig. 3 and analysed by MALDI and ESI-MS. The resulting peptide mass maps were searched against SWISS-PROT/TrEMBL.
*The spot number corresponds to those proteins that were identified by NanoES mass spectrometry.
†Identified by Blast search.

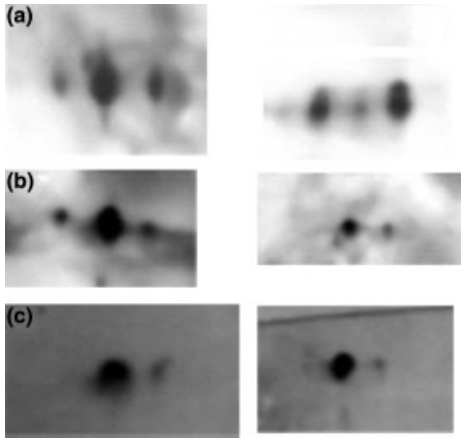

Fig. 5 Detection of GroEL (a), Hsp70 (b) and GroES (c) by Western blotting. Spots observed on the left side of the figure correspond to cells grown under noncontrolled pH and spots observed on the right side correspond to cells grown under controlled pH

Previous work suggested the involvement of stress proteins in cell adaptation to environmental stress conditions (Kilstrup *et al.* 1997; Broadbent and Lin 1999; Yan *et al.* 2000; Steiner and Sauer 2001). In this work, the total protein extracts were analysed by 2-DE. Fewer protein spots were visible in the cells grown under noncontrolled pH silver stained gel. Previous work by Hartke *et al.* (1996) had already demonstrated a reduction in the protein synthesis of *Lactococcus lactis* subsp. *lactis* as a result of an acid treatment of 30 min at pH 5·5.

Of the 60 proteins analysed by mass spectrometry only eight were identified and of these only one, GroES, corresponded to a known HSP. The other proteins identified are proteins involved in glycolysis. Induction of glycolytic enzymes during stress treatments had already been reported and also their involvement in the stress response was already demonstrated (Graumann *et al.* 1996; Wouters *et al.* 2000). Identical identification, glyceraldehyde 3-phosphate dehydrogenase, was obtained for three of the initial spots (spots number 21, 10 and 19 in Table 1). This had already been reported by other workers for *L. lactis* (Kilstrup *et al.* 1997; Willemoës *et al.* 2002) and was attributed to the presence of different forms of the protein, for e.g. deaminated, phosphorylated or modified by chemical groups (Kilstrup *et al.* 1997). All these isoforms appeared in high concentration which contributes to the poor resolution present in both gels in this region.

Previous studies on the stress response of *L. bulgaricus* demonstrated that other HSP rather than GroES were induced in response to stress conditions (Lim *et al.* 2001; Gouesbet *et al.* 2002) namely DnaK (an Hsp70) and GroEL. In the present study these proteins were not identified in the silver stained 2DE gel, probably as a result of the lower sensitivity of the silver staining in comparison with the radioactive labelling previously used. Therefore these proteins were tentatively identified in 2DE by Western blotting. Indeed, GroEL and Hsp70 were identified and it was also demonstrated that the expression of these proteins was enhanced during growth under noncontrolled pH. This suggests a relationship between acid stress occurring during growth under noncontrolled pH conditions and the increased intensity of specific proteins. This is in agreement with the results of Lim *et al.* (2001) who showed that these

proteins were induced when cells were exposed to a sudden downshift in the pH. Similarly, O'Driscoll *et al.* (1997) reported that some specific proteins over-expressed under noncontrolled pH growth conditions were involved in acid stress resistance of *Listeria monocytogenes*.

According to the results obtained, cells grown under controlled pH produced maximum biomass but lower resistance to heating, drying, storage and acid treatments. The higher resistance when cells were grown under noncontrolled pH was related with the enhanced production of heat shock proteins.

This study demonstrates that growth of *L. bulgaricus* under controlled pH (commonly used by the starter cultures production industry) results in cells more sensitive to heating, drying and storage in the dried state, stresses frequently encountered by the cells during starter cultures preparation/storage/utilization. In order to improve cellular resistance to the procedures involved in the preparation and maintenance of starters cultures and during the industrial processes in which LAB might participate, it would be advisable to optimize growth conditions in order to obtain more resistant cells.

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